Differential Effects of Small and Large Molecular Weight Wine Phytochemicals on Endothelial Cell Eicosanoid Release

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Yearly, thousands of plant extracts are screened for biochemical effects in vitro, often with little regard to the medium used or the presence of unabsorbable large molecular weight chemicals. Here, dealcoholized red wine was separated by ultrafiltration, and each fraction was investigated for effects on endothelial cell eicosanoid release. Following a 20 min incubation with cells, medium containing either 0.6% whole red wine or the large components of 0.6% red wine had more 6-ketoprostaglandin F1-α and thromboxane B_2 than control medium. Sample medium containing only the small components of 0.6% red wine had increased 6-ketoprostaglandin F1-α. The small red wine components were more effective in plasma and in whole blood than in culture medium. Therefore, we conclude that the potential for differential effects of medium type and small and large extract components should be considered in the design or interpretation of data from investigations into biological effects of plant extracts.

Keywords: *Ultrafiltration; eicosanoids; cardiovascular disease; phytochemicals; separation*

INTRODUCTION

Most nutrients larger than a few thousand daltons are not absorbed until digested (Sherwood, 1993). Data suggest that many monomeric plant phenols are absorbed by mammals but polymers are not (Butler, 1992; Jimenez-Ramsey et al., 1994; Laparra et al., 1977; Reed, 1995). Phytochemical absorption, therefore, like the absorption of other compounds, may be a function of size and structure. In addition, phytochemicals too large to penetrate plasma membranes or tight junctions of gut epithelial cells may not be absorbed to any significant extent regardless of their structure.

Cell culture and intravascular delivery systems have been used by investigators in an effort to determine mechanisms by which wine consumption could inhibit cardiovascular disease. For example, the addition of wine to culture medium stimulates endothelial cell secretion of the antithrombotic eicosanoid prostacyclin (Schramm et al., 1997), and administration of wine intravenously to animals inhibits platelet reactivity and thrombosis in stenosed coronary arteries (Demrow et al., 1995).

Similar procedures are used regularly for the examination of the effects of various plant and food extracts; however, since phytochemicals in plants and plant extracts range in size from a few hundred to a few hundred thousand daltons, phytochemicals consumed orally will reach a variety of different targets in the body. Therefore, investigation of modified as well as whole extracts would improve the likelihood that results from in vitro and intravascular assay systems reflect what happens in organisms following oral consumption of phenolic compounds. For example, endothelial cell model systems designed to model in vivo conditions should be exposed to phenolic compounds from extracts that are small enough to be absorbed. In addition, removal of most large molecular weight (MW) material (e.g., 3000 Da and above) from an extract prior to its administration intravenously would provide a phytochemical composition closer to that which would be absorbed following oral intake of a food or extract. Alternatively, removal of most of the small MW phenolic compounds from an extract will allow examination of molecules too large for absorption and may provide an extract with a composition similar to that to which a colon's cells would be exposed following oral consumption of a food or extract. Since thousands of extracts are examined in many different in vitro and intravascular assay systems each year and phenol size affects function (Jimenez-Ramsey et al., 1994; Reed, 1995; Crag et al., 1994), investigators should consider experimentation with both whole and modified extracts.

Cell culture provides the opportunity to examine biological components under conditions established by the investigator. Previously, while investigating potential mechanisms of wine mediated vasoprotection, we observed that 20 min after wine or dealcoholized wine was added to medium on confluent endothelial cells (EC), medium 6-ketoprostaglandin F1-α (6-keto-PGF1-α) concentration was increased compared to medium incubated with endothelial cells without wine (Schramm et al., 1997). Here, wine components were separated by a 3000 Da filter into small and large fractions. The effect of whole wine and each wine fraction on endothelial cell eicosanoid release was determined. Also investigated were the effects of incubation time and

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S0021-8561(97)00905-9 CCC: $15.00
Published on Web 04/17/1998

medium type on wine-induced endothelial cell prostacyclin release.

MATERIALS AND METHODS

Materials. Bovine aortic endothelial cells (BAEC) were a gift from M. E. O'Donnell, University of California, Davis. Eagle's minimal essential medium for culture (EMEM) was purchased from Mediatech (Herndon, VA), and the remaining cell culture materials were purchased from Sigma Scientific (Orlando, FL). Indomethacin plus 6-keto-PGF1-α and thromboxane B_2 immunoassay kits were purchased from Cayman (Ann Arbor, MI). Adenosine triphosphate was purchased from Calbiochem (La Jolla, CA). Microcon YM 3000 Da MW filters were purchased from Amicon (Beverly, MA). Size exclusion for filters is defined in daltons as described in Amicon Publication 277. Siliconized microtubes (1.5 mL) were purchased from Fisher (Pittsburgh, PA). Acrodisc 13 mm, 0.45 μm PTFE syringe tip filters were purchased from Gelman Sciences (Ann Arbor, MI). Standards were as follows: (+)-catechin, (−)-epicatechin, *p*-coumaric acid (Aldrich Chemical Co., Inc., Milwaukee, WI); rutin, caffeic acid (Sigma Chemical Co., St. Louis, MO); gallic acid (MCB Manufacturing Chemists Inc., Cincinnati, OH); and malvidin 3-glucoside (Extrasynthese, Genay, France). Cabernet Sauvignon and Chardonnay were produced at the Department of Viticulture and Enology (University of California, Davis) and donated after 1 year of aging.

Separation of Wine Compounds. Wine containing 12% alcohol was dealcoholized as previously described (Schramm et al., 1997). Methodology for the separation of wine by ultrafiltration is shown in Figure 1. Dealcoholized red wine (RW) (400 μL) was placed in a microtube marked "RW" and centrifuged for 10 min. All centrifugation was conducted at 8000*g* and 4 °C. The supernatant fraction was collected and placed in a Microcon YM 3000 Da MW cutoff filter tube marked soluble RW components (SRWC). Insoluble large MW phenolic compounds were removed from the bottom of the RW tube and placed in a tube marked "Tannin". The SRWC sample was centrifuged for 270 min. Filtrate containing small MW compounds was emptied into a tube marked "SMW". To ensure the passage of wine components with a MW of <3000 into the filtrate fraction, retentate was washed twice with 200 μL of deionized water and centrifuged for 210 min after each wash. Following each centrifugation, filtrate was added to the SMW tube. The filter was removed from the microfuge tube, and 225 μL of deionized water was added to the retentate and mixed with a vortex mixer for 30 s. The rinse was added to the tannin sample. Next, the filter was soaked for 4 h in 250 μL of deionized water at 4 °C. After 5 min of mixing on a vortex mixer, the wash was added to the tannin sample. Finally, the filter was covered with 250 μL of deionized water and brushed gently with a rubber spatula to remove adherent phenolic compounds. Washes were added to the tannin sample. Following determination of the necessary centrifugation and wash cycles, the method was conducted on three samples in parallel.

Final volumes of SMW and RW samples were between 720 and 740 μL; final volumes of tannin samples were between 735 and 750 μL. Prior to analysis of separation and recovery, RW, SMW, and tannin samples were equalized by dilution to a volume of 750 μL. Sample phenol concentrations were then converted to the amount present in the 400 μL original volume.

HPLC Analysis of Small MW Phenolic Compounds. HPLC was conducted as described previously (Lamuela-Raventos and Waterhouse, 1994). Prior to analysis, samples were filtered through Acrodisc 0.45 μm PTFE syringe tip filters. Analysis was conducted using a Hewlett-Packard (Palo Alto, CA) model 1090 high-performance liquid chromatograph with three low-pressure solvent pumps and a photodiode array UV−visible detector coupled to HPChemstation software. Commercial (+)-catechin, (−)-epicatechin, *p*-coumaric acid, rutin, caffeic acid, gallic acid, and malvidin 3-glucoside were used as external standards. Each compound's concentration

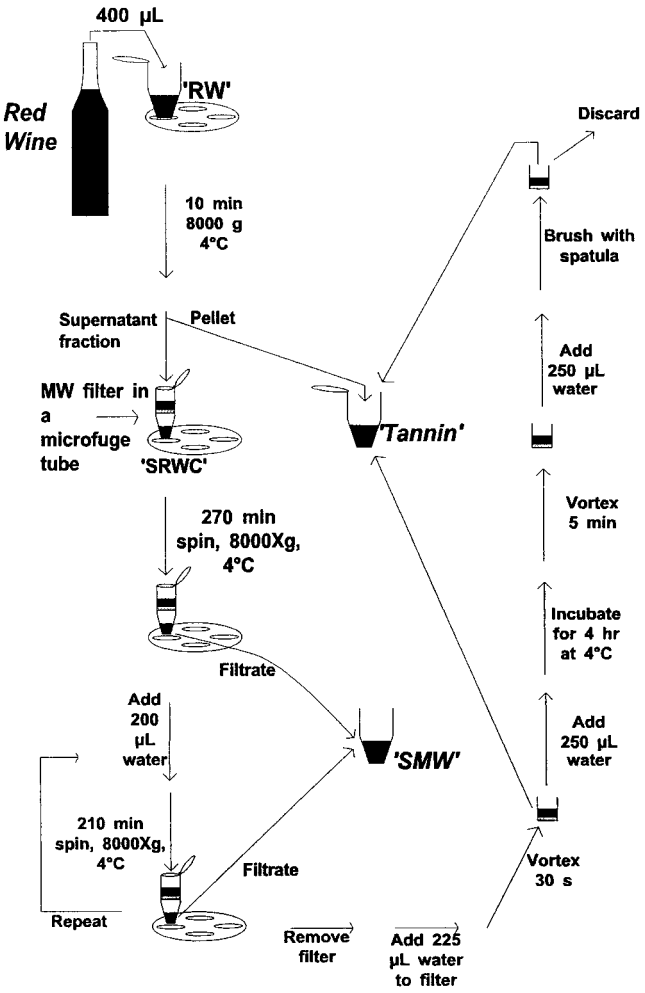

Figure 1. Diagrammatic representation of the methodology used to separate phenolic compounds by MW. All centrifugations were conducted at 8000*g* and 4 °C. RW represents red wine, SRWC represents the soluble RW components, SMW represents the small MW compounds that passed through the 3000 Da filter, and tannin represents compounds that remained in the retentate.

was determined by quantitation at approximately the wavelength of maximum absorbency: 280 nm for flavan-3-ols, 316 nm for hydroxycinnamates, 365 nm for flavonols, and 520 nm for anthocyanins. Compounds that could not be identified were assigned into a class according to their UV−visible spectrum and quantified using the peak area of a commercial standard in that class. Unidentified benzoic acids were quantified and reported in gallic acid equivalents, unidentified hydroxycinnamates in caffeic acid equivalents, unidentified flavan-3-ols in catechin equivalents, unidentified flavonols in rutin equivalents, and unidentified anthocyanins in malvidin 3-glucoside equivalents.

Determination of Phenol Content. Phenol concentration was determined according to the Folin−Ciocalteu method as previously described (Singleton and Rossi, 1965). The total amount of phenolic compounds was calculated from a gallic acid standard curve and reported as gallic acid equivalents (GAEq).

Cell Cultures and Treatments. Cell culture was conducted as previously described (Schramm et al., 1997). BAECs (passage 9) were seeded onto 24-well plates with EMEM containing 20 mmol/L L-glutamine, 10% fetal bovine serum, 100 units of penicillin/mL, and 0.1 mg of streptomycin/mL. Confluent cells were washed with sterile phosphate-buffered saline and incubated in 250 μL of phenol red-free EMEM culture medium, 80% EMEM/20% human plasma, or 100% whole blood. Blood and plasma were collected in heparinized vacutainers. Treatment compounds were added to medium

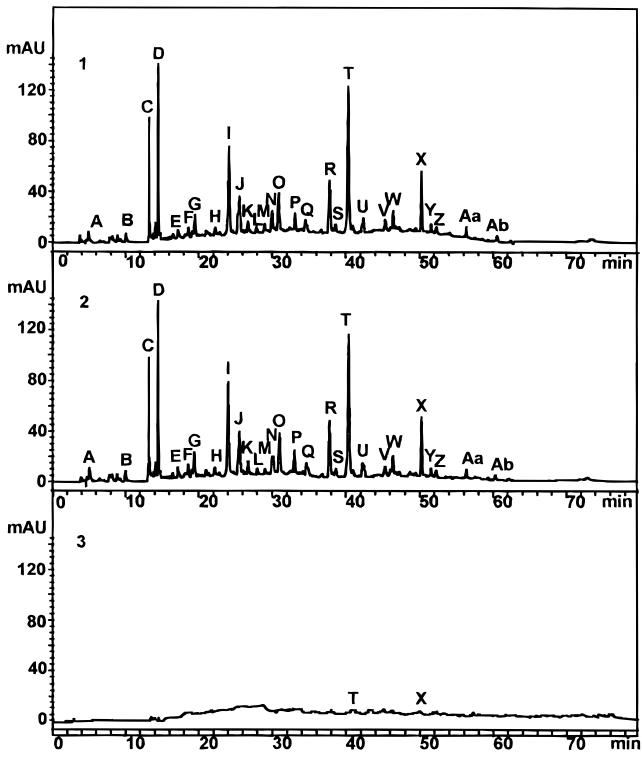

Figure 2. HPLC analysis of small MW phenolic compounds in samples reported as absorbency at 280 nm: (1) RW; (2) SMW; (3) tannin. Integrated peaks were detected with a diode array UV−visible detector coupled to HPChemstation software. mAU is milliamp units.

immediately prior to application of 250 μL of medium to each cell well. Incubation proceeded for 20 or 180 min. Two hundred microliters of medium was removed and frozen at −80 °C until analyzed. Endothelial cell integrity was monitored by trypan blue exclusion as described previously (Bioadjieras et al., 1984). Inhibition of wine-induced eicosanoid release by indomethacin was examined at 20 μmol/L.

Measurement of Thromboxane A_2 (TXA_2) and Prostacyclin (PGI_2). Measurement of eicosanoid production was conducted as described previously (Schramm et al., 1997). PGI_2 metabolite 6-ketoprostaglandin F1-α was measured in cell medium with Cayman enzyme immunoassay # 515211, and TXA_2 metabolite thromboxane B_2 was determined with Cayman immunoassay # 519031. Procedures followed immunoassay principles described previously (Pradelles et al., 1985). In addition to conducting standard curves for these assays as instructed, standard curves were conducted in EMEM, EMEM with 20% human plasma, and 100% human blood.

Statistical Analysis. Experimental data were compared by one-way analysis of variance (ANOVA) testing the hypothesis that means from samples were equal and F test with significance assigned at a level of $P < 0.05$.

RESULTS

Passage of Small Molecular Weight Wine Phenols into Filtrate: Verification and Percent Recovery Determined by HPLC. Compounds detected in RW and SMW samples included four flavonols, five hydroxycinnamic acids, six phenolic acids, six flavan-3-ols, and seven anthocyanins. HPLC detected 468 ± 46 mg/L of small MW compound phenolic material in the RW samples and 444 ± 31 mg/L in SMW samples, making recovery of the small MW components 96.7%. The content of the 28 small MW compounds in the SMW samples did not differ significantly from those in the RW samples (Figure 2; Table 1). Ninety-eight percent of the small MW compounds were removed from tannin samples. Twenty-six of the 28 small MW phenolic compounds were undetectable in all of the tannin samples. Malvidin 3-glucoside was retained at 3.9%, and an unidentified anthocyanin was retained at 4.6%.

Table 1. Phenol Identification by HPLC

code	identity	wine (RW) (mg/L)	SMW fraction (mg/L)	tannin fraction (mg/L)
A	phenolic acid	3.4 ± 0.9	3.6 ± 0.2	nd
B	phenolic acid	2.1 ± 0.4	2.1 ± 0.2	nd
C	phenolic acid	3.4 ± 0.8	5.4 ± 0.4	nd
D	gallic acid	19.7 ± 1.7	18.4 ± 1.5	nd
E	phenolic acid	1.5 ± 0.8	1.5 ± 0.2	nd
F	phenolic acid	2.8 ± 0.4	2.4 ± 0.2	nd
G	hydroxycinnamate	3.2 ± 1.0	2.6 ± 0.2	nd
H	*cis*-caftaric acid	1.3 ± 0.2	0.9 ± 0.0	nd
I	*trans*-caftaric acid	25.7 ± 2.3	24.2 ± 0.1.5	nd
J	catechin	65.3 ± 4.7	60.4 ± 6.2	nd
K	flavan-3-ol	12.2 ± 4.7	11.8 ± 2.9	nd
L	flavan-3-ol	7.5 ± 0.5	7.7 ± 1.7	nd
M	anthocyanin	4.8 ± 0.3	5.2 ± 0.4	nd
N	*cis*-coutaric acid	3.9 ± 0.8	3.8 ± 0.2	nd
O	*trans*-coutaric acid	10.5 ± 1.1	9.8 ± 1.3	nd
P	epicatechin	32.3 ± 1.3	27.4 ± 7.3	nd
Q	anthocyanin	6.2 ± 0.5	6.2 ± 0.7	nd
R	flavan-3-ol	69.8 ± 5.8	67.1 ± 6.2	nd
S	anthocyanin	3.2 ± 0.2	3.4 ± 0.2	nd
T	mavidin 3-glucoside	98.5 ± 8.2	97.9 ± 5.0	6.4 ± 10.9
U	anthocyanin	7.2 ± 1.6	6.2 ± 1.7	nd
V	flavonol	2.3 ± 1.1	2.1 ± 1.3	nd
W	flavonol	16.3 ± 0.6	15.8 ± 0.6	nd
X	anthocyanin	34.0 ± 2.5	31.5 ± 1.9	2.5 ± 4.2
Y	flavonol	9.6 ± 1.1	8.6 ± 0.0.4	nd
Z	flavan-3-ol	18.0 ± 5.3	12.4 ± 5.8	nd
Aa	anthocyanin	2.7 ± 1.4	4.7 ± 0.2	nd
Ab	quercetin	0.9 ± 0.4	1.1 ± 0.6	nd
total		468 ± 46	444 ± 31	8.9 ± 7.5

[a] Fraction codes correspond to fractions in Figure 2. Concentration of compounds was determined as described under Materials and Methods. nd stands for not detectable.

Percent Recovery of Large Molecular Weight Phytochemicals. RW samples had 1904 ± 127 mg/L GAEq. SMW sample groups averaged 791 ± 108 mg/L GAEq, and tannin samples averaged 957 ± 98 mg/L GAEq. Although the 28 SMW compounds analyzed by HPLC passed completely through the filters into the filtrate, 11.9% of the compounds in the retentate was not recovered from the filter surface [100% − [(791 + 957)/1904] × 100%].

Effect of Wine and Wine Constituents on Basal EC Eicosanoid Release. 6-Keto-PGF1-α concentration in control medium was 50 ± 9.8 pg/mL following a 20 min incubation with endothelial cells. TXB_2 concentration in control medium was 492 ± 48 pg/mL following a 20 min incubation with endothelial cells. Cell culture medium incubated with ECs for 20 min and containing 0.6% dealcoholized RW, SMW, or tannin sample had more 6-keto-PGF1-α than control culture medium incubated with ECs for 20 min (Figure 3). SMW samples had 42% of wine's phenols and 44% of wine's efficacy. Dealcoholized white wine did not significantly affect medium 6-keto-PGF1-α concentration. Phenol concentration in white wine, red wine, SMW wine samples, and tannin wine samples correlates with the ability of the samples to alter EC 6-keto-PGF1-α release (Figure 4).

Although an increase in cell medium 6-keto-PGF1-α concentration was observed following the application of RW or either wine fraction to BAECs, only RW and tannin samples induced a significant increase in cell medium TXB_2 (Figure 5). The ratio of 6-keto-PGF1-α/

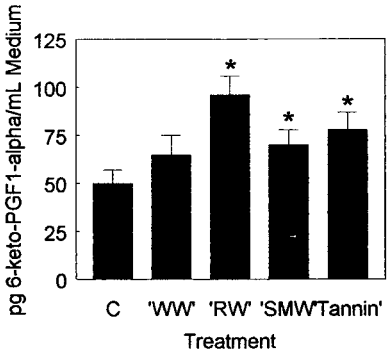

Figure 3. Effect of wine and wine components on basal (C) prostacyclin release from endothelial cells. Culture medium was replaced with new medium before experimentation and supplemented at 0.6% with one of the following as shown: WW, white wine; RW, red wine; SMW, small molecular weight wine components; tannin, large molecular weight wine components. Incubation proceeded for 20 min, and medium was collected and analyzed by immunoassay for 6-keto-PGF1-α. Twelve confluent wells were used for each group. *, significantly different from control at $P < 0.05$.

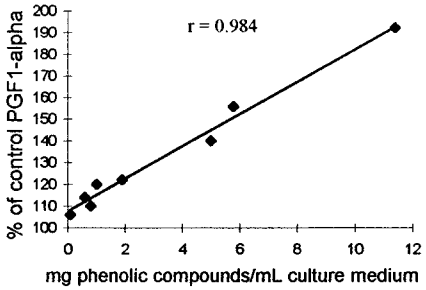

Figure 4. Dependence of wine's efficacy on phenolic compounds. The *x*-axis contains medium phenol content when white wine, red wine, and red wine component groups were added at 0.1 and 0.6%. The *y*-axis contains medium 6-keto-PGF1-α concentration as a percentage of control.

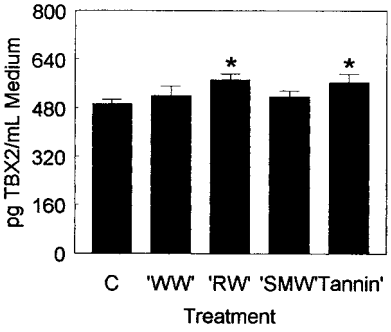

Figure 5. Effect of wine and wine components on basal (C) thromboxane release from endothelial cells. Culture medium was replaced with new medium before experimentation and supplemented at 0.6% with one of the following as shown: WW, white wine; RW, red wine; SMW, small molecular weight wine components; tannin, large molecular weight wine components. Incubation proceeded for 20 min, and medium was collected and analyzed by immunoassay for TXB_2. Twelve confluent wells were used for each group. *, significantly different from control at $P < 0.05$.

TXB_2 was 0.102 in control groups, 0.269 in ATP (200 μmol/L) treatment groups, 0.129 in groups treated with 0.6% white wine, 0.170 in medium from cells incubated with 0.6% RW, 0.135 in medium from cells incubated with 0.6% SMW wine components, and 0.138 in medium from cells incubated with 0.6% tannin wine components.

Effect of Medium Type and Incubation Time on Wine-Induced Basal EC 6-Keto-PGF1-α Release.

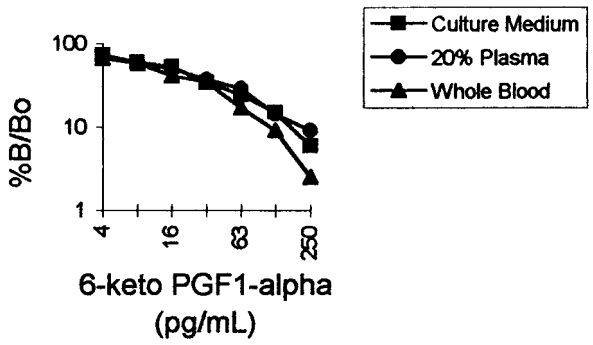

Figure 6. Standard curves used to determine 6-keto-PGF1-α concentration in phenol red-free EMEM culture medium, 80% EMEM/20% human plasma, or 100% whole blood.

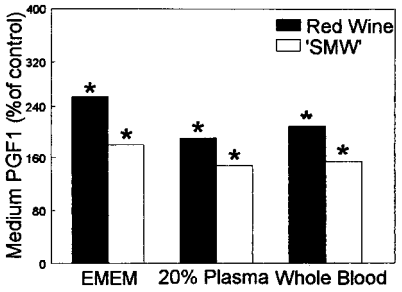

Figure 7. Effect of red wine vs small molecular weight wine components on endothelial cell prostacyclin release during a 20 min incubation. Culture medium was replaced with new medium before experimentation and supplemented at 0.6% with either red wine RW or SMW wine components as shown. Incubation proceeded for 20 min, and medium was collected and analyzed by immunoassay for 6-keto-PGF1. Twelve confluent wells were used for each group. *, significantly different from control at $P < 0.05$.

Figure 6 shows standard curves of 6-keto-PGF1-α in culture medium, 20% plasma/80% culture medium, and whole blood. Addition of dealcoholized RW and SMW samples to medium prior to incubation of culture medium with ECs resulted in increased 6-keto-PGF1-α concentration following a 20 min incubation (Figure 7). In contrast, culture medium with dealcoholized RW and SMW samples had less 6-keto-PGF1-α than culture medium alone following a 180 min incubation with ECs (Figure 8). Twenty percent plasma or 100% whole blood medium with either dealcoholized RW or SMW samples had higher amounts of 6-keto-PGF1-α following incubation for 20 and 180 min periods when compared to said mediums without dealcoholized RW or SMW samples.

Cell Viability and Positive/Negative Controls. Cell viability in all treatment groups was ≥92%. ATP induced a significant increase in cell medium 6-keto-PGF1-α and TXB_2 when added to culture medium incubated for 20 min with ECs. 6-Keto-PGF1-α concentration was increased to 364% of control and TXB_2 concentration to 138% of control. Indomethacin (20 μmol/L) inhibited wine-induced increases in culture medium 6-keto-PGF1-α by 71% and in TXB_2 by 82%.

DISCUSSION

Grapes are important in our society for at least two reasons. First, the world's largest fruit crop is the grape. Grape solids are consumed foremost through wine consumption, second through table grape consumption, and additionally through consumption of raisins, juice, and other products (Mazza, 1995). Second, the periodic consumption of red wine can reduce

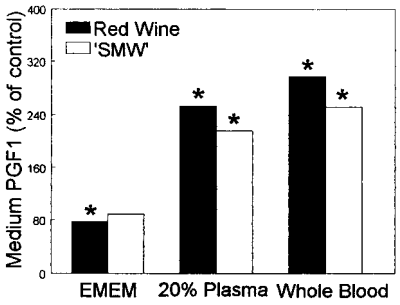

Figure 8. Effect of red wine vs small molecular weight wine components on endothelial cell prostacyclin release between 20 and 180 min of incubation. Culture medium was replaced with new medium before experimentation and supplemented at 0.6% with either red wine RW or SMW wine components as shown. Incubation proceeded for 180 min, and medium was collected at 20 and 180 min and analyzed by immunoassay for 6-keto-PGF1. Medium concentration at 20 min was subtracted from medium concentration at 180 min to give the prostacyclin release between 20 and 180 min of incubation. Twelve confluent wells were used for each group. *, significantly different from control at $P < 0.05$.

one's risk for developing cardiovascular disease (Renaud and de Lorgeril, 1992; Gronbaek et al., 1995). Red wine contains large quantities of phenolic compounds (e.g., tannins, flavonols, anthocyanins, cinnamic acids, and phenolic acids) that may mediate its vasoprotective effects.

The enabling technique of ultrafiltration described here should facilitate research and development in many areas of food science, nutrition, and toxicology because it will allow separation of phytochemicals extracted from plants and plant-derived foods. In this investigation, 96.7% of the small MW phenolic compounds in wine, detectable by HPLC, were recovered in sample filtrate (SMW). If recovery of the 28 small MW phenolic compounds detected represents recovery of all small MW compounds in the wine, then the ultrafiltration procedure described here enabled effective separation and recovery of small MW wine compounds. Without the washing steps (Figure 1), only 90% of the small MW phenols passed through the filter into the filtrate. Of the 791 mg of phenolic compounds/L of SMW sample, flavonoids and low MW phenols accounted for only 444 mg/L, suggesting the presence of large quantities of short-chain phenol polymers in red wine.

Although 2%, by weight, of the small molecular weight phenols present in red wine were retained in tannin samples, this phenolic material was attributed to the retention of only two compounds. Both retained compounds were anthocyanins. Anthocyanins may have interacted with either the cellulose ultrafilter membranes or large MW phenolic compounds more than the other small phenolic compounds did. Alternatively, retention of detectable amounts of the 2 anthocyanins may reflect their relative abundance in wine since they were present at higher concentrations than the other 26 phenolic compounds.

Previously, we determined that wine and dealcoholized wine induce endothelial cell prostacyclin release without affecting nitric oxide production. Shown here is the effect of wine and wine components on EC release of prostacyclin and thromboxane. Data suggest that if only the small MW phytochemicals in wine are absorbed, wine consumption will induce selective alterations in endothelial cell eicosanoid release. Although only the large MW fraction of wine induced EC thromboxane release, both the small and large MW fractions stimulated EC prostacyclin release (Figure 4). We hypothesize that the effects of wine on EC prostacyclin release are mediated through a structure that is common to many phenolic acids, flavonoids, and condensed tannins [e.g., a vicinal (adjacent) hydroxyl group structure]. If so, phenolic phytochemicals from a variety of plant sources should mediate similar vasoprotective effects, and the identification of these sources will give individuals the opportunity to choose food items that offer protection and are palatable.

The ultimate objective of this line of work is the determination of the most effective dietary vehicle for vasoprotective phytochemicals. Before this objective can be met, however, the active components of wine must be identified and mechanisms through which they act thoroughly understood. Results here illustrate that large and small molecular weight wine components can have different effects on cellular biochemistry. In addition, data from experimentation in plasma and whole blood suggest that the efficacy of wine components is greater in vivo than in vitro. This information needs to be considered in the design of experiments with plant extracts and in the interpretation of their results.

ACKNOWLEDGMENT

We thank Heather E. Collins for blood, editing, and support and Cora Dillard for editing.

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Received for review October 21, 1997. Revised manuscript received February 27, 1998. Accepted March 5, 1998. This investigation was supported in part by USDA/BARD Grant IS2260-93RC and NIH Grant 1 U24 AI37627 from the Office of Alternative Medicine, NIH.

JF9709055