Chapter 4

Identification and Assay of Trihydroxystilbenes in Wine and Their Biological Properties

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We have developed a series of methods to assay the isomers and glucosides of resveratrol including GC-MS techniques by direct-injection and derivatisation procedures, and HPLC techniques using normal phase isocratic elution with UV detection and reverse phase gradient elution with diode array detection. Their application to commercial wines has revealed major differences in resveratrol isomer and glucoside concentrations showing specific influences of cultivar and climate. Prospective studies performed during fermentation and subsequently have defined the kinetics of release of resveratrol isomers and demonstrated the varying roles of duration of skin contact, oak ageing and filtration. Resveratrol alters the synthesis and secretion of lipids and lipoproteins by a human liver cell line; blocks human platelet aggregation *in vitro*; and inhibits the synthesis of pro-aggregatory and pro-inflammatory eicosanoids by platelets and neutrophils respectively. This spectrum of effects places it among the most powerful anti-atherosclerotic phenolics yet identified as a constituent of wines.

Current interest in the potential health benefits of the phenolic constituents of wine stimulated an explosion of research activities focused upon these compounds. The most significant results to emerge from this research have been well described by earlier contributors to this Symposium, and no further elaboration is required in the present report. Indeed, an adequate introductory account of the trihydroxystilbene resveratrol and its glucoside polydatin has already been provided. Further information relevant to resveratrol and its biological activities is recorded in two recent review publications by our group which also present the rationale underlying

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our current research program (1, 2). This is targeted towards the following objectives:

1. Identification of the biologically active components of wine that are particularly relevant to the prevention of coronary heart disease (CHD) and cancer.
2. Development of assays to allow their quantitation in wine and other beverages.
3. To survey their concentrations in wines from different regions and countries with a view to defining the enological factors that can lead to their enrichment.
4. Testing the biological potency of these compounds in laboratory experiments involving cultured cells.
5. Performance of clinical studies validating their effectiveness in human subjects.

In this paper, we will review the progress that has been accomplished in the past three years with particular reference to resveratrol and related trihydroxystilbenes. Much of this work has already been published, and other findings have been presented at scientific meetings such as the present, but we will also describe some very recent results which are being reported for the first time.

Assay of Resveratrol

Direct-Injection GC-MS Procedure. At the time we started this work, the reference method for *trans*-resveratrol was that of Siemann and Creasy (3). This was time-consuming and called for considerable technical expertise, since it required multiple solvent extractions followed by two sequential HPLC procedures culminating in differential spectrophotometry after UV-irradiation. Our first approach was based upon direct injection of an ethyl acetate eluate, derived from a 1 mL sample of wine that had been extracted on a C-18 column, into a gas-chromatograph fitted with a highly thermostable capillary column, DB-5 and subsequently DB-17 (4). Detection and quantitation was by mass-spectrometry with selective ion monitoring using the molecular ion at mass 228; ions at mass 229 (carbon isotope) and 227 (M-H) were employed as qualifiers, and pure *trans*-resveratrol as standard, initially prepared by organic synthesis and subsequently obtained commercially. The method was linear over a wide range (0.05 - 10 mg/L) with recovery averaging 100% and precision varying from 5.3 - 7.4% depending on concentration. The same principles permitted the determination of *cis*-resveratrol which generated an identical mass spectrum but eluted several minutes earlier than *trans*-resveratrol (5). Both isomers could be analyzed simultaneously in 1 mL of wine at a rate of 20 min per sample. A disadvantage of the method is the high cost of the column which needs to be replaced after 150-300 assays due to increased baseline and some degradation of peak contours occasioned by the very high temperatures (290-350°C) at which the chromatography is performed, causing more rapid 'bleeding' than occurs when conventional temperatures are used.

Derivatisation GC-MS Procedure. Simultaneously, we developed a GC-MS method in which 1 mL of wine was subjected to solid phase extraction and the dried residue was derivatised with BSTFA before chromatography on a DB-5 HT column (6). The target ion at mass 444 was used for quantitation with ions at mass 445 and

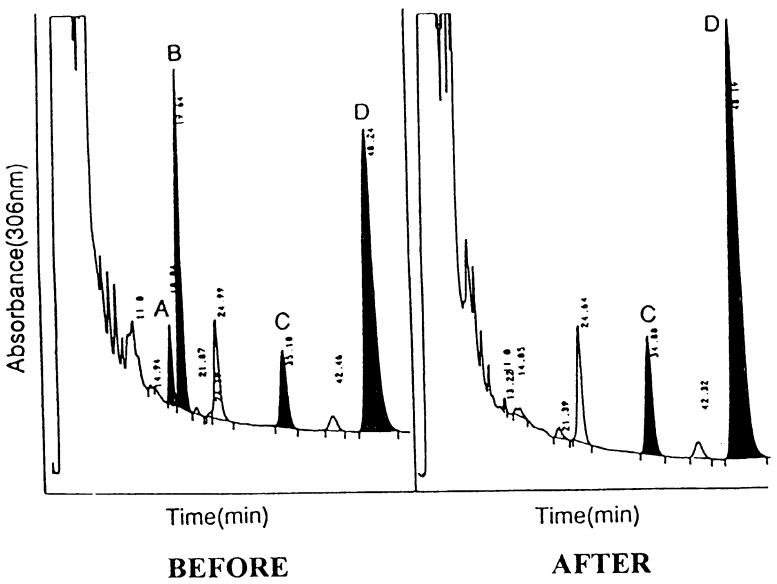

Figure 1. HPLC separation of *cis*-polydatin (A), *trans*-polydatin (B), *cis*-resveratrol (C) and *trans*-resveratrol (D) before and after treatment of a red wine sample for 12 hours at room temperature. Adapted from ref. 8.

443 employed as qualifiers. Identical mass ion spectra were yielded by *trans* and *cis*-resveratrol which eluted approximately 3-min apart. The method was linear from 10-3,000 μg/L, gave recoveries in the range 91-98%, with precision varying from 2.6 - 9.9% depending upon the concentration. Although it is more time-consuming than the direct method, since evaporisation and derivatisation are required, the method is very versatile and has been further elaborated into a multi-residue method capable of simultaneously analyzing up to 15 biologically active phenolics by on-line selective ion monitoring. These include *trans* and *cis*-polydatin, quercetin, catechin, epicatechin, morin, and p- and m- coumaric acid (Soleas *et al*, In Preparation).

Assay of Resveratrol Glucosides

The next phase of our work was devoted to the development of methods to assay the β-glucosides of resveratrol, *cis* and *trans*-polydatin (piceid). These have been shown to share at least one major biological property with the free isomers, namely, inhibition of protein kinase activities (7). Their solubility and susceptibility to enzymatic hydrolysis by intestinal glucosidases suggest that they may have higher bioavailability than the hydrophobic free isomers, and they hold further interest as possible sources of the latter during fermentation.

Normal-Phase HPLC. Our first approach involved normal-phase HPLC with isocratic elution of the polyphenols in 20 μL of wine directly injected onto a LiChrospher 100-CN column by a mobile phase of water-acetonitrile-methanol (8). In this system, the glucosides eluted between 16 and 18 min while the isomers of resveratrol appeared much later: around 35 min for *cis* and 45 min for *trans* (Fig 1). All four were well resolved from each other and from neighbouring peaks and could therefore be quantitated simultaneously in the same sample over a 50-min run. Detection was at 306 nm, corresponding to the peak absorbance of *trans*-resveratrol and although the peak of *cis*-resveratrol was around 280 nm, the former wavelength could be successfully used for all four compounds. The spectra of the glucosides were almost identical to those of the free isomers, with a minor shift to the left approximating 2.5 nm. Thus, pure *trans*-resveratrol could be used to standardize both the free isomer and the glucoside, while *cis*-resveratrol prepared by UV-irradiation of *trans*-resveratrol was the only other standard required. The polydatins were quantitatively converted to the corresponding isomers on treatment with β-glucosidase (Figure 1). GC-MS criteria were also used to identify and validate the 4 peaks. Excellent linearity, recovery and precision were obtained for all constituents.

Reverse-Phase HPLC. More recently, we have developed a reverse-phase HPLC method utilizing diode array detection to measure a wide array of wine phenolics (9). ODS-Hypersil serves as the stationary phase, and gradient elution is accomplished in 40 min with a mobile phase of water-methanol-acetic acid. 20 μL of sample is applied directly through a guard column of LiChrospher 100 RP-18. The isomers of resveratrol and polydatin are well-resolved in this system which

allows their determination with excellent analytical characteristics. Virtually all other wine phenolics for which standards are available can be adequately resolved and quantitated. A powerful feature of the software developed for these assays, which routinely records the absorbance at 5 wavelengths (265, 280, 306, 317 and 369 nm), is the ability to utilize purity checks and match-factor analysis to authenticate each peak and eliminate interference. Although HPLC analyses of wine phenolics with diode array detection have been described earlier, the authors have not stressed the importance of validating the purity and authenticity of each peak. In our experience, 5% of all peaks obtained during the analysis of commercial wines which match the retention times of the relevant standards demonstrate unacceptable spectral properties due to impurities and should not be reported (Goldberg *et al*, In Preparation).

Method Comparison. The availability of several methods for the analysis of trihydroxystilbenes raises the issue of which ought to be adopted for general use. Unfortunately, there is no easy answer to this question. While all methods provide results which demonstrate good inter-method correlation, similar relative differences between wines, and virtually identical conclusions of a general nature, the absolute values obtained reveal systematic variations which are method-dependent and cannot be attributed to the use of different standards or standardization procedures (10). Higher results for *trans*-resveratrol with direct injection GC-MS may be due to fragmentation of *trans*-polydatin which will release the same ion spectrum. The two-hour evaporation and derivatization steps in our second GC-MS procedure may allow some *trans:cis* isomerization, with underestimation of the first and over-estimation of the second. The normal-phase HPLC method with single wavelength monitoring is susceptible to significant peak contamination which defies visual inspection but can be identified by diode array detection and use of appropriate software for purity checks and spectral comparisons. It has already been shown that liquid phase extractions as used in several methods (3,11,12) are associated with poor recovery and losses due to oxidation (13). The choice of method will therefore be governed by considerations of capital and running costs and whether the method selected can also measure other compounds of interest.

Regional Differences in Trihydroxy Stilbene Content

***Trans*-Resveratrol Concentrations.** Our first survey, involving approximately 1,000 wines, was restricted to *trans*-resveratrol (14). There were striking differences based on cultivar and region. The salient findings were the following:

a) Highest concentrations were encountered in wines from Pinot Noir grapes. These included the wines of Burgundy, Oregon and Switzerland whose most prominent red wine, Dole, is vinted from a clone of Pinot Noir. Wines made from this cultivar in other regions of France (Alsace and Loire Valley) or in other countries (California, Australia, Italy, Central Europe) invariably had much higher concentrations than wines made in the same regions by the same producers from different cultivars. There were no apparent temperature-dependent influences upon the *trans*-resveratrol content of Pinot Noir wines.

b) Wines vinted from Cabernet Sauvignon grapes had much lower *trans*-resveratrol concentrations with the exception of red Bordeaux and Canadian wines. Especially low values were found in Cabernet Sauvignon wines from countries noted for warmer drier climates such as California, Australia, South America, South Africa, Italy and Spain, but similar wines from Central Europe and the Midi region of France had moderately high concentrations. Thus, in general, this cultivar generated wines of higher *trans*-resveratrol content when grown under unfavorable climatic conditions.

c) Whether as the predominant cultivar (e.g., St. Emilion), or the exclusive cultivar as in many Italian and New World wines, Merlot generated wines that tended to span a narrower range than those of Cabernet Sauvignon and showed a less profound impact of climatic conditions. In all countries and regions (including the Northern Rhone Valley), those wines vinted from Shiraz grapes had low *trans*-resveratrol concentrations and climate seemed to exercise no effect.

d) Wines from the Southern Rhone Valley, Midi and Provence had moderately high concentrations of *trans*-resveratrol. The predominant cultivars contributing to the cepage in these regions include Carignan, Cinsault, Mourvedre and Grenache which appear to be quite high in trihydroxystilbenes (15). It is important to emphasize, as will become apparent subsequently, that these relatively simple wines do not receive the length of oak ageing accorded the more prestigious wines of the Northern Rhone, or those of the Rioja region of Spain which utilize some of these cultivars as well as the indigenous Tempranillo grape.

e) The lowest *trans*-resveratrol concentrations of any of the commercial wines tested were found in Italian wines. It mattered little whether these were from Piemonte where the Nebbiolo grape predominates, Tuscany which is the home of Sangiovese, or Veneto where a wide range of cultivars are used in red wine production. The red wines of Spain and Portugal also had relatively low *trans*-resveratrol concentrations. The data collected at that time did not define the predominant influence as due to climate, wine-making techniques such as fining and oak-ageing, or the intrinsic genetic properties of the cultivars from which the wines were produced.

f) In confirmation of many reports that white wines are almost invariably low in *trans*-resveratrol, few if any such wines had concentrations within 10% of those found in the lowest range of red wines. Rose wines were almost as low, and so were fortified red wines such as Ports and Madeiras. Among white wines, only the occasional German Riesling or Swiss (e.g. Neuchatel or Fendant) had modest concentrations which overlapped the lower range of concentrations for red wines.

***Cis*-Resveratrol Concentrations.** Once the methods for *cis*-resveratrol assay had been developed (5), we were able to measure the concentrations of both isomers in commercial wines, and we have recently described our findings in considerable depth (Goldberg, D.M.; Ng, E.; Yan, J.; Karumanchiri, A.; Soleas, G.J.; Diamandis, E.P. *J. Wine Res*, in press). The same generalizations that applied to *trans*-resveratrol were largely true of the *cis*-isomer. Thus, Pinot Noir wines (Burgundy,Oregon), those of Switzerland, and red Bordeaux had highest concentrations, with wines from Canada, the Southern Rhone Valley, Beaujolais and the Midi next in line. Wines from warm New World regions and the Mediterranean basin had very low concentrations, with Cabernet Sauvignon wines showing a definite temperature-dependence.

A few unexpected findings are worth emphasizing. Although the *cis*-resveratrol concentrations generally averaged 50-60% those of the *trans*-isomer, there were some notable exceptions. The ratio of *cis:trans* averaged 0.34 in South African and 0.46 in South American wines. By contrast, very high ratios characterized Pinot Noir wines from Burgundy (0.87) and Oregon (0.95) as well as those of Beaujolais (0.92). These trends were reflected in the wines from individual cultivars. For example, among wines from Cabernet Sauvignon, the lowest ratios occurred in those from South Africa (0.33) and South America (0.36). Variations were seen among the different regions of Italy with the ratio averaging 0.35 in Tuscan wines, 0.40 in those from Piemonte, rising to 0.62 in the Veneto and 0.81 in wines grouped together from areas including Umbria, Campagna, Sardegna and Puglia, i.e., further South than the first three regions listed.

Resveratrol Glucoside Concentrations. The most striking and unexpected results were uncovered by our analyses of commercial wines in which their concentrations of resveratrol isomers and glucosides were compared (16). To summarize briefly:

a) Those countries whose wines were especially high in *trans* and *cis*-resveratrol (e.g. Burgundy, Bordeaux, Oregon) had low concentrations of both *trans* and *cis*-polydatin, whereas many wines with low concentrations of the free isomers (South America, South Africa, Italy, Spain and Portugal) had very high concentrations of both glucosides. However, some countries such as Australia and California whose wines were low in free resveratrol isomers also had low concentrations of both glucosides. Canadian wines and those from the Rhone Valley were unique in having very high glucoside concentrations as well as above average concentrations of the free isomers.

b) With most wines, *trans*-polydatin was present in higher concentrations than the *cis*-isomer, but in Canadian wines in particular, and to a lesser extent in red Burgundy, Australian and South American wines, higher concentrations of *cis*-polydatin were present.

c) Fortified wines such as Ports, Madeiras and Sherries that were virtually devoid of free resveratrol had significant concentrations of the glucosides, often exceeding those present in regular table wines.

d) The ratio of total glucoside to total isomer concentrations was absolutely characteristic for several wines. This ratio averaged 4.0 in wines from Spain and Portugal, 3.0 in wines from Italy, and <1 in wines from California, Bordeaux, Burgundy and Australia (Fig 2).

e) The results are consistent with the notion that the overall content of resveratrol isomers and glucosides in wine is a function of genetic factors (e.g., cultivar) and acquired factors (e.g., climate, soil, vinification techniques), and that to a certain extent there is a reciprocal relationship between the glucoside and free isomer content of wines, possibly due to the fact that the latter originate (at least in part) from the former, and this conversion is influenced by viticultural and enological practices characterictic of each wine-producing region.

Influence of Enological Procedures

We have been able to conduct a very detailed investigation of the enological factors that influence the resveratrol isomer concentrations of wines produced in the Niagara region of Southern Ontario (17) and we believe that the conclusions drawn from this study are generally applicable to the production of wines elsewhere. These include the following observations:

a) During fermentation there is negligible isomerization of *trans* to *cis*-resveratrol, presumably due to absence of light and oxygen.

b) The extraction of resveratrol isomers during skin fermentation follows one of two patterns which seem to be characteristic of the cultivar concerned. With Cabernet Franc, Villard Noir and Chambourcin, a rapid increase in *trans*-resveratrol occurred within 24 hours of fermentation reaching a maximum over the next 2 days and followed by a plateau and, in some cases, by a slight fall in concentration (Fig 3a). Simultaneously or one day later, the *cis*-resveratrol concentration started to increase, but much more slowly and throughout the period of observation, over which time neither a plateau or a decrease was observed. The second pattern seen with De Chaunac, Pinot Noir, Cabernet Sauvignon and Merlot (Fig 3b) was characterized by a more gradual and parallel increase in the concentrations of both isomers, accelerating after day 3 of fermentation, reaching a peak around day 6 and declining thereafter.

c) Striking differences in resveratrol content were noted in wines from different vintages. For example, the *cis*-resveratrol concentrations of Pinot Noir wines of the 1992 vintage were one-quarter those of the 1993 vintage; the *trans*-resveratrol concentrations of 1993 Niagara Pinot Noir wines were only about 50% higher than those of the 1992 vintage. In contrast to Pinot Noir wines which were higher in

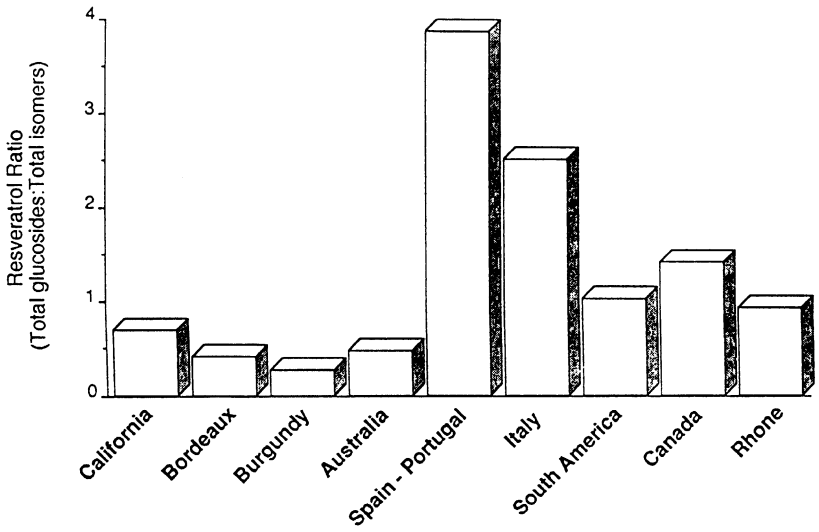

Figure 2. Mean ratio of total resveratrol glucosides to total resveratrol free isomers in red wines from different regions.

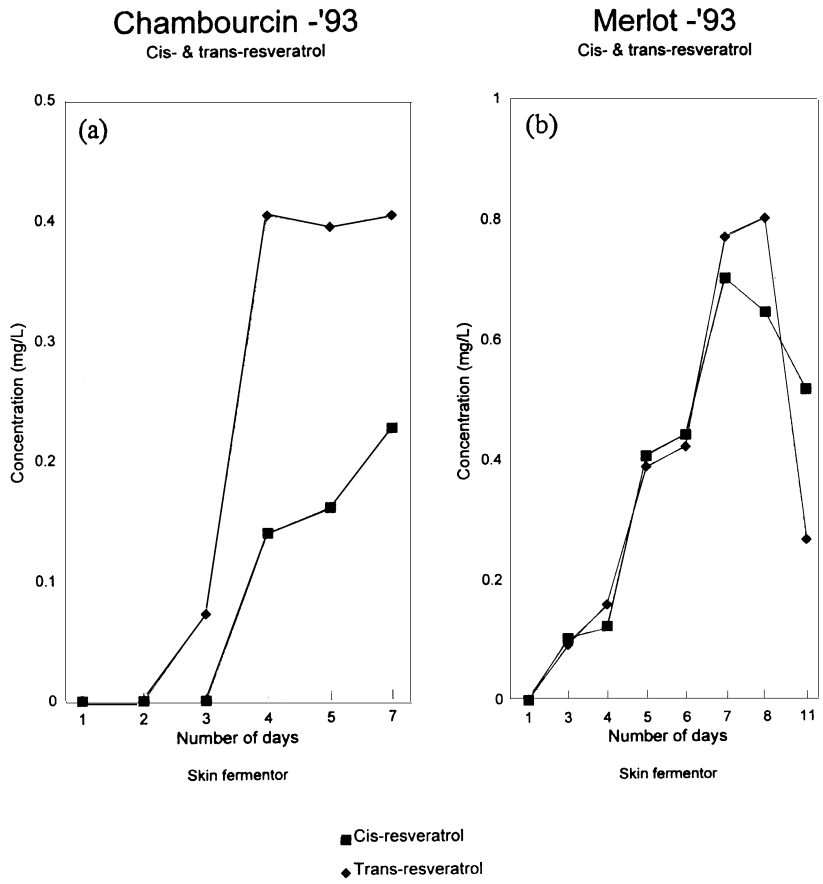

Figure 3. Concentrations of *trans-* and *cis-* resveratrol during skin fermentation of (a) Chambourcin and (b) Merlot grapes. Reproduced with permission from ref. 17. Copyright 1995, Carfax Publishing.

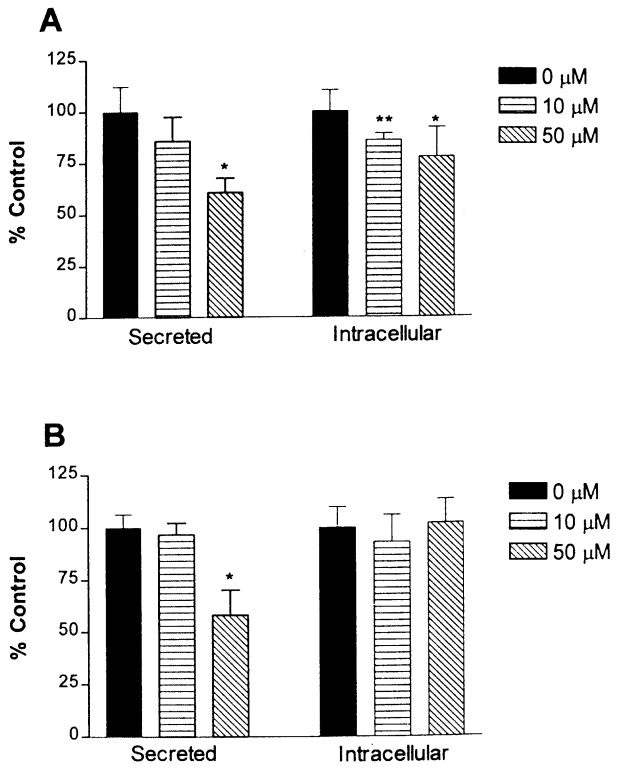

Figure 4. Effect of *trans*-resveratrol on the secretion and intracellular concentrations of cholesteryl esters (A) and triglycerides (B). Confluent Hep G2 cells were incubated for 24 hours at the stated concentrations. Results are mean ± SD of 4 plates assayed in duplicate and expressed as a percentage of control cultures (no resveratrol). *P < 0.05; ** P< 0.01.

1993 than in 1992, Niagara wines from Merlot and Cabernet Sauvignon cultivars were slightly lower in resveratrol free isomer concentrations in 1993.

d) We were unable to detect *cis*-resveratrol in the skins of any wine-producing cultivars. Surprisingly, the *trans*-resveratrol concentrations of white grape skins were in the range of those from most red grapes. Clearly, the very low resveratrol concentrations that we observed in white wine and which have been consistently noted by ourselves and others (3,6,12,14) are due to the absence of skin contact during fermentation. It should also be emphasized that for most grapes, the *trans*-resveratrol concentrations of the finished wines were less than those of the corresponding skins, indicating either incomplete extraction or loss during vinification and processing. Length of skin contact was an important variable. Doubling of the skin fermentation time increased *trans*-resveratrol nearly 3-fold, and *cis*-resveratrol by about 150%.

e) Wide variations occurred in the *trans*-resveratrol content of skins from the same cultivar harvested at the same time but grown in regions of Niagara up to 20 miles apart at different elevations and degrees of exposure.

f) Certain materials used in clarification, but not others, caused reduced concentrations of resveratrol isomers. Losses tended to be greater with *trans* than with *cis*-resveratrol. Bentonite and gelatin had little effect. Silica caused minor losses and charcoal major losses in both isomers. Interestingly, cellulose filter pads retained *trans* but not *cis*-resveratrol to the extent that the two isomers could be separated by replicate passage. Oak ageing led to major reduction in both isomers even though the wines were kept cool and free of light.

In Vitro Studies

Our objectives in these investigations were to examine the effect of resveratrol on: a) lipid and lipoprotein metabolism; b) platelet aggregation; c) eicosanoid metabolism. The first objective was accomplished by utilizing the human hepatocarcinoma cell line, Hep G2, which has preserved the major functions of human liver parenchymal cells (18). The third involved the impact of resveratrol upon thromboxane and hepoxillin synthesis by platelets, and the 5- and 15-lipoxygenase pathways in leukocytes.

Lipid Metabolism. A slight but significant reduction of apolipoprotein B content and secretion takes place when Hep G2 cells are grown in the presence of *trans*-resveratrol in the 1-50 μM concentration range, although a true dose-dependent response was not demonstrated (2). The intracellular content and the rate of secretion of cholesteryl esters as well as the rate of secretion of triglycerides were reduced by resveratrol in a dose-dependent manner (Fig 4), but the intracellular triglyceride content was unaffected. Overall, these changes would tend to diminish the rate of secretion of VLDL which are converted to atherogenic LDL in the circulation, and are therefore consistent with an anti-atherogenic role of resveratrol.

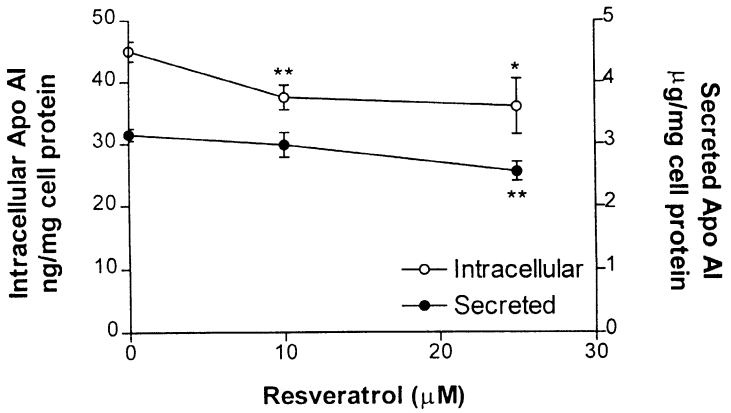

Figure 5. Effect of *trans*-resveratrol on the secretion and intracellular concentration of apolipoprotein AI. Confluent Hep G2 cells were incubated for 24 hours at the stated concentrations. Results are mean ± SD of 4 plates assayed in duplicate * $P < 0.05$; ** $P < 0.01$.

The effects of *trans*-resveratrol on apo-AI metabolism of Hep G2 cells were not entirely reproducible, and seemed to depend upon the duration of exposure, but in general there was reduced intracellular content and secretory rate of this apolipoprotein when the cells were grown in its presence, the results of a typical experiment being exhibited in Fig 5. We were unable to demonstrate a clear dose-response relationship in 4 independent experiments over the range of 5-50 µM, and, paradoxically, greater inhibition was encountered when cells were incubated for 24 h than for 72 h. Since apo-AI is the dominant apolipoprotein of HDL and is essential for initiating reverse cholesterol transport as well as esterification of free cholesterol (19), our results suggest that *trans*-resveratrol may lead to some inhibition of these processes and in this regard it could have deleterious consequences from the standpoint of protection against atherosclerosis.

For this reason, it was instructive to examine the effects of another phenolic constituent of wine, quercetin, upon these processes. In 4 independent experiments, there was a consistent reduction of the intracellular concentration and rate of secretion of apoB which was more pronounced than the comparable effects of *trans*-resveratrol, but which did not show a clear dose-response relationship or time-dependence. Whereas the rates of secretion of cholesteryl esters and triglycerides were decreased by quercetin, their intracellular concentrations were increased (Fig 6). Finally, the synthesis and secretion of apoAI by Hep G2 cells were reduced to a greater extent by this polyphenol than by resveratrol (Fig 7). Whereas *trans*-resveratrol over the concentrations used in these experiments had no effect upon protein synthesis as judged by incorporation of ^{14}C-leucine into TCA-precipitable protein, concentrations of quercetin >20 µM strongly inhibited this process, a finding that makes it difficult to interpret our results. It does appear, however, that the effects of quercetin upon hepatic lipoprotein metabolism are less beneficial than those of resveratrol in protecting against atherosclerosis.

Platelet Aggregation. The significance of this process in the initiation of vascular endothelial damage in atherosclerosis and in precipitating lumenal occlusion leading to acute CHD has been described by earlier contributors to this Symposium. Our experiments were designed to examine the ability of *trans*-resveratrol to inhibit this aggregation and to ascertain its contribution to the overall anti-aggregatory potential of red wine (20).

Using ADP and thrombin as agonists, we demonstrated a dose-dependent inhibition by both *trans*-resveratrol and quercetin of aggregation by human platelets provoked by these agents which was more powerful than that caused by the total phenolics derived from dealcoholized wine on a molar basis . The IC_{50} for resveratrol was a little higher than that for quercetin with both agonists, but the IC_{50} for ethanol was three orders - of - magnitude higher for thrombin-induced aggregation whereas it had no inhibitory effect upon ADP-induced aggregation in the millimolar range. None of the other wine phenolics (catechin and epicatechin) or antioxidants (α-tocopherol, hydroquinone, butylated hydroxytoluene) tested had any effect on this process. The anti-aggregatory properties of red wine are thus in

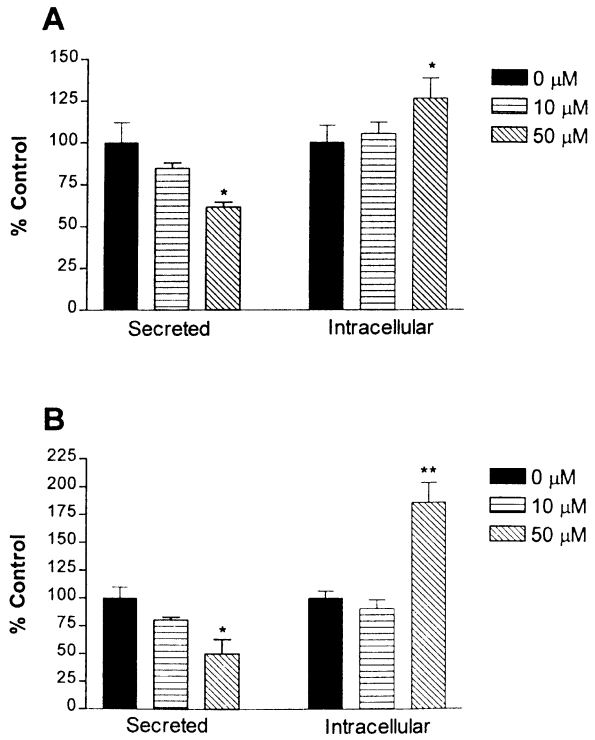

Figure 6. Effect of quercetin on the secretion and intracellular concentrations of cholesteryl esters (A) and triglycerides (B). Details as for Figure 4.

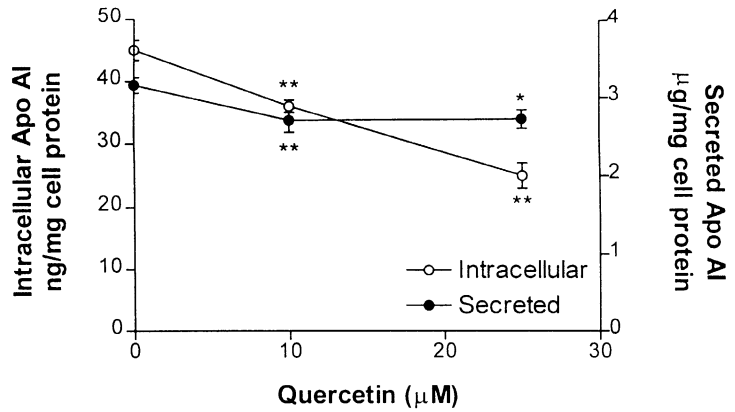

Figure 7. Effect of quercetin on the secretion and intracellular concentration of apolipoprotein AI. Details as for Figure 5.

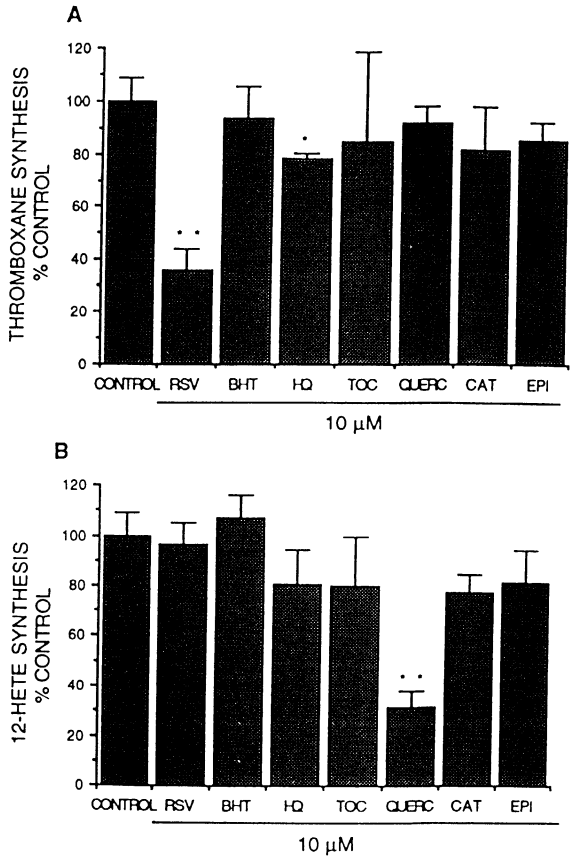

Figure 8. Effect of *trans*-resveratrol, antioxidants and other wine phenolics on the synthesis of thromboxane B_2 (A, top) and 12-HETE (B, bottom) from labelled arachidonate by washed human platelets at a fixed concentration of 10 μmol/L as a percentage of the control (DMSO) in which they were dissolved. Data are mean ± SD of 4 experiments. RSV, *trans*-resveratrol; BHT, butylated hydroxytoluene; HQ, hydroquinone; TOC, alpha-tocopherol; QUERC, quercetin; CAT, catechin; EPI, epicatechin. * $P< 0.05$; ** $P< 0.001$. Reproduced with permission from ref. 20. Copyright 1995, with kind permission from Elsevier Science - NL.

considerable measure due to these two polyphenols. Varying their content in red wine or in a de-alcoholized extracts of red wine caused marked changes in anti-aggregatory potential suggesting that the other phenolics had little or no effect, or were present in grossly sub-optimal concentrations. Indeed, applying information obtained from dose-response curves, we could compute the anti-aggregatory effect of a particular wine to approximate that expected from the sum of its concentrations of resveratrol and quercetin.

Eicosanoid Metabolism

Platelets. The effects of *trans*-resveratrol and quercetin were tested on two pathways of eicosanoid synthesis from arachidonic acid. The first was the cyclo-oxygenase pathway leading to the production of thromboxane A_2, a powerful pro-aggregatory eicosanoid which is synthesized by stimulated platelets and plays a crucial role in the propagation of aggregation once this process is initiated. Because of its very short half-life this component cannot be assayed with meaningful results, but the overall activity of the pathway and its rate of synthesis can be assessed by measuring the production from labeled arachidonate of thromboxane B_2 and HHT, two stable products of the pathway dowstream from thromboxane A_2. By these criteria, *trans*-resveratrol exercised a profound inhibitory effect upon thromboxane A_2 production, approximating 60% at a concentration of 10 μM (Fig 8a). Neither quercetin or any of the other wine phenolics or antioxidants tested had any effect at this concentration, with the exception of hydroquinone which caused a slight inhibition of 15-20%.

The other major eicosanoids produced from arachidonic acid by human platelets include the hepoxillins which are mediators of calcium mobilization, vascular permeability and neutrophil activation (21). This pathway involves the enzyme 12-lipoxygenase and one of its active and stable products is 12-HETE which has been postulated to be pro-atherogenic by virtue of impairing endothelial function and prostacyclin production (22). At a concentration of 10 μM, quercetin reduced the formation of 12-HETE by 70% (Fig 8b) but none of the other compounds tested were effective at this concentration, although at higher concentrations *trans*-resveratrol caused moderate inhibition.

Neutrophil Leukotriene Production. The production of leukotrienes by neutrophils through the lipoxygenase pathway represents the most important aspect of arachidonic acid metabolism in these cells (23). These compounds are powerful mediators of inflammatory reactions and are likely to play a role in at least some of the cellular processes that lead to the development of atherosclerosis (24,25). The two principal lipoxygenase enzymes in neutrophils are those of the 5- and 15- series, and their intracellular activity can be assessed by measuring the production from arachidonic acid of their stable products 5-HETE and 15-HETE, respectively.

In preliminary experiments, neither catechin, epicatechin, or the various antioxidants tested (hydroquinone, butylated hydroxytoluene or α-tocopherol)

altered the rates of production of 5-HETE or 15-HETE from arachidonic acid by human neutrophil homogenates. On the other hand the synthesis of both products was profoundly inhibited by both resveratrol and quercetin. Dose-response studies provided IC_{50} estimates of 22.4 μM (resveratrol) and 2.8 μM (quercetin) for inhibition of 5-HETE synthesis, and 8.7 μM (resveratrol) and 0.75 μM (quercetin) for inhibition of 15-HETE synthesis. These effects must be independent of free-radical scavenging activity since they do not occur in the presence of more powerful anti-oxidants than resveratrol, such as catechin and epicatechin.

Conclusion

Taking into account its actions on apolipoprotein B and lipid secretion by liver cells, its inhibition of platelet aggregation and of the synthesis of thromboxane A_2 and leukotrienes as delineated by the studies reported herein, as well as its moderately high antioxidant activity as reported by others (26), resveratrol, together with quercetin, ranks as the most potent wine phenolic as defined by an array of biological properties likely to prevent the development of atherosclerosis.

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