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Effect of *trans*-resveratrol, a natural polyphenolic compound, on human polymorphonuclear leukocyte function

[1,4]Serenella Rotondo, [1,3]Grazyna Rajtar, [1]Stefano Manarini, [1]Antonio Celardo, [2]Domenico Rotilio, [1]Giovanni de Gaetano, [1]Virgilio Evangelista & [1]Chiara Cerletti

Istituto di Ricerche Farmacologiche Mario Negri, [1]'G. Bizzozero' Laboratory of Platelet and Leukocyte Pharmacology, Department of Vascular Medicine and Pharmacology, and [2]'G. Paone' Environmental Health Center, Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro, Italy

1 Polymorphonuclear leukocytes (PMN) may contribute to the pathogenesis of acute coronary heart disease (CHD).

2 Epidemiological and laboratory evidence suggests that red wine, by virtue of its polyphenolic constituents, may be more effective than other alcoholic beverages in reducing the risk of CHD mortality.

3 The aim of the present study was to investigate the effects of *trans*-resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a polyphenol present in most red wines, on functional and biochemical responses of PMN, upon *in vitro* activation.

4 *trans*-Resveratrol exerted a strong inhibitory effect on reactive oxygen species produced by PMN stimulated with 1 μM formyl methionyl leucyl phenylalamine (fMLP) (IC_{50} 1.3 ± 0.13 μM, mean $\pm$ s.e.mean), as evaluated by luminol-amplified chemiluminescence.

5 *trans*-Resveratrol prevented the release of elastase and β-glucuronidase by PMN stimulated with the receptor agonists fMLP (1 μM, IC_{50} 18.4 ± 1.8 and 31 ± 1.8 μM), and C5a (0.1 μM, IC_{50} 41.6 ± 3.5 and 42 ± 8.3 μM), and also inhibited elastase and β-glucuronidase secretion (IC_{50} 37.7 ± 7 and 25.4 ± 2.2 μM) and production of 5-lipoxygenase metabolites leukotriene B_4 (LTB_4), 6-*trans*-LTB_4 and 12-*trans*-epi-LTB_4 (IC_{50} 48 ± 7 μM) by PMN stimulated with the calcium ionophore A23187 (5 μM).

6 *trans*-Resveratrol significantly reduced the expression and activation of the β_2 integrin MAC-1 on PMN surface following stimulation, as revealed by FACS analysis of the binding of an anti-MAC-1 monoclonal antibody (MoAb) and of the CBRM1/5 MoAb, recognizing an activation-dependent epitope on MAC-1. Consistently, PMN homotypic aggregation and formation of mixed cell-conjugates between PMN and thrombin-stimulated fixed platelets in a dynamic system were also prevented by *trans*-resveratrol.

7 These results, indicating that *trans*-resveratrol interferes with the release of inflammatory mediators by activated PMN and down-regulates adhesion-dependent thrombogenic PMN functions, may provide some biological plausibility to the protective effect of red wine consumption against CHD.

Keywords: Coronary heart disease; polymorphonuclear leukocytes; toxic oxygen species; proteolytic enzymes; leukotriene B_4; β_2 integrins; MAC-1; cell adhesion; red wine polyphenols; *trans*-resveratrol

Introduction

Considerable evidence implicates polymorphonuclear leukocytes (PMN) in different steps of the natural history of the coronary heart disease (CHD). Epidemiological studies in man have shown that high PMN count was associated with the risk of acute myocardial infarction (Ernst *et al*., 1987) and its recurrence (Lowe *et al*., 1985). Further studies showed increased *ex vivo* functional responsiveness as well as *in vivo* neutrophil activation in different clinical manifestations of ischaemic heart disease and suggested an active role for these cells in the progression of vascular occlusion (Mehta *et al*., 1989; Mazzone *et al*., 1993). In animal models of myocardial infarction, PMN depletion, pharmacological suppression of PMN activation as well as inhibition of PMN-endothelial cell adhesion, reduced the extent of acute tissue injury and mortality following ischaemia and reperfusion (Romson *et al*., 1983; Mullane *et al*., 1984; Simpson *et al*., 1988).

Biological plausibility for the possible role of PMN in the acute manifestations of ischaemic heart disease has been provided by several studies *in vitro*. Particularly, activated PMN produce and release reactive oxygen species, inflammatory leukotrienes and proteolytic lysosomal enzymes, which can directly induce vascular damage and influence the activation state of platelets and endothelial cells (Harlan, 1987; Cerletti *et al*., 1995).

Epidemiological studies have shown some benefit of moderate alcohol consumption on reducing CHD (Renaud & De Lorgeril, 1992) and recent findings have suggested that the benefit of consuming alcohol in the form of red wine might be higher than that associated with other alcoholic beverages (Grønbaek *et al*., 1995). Although these studies are not conclusive, as shown by a recent metanalysis of available data (Rimm *et al*., 1996), several laboratory studies (Corvazier & Maclouf, 1985; Seigneur *et al*., 1990; Frankel *et al*., 1993a; Demrow *et al*., 1995; Andriambeloson *et al*., 1997) indicate that compounds found in red wine might render it cardioprotective to an extent over and above that expected from its alcohol content alone (Goldberg *et al*., 1995a).

A number of polyphenols have recently attracted interest as constituents of red wine in this respect. Among the non flavonoid polyphenolic compounds, *trans*-resveratrol (3,4′,5-

[3]On leave of absence from Medical Academy, Lublin, Poland.
[4]Author for correspondence.

trihydroxy-*trans*-stilbene), used for analgesic and therapeutic purposes in oriental folk medicine (Pace-Asciak *et al*., 1995), has been proposed as one of the components in red wine that might confer specific protection against CHD. Preliminary evidence from experimental animals (Kimura *et al*., 1983) and more recent *in vitro* studies on human plasma (Frankel *et al*., 1993b) suggest that its antioxidant activity might also be relevant *in vivo*. Moreover, *in vitro* anti-platelet activity of *trans*-resveratrol has been observed (Chung *et al*., 1992) and subsequently confirmed (Pace-Asciak *et al*., 1995; Rotondo *et al*., 1996).

In this study we have concentrated on the potential of *trans*-resveratrol to moderate the acute manifestations of CHD possibly linked to PMN activation.

Methods

Preparation of platelet-rich plasma (PRP) and PMN

Venous blood was obtained, under informed consensus, from healthy volunteers, who had not received any medication for at least two weeks. After removal of PRP by centrifugation of citrated blood, PMN were isolated by dextran sedimentation followed by Ficoll-Hypaque gradient and hypotonic lysis of erythrocytes, washed and resuspended in HEPES-Tyrode buffer containing 1 mM $CaCl_2$, as described by Evangelista *et al*. (1991). Cellular suspensions contained 95% of PMN and an average of 1 platelet/30 PMN.

Cell viability

Trypan blue exclusion and release of cytoplasmic lactate dehydrogenase (LDH) were monitored to assess PMN viability over a 30 min period of treatment with dimethyl sulphoxide (DMSO; control) or with the highest concentration of *trans*-resveratrol (440 μM). After 30 min, trypan blue exclusion was 96.3 ± 2 and 96 ± 1% (mean $\pm$ s.e.mean, $n = 3$) in control and in *trans*-resveratrol-treated cells, respectively. LDH release, expressed as percentage of total activity measured in supernatant from cells lysed with Triton X-100 (0.1%), was 9.1 ± 1.5% in control *vs* 7.0 ± 1.3% in *trans*-resveratrol-treated cells (mean $\pm$ s.e.mean, $n = 4$).

Measurement of reactive oxygen species

Preliminary time course experiments, over a 30 min period, showed that the effects of *trans*-resveratrol were maximal after 2 min of preincubation with PMN suspension at 37°C (not shown). Two minutes of preincubation have been therefore used throughout the study.

Total reactive oxygen species were determined by chemiluminescence (CL) assay in the lumiaggregometer (Platelet Ionized Calcium Aggregometer, PICA, ChronoLog, Mascia Brunelli) (Bazzoni *et al*., 1991). CL was measured as peak height in cm and results presented as % of values obtained for PMN stimulated in the presence of DMSO (% of control).

Superoxide anion production was determined by measuring the reduction of ferric cytochrome c. Values, calculated as nmol of ferric cytochrome c reduced by 1×10^6 PMN in 40 min of stimulation, are presented as % of control (Bazzoni *et al*., 1991). At concentrations of *trans*-resveratrol higher than 44 μM, an increase in reduced ferric cytochrome c was observed in both unstimulated and stimulated PMN. This increase was due to non-specific ferric cytochrome c reduction by *trans*-resveratrol, since it was not inhibited by superoxide dismutase (SOD, 50 u ml^{-1}) and catalase (200 u ml^{-1}). This was confirmed by incubating *trans*-resveratrol with cytochrome c in a cell-free system. Concentration-dependent reduction of ferric cytochrome c by *trans*-resveratrol, not inhibited by SOD and catalase, was observed also in this condition (not shown). The maximal concentration of *trans*-resveratrol used in this assay was therefore 44 μM.

Measurement of enzyme release

β-Glucuronidase and elastase were assayed in supernatants from activated PMN (see below), with phenolphthalein glucuronate and N-succinyl-Ala-Ala-Val-p-nitroanilide, respectively, as substrates. Elastase was calculated in nmol l^{-1} and β-glucuronidase as μg ml^{-1} of substrate cleaved; results are presented as % of control (Evangelista *et al*., 1991).

Measurement of 5-lipoxygenase metabolites

5-Lipoxygenase metabolites were measured by high performance liquid chromatography (h.p.l.c.; Celardo *et al*., 1994). Briefly, 1.25×10^7 PMN in 500 μl volume were preincubated 2 min with DMSO or *trans*-resveratrol in the presence of 2.5 μg ml^{-1} cytochalasin B and stimulated for 5 min with 5 μM calcium ionophore A23187. Reaction was stopped by rapidly centrifuging PMN suspension at $7{,}000 \times g$ and supernatant transferred in 8 volumes of ice-cold ethyl acetate. Prostaglandin B_2 (PGB_2) was added as internal standard. The extracts were reconstituted in the mobile phase (methanol:0.1% acetic acid in deionized water:acetonitrile 58 : 34 : 8, v/v, adjusted to pH 5.6 with NH_4OH) and injected into an automated reverse phase (r.p.)-h.p.l.c. system (System Gold; Beckman Instr., San Ramon, U.S.A.), with a 4 μm Superspher 100 RP18 Lichrocart column (250×4.6 mm i.d., Merck, Darmstadt, Germany). Leukotriene B_4 (LTB_4) and its *trans*-isomers were identified on the basis of retention times and u.v. spectra at 280 nm of authentic leukotriene standards.

PMN aggregation study

Samples of washed PMN (5×10^6 cells ml^{-1}), in the presence of 2.5 μg ml^{-1} cytochalasin B, were preincubated in an Elvi 840 lumiaggregometer (Elvilogos, Milano, Italy) with DMSO or *trans*-resveratrol for 2 min at 37°C under constant stirring at 1,000 r.p.m. and then stimulated. Changes in light transmission were recorded for 3 min and aggregation quantified by measuring peak height in cm. Aliquots of the same PMN suspension were fixed with one volume of 2% paraformaldehyde (PFA) and the single, non-aggregated PMN counted under contrast light microscopy.

The remaining PMN suspension was immediately centrifuged for 2 min at $7{,}000 \times g$ and the supernatant stored at -20°C for measurement of β-glucuronidase and elastase release.

Cytofluorimetric analysis of MAC-1 expression and activation

PMN (5×10^6 cells ml^{-1}), preincubated with *trans*-resveratrol or DMSO, were stimulated for 1 min with 1 μM formyl methionyl leucyl phenylalamine (fMLP); reaction was stopped by adding an equal volume of ice-cold HEPES-Tyrode buffer containing PGI_2 3.3 μM. PMN were incubated for 30 min at room temperature with a fluorescein isothiocyanate (FITC)-

conjugated anti-MAC-1 mouse MoAb or with the CBRM1/5 mouse MoAb, recognizing an activation-dependent epitope on MAC-1 α-chain, followed by a FITC-conjugated anti-mouse IgG. PMN fluorescence was analysed by a FACStar flow cytometer (Becton Dickinson), as previously described (Evangelista *et al*., 1996). Labelling with anti-MAC-1 and CBRM1/5 MoAbs was evaluated as mean fluorescence (MF) intensity in arbitrary units and presented as percentage of the value expressed by resting PMN.

Double colour cytofluorimetric assay of platelet-PMN adhesion

A previously described methodology has been used (Evangelista *et al*., 1996). Briefly, PMN were stained with the vital red fluorescent dye hydroethidine (HE, 20 μg/5×10^7 cells ml^{-1}) for 30 min at 4°C. Platelets were loaded by incubating PRP with 2 μg ml^{-1} of the green fluorescent dye, 2′,7′-bis-(2 carboxyethyl)-5(6)-carboxy-fluorescein (BCECF)-triacetoxymethyl ester for 30 min at 37°C. After two washes, the BCECF-loaded platelets were resuspended in HEPES-Tyrode with 1 mM $CaCl_2$ at 1×10^9 platelets ml^{-1}. Platelets were stimulated with thrombin (0.5 u ml^{-1}) for 2 min at room temperature, fixed with one volume of 2% PFA for 1 h. After fixation, platelets were washed and resuspended in the same medium with 1 mM $CaCl_2$ at 1×10^8 cells ml^{-1}.

HE-loaded PMN (1×10^7 ml^{-1}) were incubated with DMSO or *trans*-resveratrol at 37°C for 2 min in the lumiaggregometer and an equal volume of BCECF-loaded, thrombin-activated fixed platelets (1×10^8 ml^{-1}) was then added. Cell interaction was stopped after 1 min with one volume of 2% PFA and samples kept at 4°C in the dark and analysed by flow cytometry within 1 h.

PMN were identified on the basis of forward and side scatter alone or in combination with the specific red fluorescent marker. Gating on events identified as PMN was performed in order to exclude single platelets. For each experiment, the threshold of adhesion was set on the green fluorescence scale by use of a sample of mixed cells in the presence of 10 mM EGTA. The percentage of PMN showing green fluorescence above the threshold represents the percentage of PMN binding platelets (%[+] PMN). Platelet adhesion was also quantified by evaluating the relative number of platelets bound to one hundred PMN (platelets/100 PMN), as described by Evangelista *et al*. (1996).

Measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

PMN, resuspended in HEPES-Tyrode, Ca^{2+}/Mg^{2+}-free, at 10^7 cells ml^{-1}, were incubated with 1 μM fura-2 acetoxymethyl ester (fura 2-AM) for 25 min at 37°C, washed twice and finally resuspended at 5×10^6 ml^{-1} in HEPES-Tyrode with 1 mM $CaCl_2$. Fura 2-loaded PMN fluorescence was measured in a Perkin Elmer LS-5B fluorescence spectrometer (Perkin-Elmer Corp., Norwalk, NJ). Monochromator settings of 340 nm (excitation) and 500 nm (emission) were used. A 1 ml portion of PMN suspension was preincubated for 2 min under continuous stirring at 37°C, before agonist addition. $[Ca^{2+}]_i$, after stimulation by fMLP 100 nM, was calculated according to the formula (Fabiato & Fabiato, 1979): $[Ca^{2+}]_i = K_d[(F-F_{min})/F_{max}-F)]$, where K_d is the Ca^{2+} binding dissociation constant (224 nM for fura 2) and F the fluorescence recorded at resting conditions. F_{max} was obtained by lysing the cells with 0.1% Triton X100 in the presence of 1 mM Ca^{2+}; F_{min} was determined by exposing the lysed PMN to 10 mM EGTA after the pH had been adjusted to 8.5 with 20 mM Tris base. $[Ca^{2+}]_i$ was expressed in nM.

Tyrosine phosphorylation experiments

PMN (2×10^7 cell ml^{-1}) were incubated with *trans*-resveratrol or DMSO for 2 min before addition of 1 μM fMLP. The reaction was stopped after 1 min by adding an equal volume of 2× reducing Laemmli's buffer (Laemmli, 1970), added with 2 mM sodium orthovanadate, 5 mM EGTA, 5 mM EDTA, 10 mM sodium pyrophosphate, 10 mM iodacetic acid, 1 mM phenylmethylsulphonyl fluoride, 10 mM sodium fluoride, 10 μg ml^{-1} leupeptin and aprotinin, 1 mg ml^{-1} eglin C and trypsin-chymotrypsin inhibitor. Samples were boiled for 7 min and centrifuged for 10 min at $7{,}000\times g$. Aliquots of 100 μl, corresponding to 2.5×10^6 total cell lysate, were loaded into 7.5–12.5% gradient SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose sheets (Towbin *et al*., 1979) and non-specific sites blocked with 1% BSA in Tris-buffered saline for 1 h at room temperature on a horizontal shaker. The nitrocellulose sheets were then incubated with the recombinant horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20 (0.1 μg ml^{-1}, 30 min at 37°C). Detection was performed by chemiluminescence with ECL-kit. Phosphotyrosine bands were visualized by autoradiography. For each experiment basal level of protein tyrosine phosphorylation was assessed on unstimulated PMN in the presence of DMSO. Selected autoradiograms were analysed with a UltroScan XL densitometer (LKB Instr., Sweden), values in arbitrary units corrected for background and expressed as % of control.

Statistical analysis

Data are presented as mean ± s.e.mean, for the indicated number of independently performed experiments. Statistical analysis was done by one way ANOVA-Dunnett's test; a P value of 0.05 or less was considered significant. IC_{50} values (i.e. the concentration of *trans*-resveratrol required to produce 50% of inhibition of maximal response) were calculated by use of a Mac Allfit program.

Materials

trans-Resveratrol (*trans*-3,4′,5-trihydroxystilbene), ferric cytochrome c, prostaglandin E_1 (PGE_1), cytochalasin B, N-2-hydroxyethyl piperazine-N′-2-ethanesulphonic acid (HEPES), ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), ethylene-diamine-tetraacetic acid (EDTA), phenolphthalein, phenolphthalein glucuronic acid, thrombin from human plasma (2,000 NIH units mg^{-1} protein), N-succinyl-Ala-Ala-Val-p-nitroanilide, superoxide dismutase, catalase, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), fura-2 acetoxymethyl ester (fura-2-AM), 2′,7′-bis-(2 carboxyethyl)-5(6)-carboxy-fluorescein triacetoxy methyl ester (BCECF-AM), fluorescein isothiocyanate (FITC)-conjugated antimouse IgG (whole molecule), anti-MAC-1 mouse monoclonal antibody (MoAb), were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.); hydroethidine (HE) was purchased from Molecular Probes Europe (Leiden, The Netherlands); Tween-20, paraformaldehyde (PFA) and Triton X100 were from Merck (Milano, Italy); dextran T500 and Ficoll-Hypaque were from Pharmacia Fine Chemicals (Uppsala, Sweden). The recombinant horseradish peroxidase-conjugated anti-phosphotyrosine MoAb RC20 was from Transduction Laboratories (Exeter, U.K.). Enhanced chemi-Luminescence Western blotting system (ECL-kit) was from

Amersham Life Science (Little Chalfont, Buckinghamshire, U.K.). The mouse MoAb CBRM1/5 against the activation epitope of MAC-1 α-chain was kindly provided by Dr T.A. Springer (The Center for Blood Research, Boston, MA). Reagents for electrophoresis and Western blot analysis were pure grade.

n-Formyl-methionyl-leucyl-phenylalanine (fMLP), the complement fragment 5a (C5a) and the calcium ionophore A23187 (all from Sigma) were dissolved in dimethyl sulphoxide (DMSO), stored at −20°C, and diluted in isotonic saline just before use. Thrombin was dissolved in saline at concentrations of 50 u ml^{-1}, and stored at −20°C until use. *trans*-Resveratrol was dissolved in DMSO just before use. Final concentration of DMSO in samples was always ⩽0.5% (v/v).

Results

Antioxidant activity: effect of trans-*resveratrol on reactive oxygen species produced by stimulated PMN*

The antioxidant effect of *trans*-resveratrol was first investigated on fMLP-induced luminol-dependent chemiluminescence, which is an index of the generation of multiple reactive oxygen species, mainly superoxide anion, hydrogen peroxide and hypochlorous acid. Preincubation of PMN suspension for 2 min with *trans*-resveratrol strongly inhibited the increase of chemiluminescence subsequent to stimulation with 1 μM fMLP, with an IC_{50} of 1.3±0.13 μM (Figure 1a). Superoxide dismutase-inhibitable ferric cytochrome c reduction was used to assess specifically the effect of *trans*-resveratrol on superoxide anion. At 44 μM, *trans*-resveratrol inhibited ferric cytochrome c reduction by fMLP-stimulated superoxide anion to 20±2% of control (Figure 1b).

Effect on granule release

The effect of *trans*-resveratrol on granule secretion was investigated by measuring the release of elastase and β-glucuronidase in supernatants from PMN stimulated for 3 min with 1 μM fMLP (Figure 2), 0.1 μM C5a or 5 μM A23187. *trans*-Resveratrol significantly inhibited enzyme release induced by these agonists in a concentration-dependent manner, with very similar IC50 values (Figure 2, inset).

Effect on 5-lipoxygenase metabolite production

LTB_4, the main active product of 5-lipoxygenase pathway in purified PMN, is synthesized from LTA_4 by the action of intracellular LTA_4 hydrolase; LTA_4 is also released in the extracellular space where it is non-enzymatically converted to the inactive 6-*trans*-LTB_4 and 12-*trans*-epi-LTB_4 isomers (Lewis *et al*., 1990). Total LTA_4 production, i.e. the sum of LTB_4 and its *trans*-isomers, is therefore taken as index of total 5-lipoxygenase activity.

PMN activated by 5 μM A23187 produced 38.6±4.4 ng/10^6 cells (mean±s.e.mean, $n=3$) of LTB_4, 6-*trans*- and 12-*trans*-epi-LTB_4. Production of 5-lipoxygenase metabolites was strongly inhibited by *trans*-resveratrol (IC_{50} 48.2±7 μM) and virtually abolished at 220 μM.

Effect on MAC-1 expression and activation

Stimulation of PMN by fMLP (1 μM) increased the basal level of MAC-1 expression, monitored by the binding of the anti-MAC-1 MoAb and evaluated as mean fluorescence intensity in arbitrary units, to 250±78% (mean±s.e.mean, $n=4$). Preincubation of PMN with increasing concentrations of *trans*-resveratrol resulted in a concentration-dependent inhibition of fMLP-induced MAC-1 expression (Figure 3a). Binding to PMN of MoAb CBRM1/5, which specifically recognizes an activation-dependent epitope on MAC-1 α-chain, increased its basal level to 440±139% (mean±s.e.mean, $n=4$) following fMLP stimulation and was also strongly inhibited by *trans*-resveratrol (Figure 3b).

Effect on PMN homotypic and heterotypic aggregation

Stimulation of PMN suspension with 1 μM fMLP for 3 min, under constant stirring at 1,000 r.p.m. induces formation of homotypic aggregates, monitored as increase in light transmission in a lumiaggregometer, which was completely prevented by the specific anti-MAC-1 α-chain MoAb LPM19c (kindly provided by Dr Karen Pulford, Oxford University, U.K., unpublished observation). The increase of light transmission induced by fMLP was inhibited in a concentration-dependent manner by *trans*-resveratrol with an IC_{50} of 43.8±8.8 μM ($n=5$). PMN aggregation was also evaluated in a more stringent way, by counting single, non-aggregated PMN under

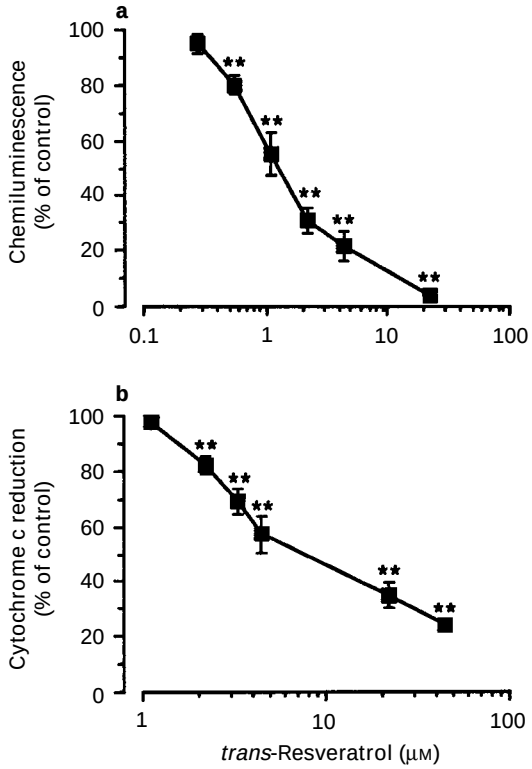

Figure 1 Effect of *trans*-resveratrol on total reactive oxygen species (a) and superoxide anion (b) produced by PMN stimulation with fMLP. (a) PMN, were preincubated with DMSO (control) or *trans*-resveratrol for 2 min at 37°C before stimulation with 1 μM fMLP in the presence of 10 μM luminol and 2.5 μg ml^{-1} cytochalasin B. Changes in the chemiluminescence (CL) were recorded for 3 min. CL was measured as peak height in cm and values presented as % of control (11±2 cm, mean±s.e.mean, $n=5$). (b) PMN, were preincubated with DMSO (control) or *trans*-resveratrol for 2 min at 37°C in the presence of 2.5 μg ml^{-1} cytochalasin B and 20 nmol of ferric cytochrome c before stimulation with 1 μM fMLP for 40 min. Values, calculated as nmol of ferric cytochrome c reduced by 1×10^6 PMN in 40 min of stimulation, are presented as % of control (10.2±1.7 nmol, mean±s.e.mean, $n=7$). $^{**}P<0.01$, significantly different as compared to control, by one way ANOVA-Dunnett's test.

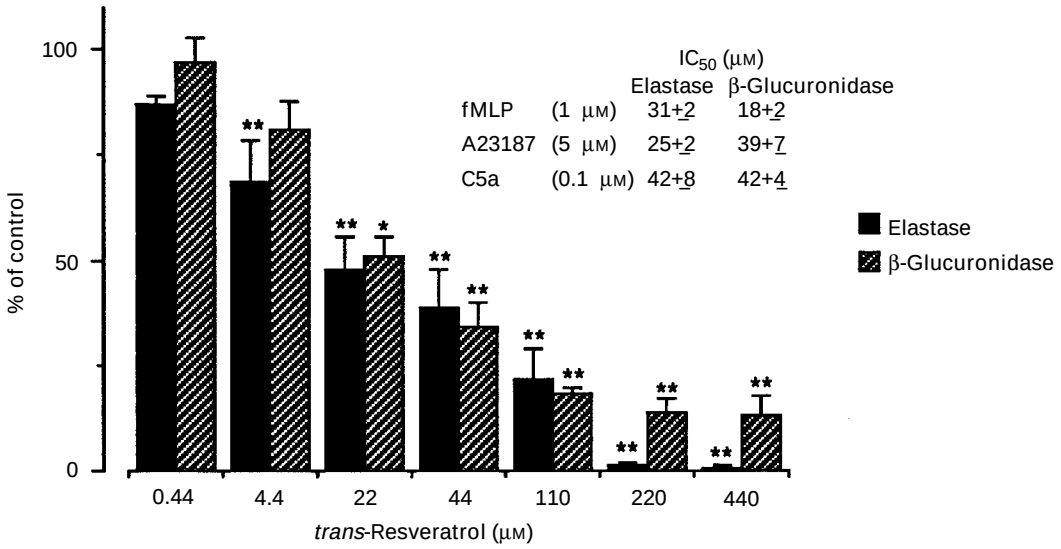

Figure 2 Effect of *trans*-resveratrol on elastase and β-glucuronidase released by PMN stimulated with fMLP. PMN (5×10^6 ml^{-1}) were preincubated with DMSO (control) or *trans*-resveratrol for 2 min at 37°C, in the presence of 2.5 μg ml^{-1} cytochalasin B, before stimulation with 1 μM fMLP; after 3 min samples were centrifuged and supernatants used to measure enzyme release as described under Methods. Results are presented as % of control values (196 ± 18 nmol l^{-1} of elastase; 29 ± 3.5 μg ml^{-1} of β-glucuronidase-substrate cleaved, mean$\pm$s.e.mean, $n=6$ and 3). *$P<0.05$; **$P<0.01$ significantly different as compared to control, by one way ANOVA-Dunnett's test. Inset: IC_{50} (μM, means$\pm$s.e.mean, $n=3-6$) of *trans*-resveratrol for elastase and β-glucuronidase release from PMN stimulated by fMLP, A23187 and C5a.

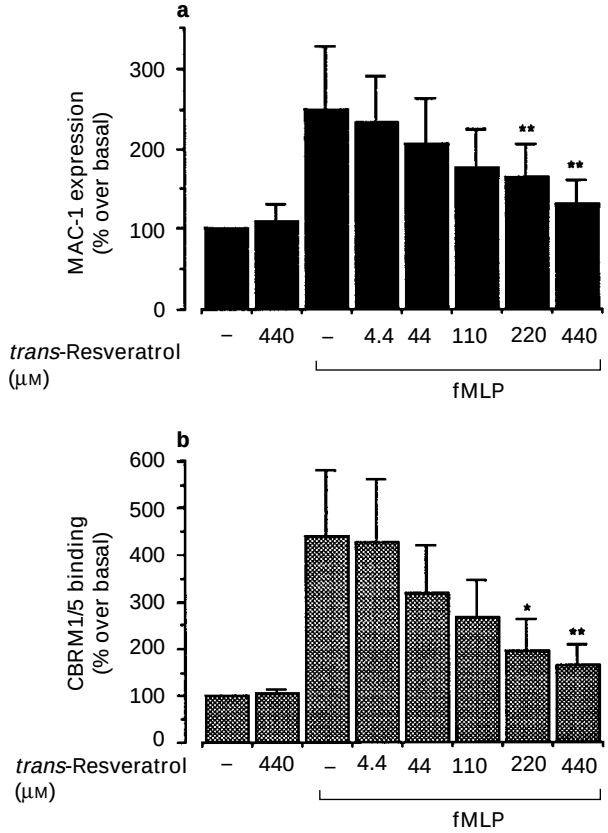

Figure 3 Effect of *trans*-resveratrol on the expression (a) and activation (b) of the β_2 integrin MAC-1 on PMN surface upon stimulation. PMN, preincubated for 2 min at 37°c with DMSO or *trans*-resveratrol were stimulated with 1 μM fMLP for 1 min; binding of the FITC-conjugated anti-MAC-1 mouse MoAb (a) and of the CBRM1/5 MoAb (b), specifically recognizing an activation-dependent epitope on MAC-1, were analysed by FACS, as described under Methods. Results, evaluated as mean fluorescence intensity in arbitrary units, are presented as percentage of increase above the basal values expressed by unstimulated PMN (107 ± 38 and 50 ± 21, respectively, mean$\pm$s.e.mean, $n=4$). *$P<0.05$; **$P<0.01$ significantly different as compared to control, by one way ANOVA-Dunnett's test.

optical microscope, after fixation of aliquots of the same PMN suspension with 2% PFA (1/1, v/v). The number of single PMN dropped from a resting value of $5.1\pm0.6\times10^6$ ml^{-1} to $0.5\pm0.1\times10^6$ ml^{-1} (mean$\pm$s.e.mean, $n=4$) 3 min after stimulation with 1 μM fMLP. Preincubation of PMN with *trans*-resveratrol determined a concentration-dependent rise of the number of single PMN towards resting values with an IC_{50} of 74.5 ± 8.8 μM.

PMN-platelet adhesion was studied by cytofluorimetry. PMN were incubated with different concentrations of *trans*-resveratrol for 2 min before addition of resting or thrombin-stimulated fixed platelets. According to a previous study (Evangelista *et al*., 1996), activated platelets adhere to non-exogenously-stimulated PMN in a time-dependent manner, reaching a maximum at 1 min. At this time, the number of platelets adherent to 100 PMN (platelets/100 PMN) was 331 ± 58 and the percentage of PMN carrying platelets (%[+] PMN) was 64.4 ± 8.4 (mean$\pm$s.e.mean, $n=3$). *trans*-Resveratrol reduced in a concentration-dependent manner the relative number of platelets/100 PMN (IC_{50} 32.4 ± 4.4 μM) as well as the %[+] PMN (IC_{50} 46.0 ± 5.3 μM). In the presence of fMLP, the relative number of platelets/100 PMN rose to 861 ± 225 and the %[+] PMN to 78 ± 8.4 (mean$\pm$s.e.mean, $n=3$), both parameters being inhibited in a concentration-dependent manner by *trans*-resveratrol, with IC_{50} of 118 ± 31 μM and 215 ± 13 μM, respectively (Figure 4a and b).

Effect on signalling events

Cytoplasmic calcium mobilization Ca^{2+} levels in fura-2-loaded PMN rapidly increased from a basal value of 180 ± 16 nM to a maximum of 1215 ± 110 nM (mean$\pm$s.e.mean, $n=3$) following stimulation with 100 nM fMLP and returned towards resting levels within 3 min. *trans*-Resveratrol inhibited fMLP-stimulated intracellular Ca^{2+} rise in a concentration-dependent manner, without affecting basal levels. At the maximal concentration tested (110 μM)

it reduced the peak of cytoplasmic calcium level reached after fMLP stimulation to 21.5±3% of control values (Figure 5).

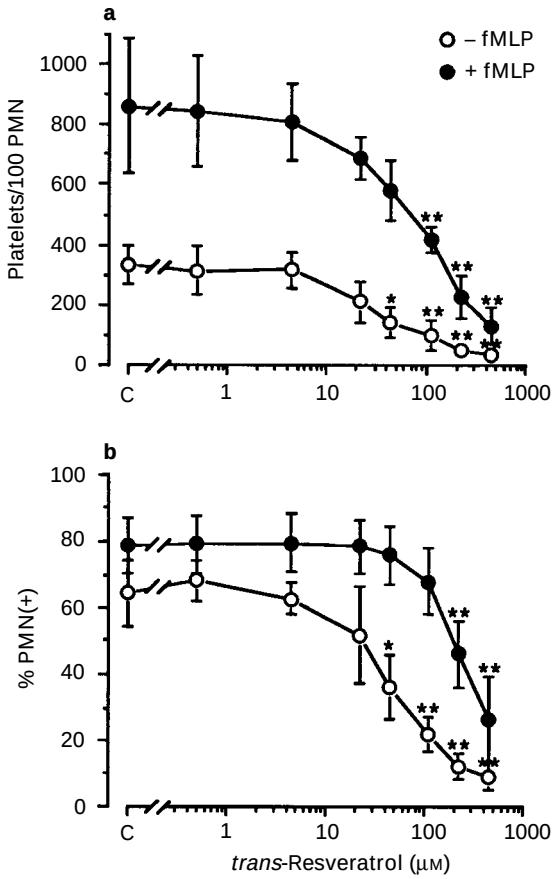

Figure 4 Effect of *trans*-resveratrol on the adhesion between unstimulated or fMLP-stimulated PMN and thrombin-stimulated, fixed platelets. HE-PMN (1×10^7 ml^{-1}), preincubated with DMSO or *trans*-resveratrol for 2 min, were mixed with thrombin-stimulated PFA-fixed BCECF-platelets (1×10^8 ml^{-1}) by stirring at 1000 r.p.m., 37°C, without or with 1 μM fMLP. Cell interaction was stopped after 1 min by adding an equal volume of 2% PFA and samples analysed by FACS. The formation of activated platelet/PMN mixed conjugates was evaluated and presented as (a) relative number of platelets carried by 100 PMN (platelets/100 PMN) and as (b) percentage of PMN carrying the platelet marker (%[+]PMN). $^*P<0.05$; $^{**}P<0.01$ significantly different as compared to control, by one way ANOVA-Dunnett's test. Results are means±s.e.mean, $n=3$.

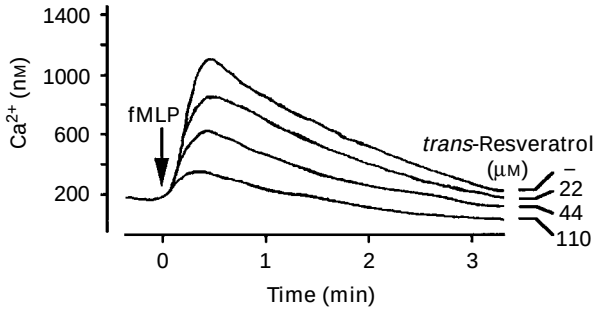

Figure 5 Effect of *trans*-resveratrol on cytoplasmic Ca^{2+} increase stimulated by 100 nM fMLP in fura 2-loaded PMN. Fura 2-loaded PMN (5×10^6 ml^{-1}) were preincubated with DMSO or *trans*-resveratrol for 2 min at 37°C before stimulation with 100 nM fMLP and fluorescence signal recorded for at least 5 min. $[Ca^{2+}]_i$ traces, calculated as described in Methods, are representative of 3 experiments.

Protein tyrosine phosphorylation Following stimulation with 1 μM fMLP, enhancement of the basal level of phosphoproteins in the range between 40 and 80 kDa and appearance of a major phosphoprotein of ~110 kDa (P~110) were observed (Figure 6). The extent of protein phosphorylation was maximal at 1 min after stimulation and declined to resting levels in 5 min (not shown). Preincubation of PMN for 2 min with *trans*-resveratrol caused a concentration-dependent reduction of the fMLP-induced accumulation of several tyrosine-phosphorylated proteins.

In particular, densitometric analysis of selected autoradiograms revealed that inhibition of P~110 phosphorylation was significant ($P<0.05$, $n=5$) at concentrations of *trans*-resveratrol ⩾110 μM.

Discussion

In this study we show that *trans*-resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) is a potent inhibitor of several PMN functions considered to mediate the contribution of these cells to the pathogenesis and evolution of acute CHD (De Servi *et al*., 1991). We found that *trans*-resveratrol exerted a potent antioxidant effect on multiple reactive oxygen species produced by fMLP-stimulated PMN. Toxic oxygen metabolites, which are considered critical mediators of cellular injury by initiating membrane lipid peroxidation, have been shown to induce endothelial cell damage, alteration of vascular permeability (Harlan, 1987) and activation of both endothelial cells (Patel *et al*., 1991) and platelets (Praticò *et al*., 1993). Toxic oxygen metabolites may mediate coronary flow reduction and myocardial dysfunction in experimental models of coronary occlusion and reperfusion (Ambrosio *et al*., 1987).

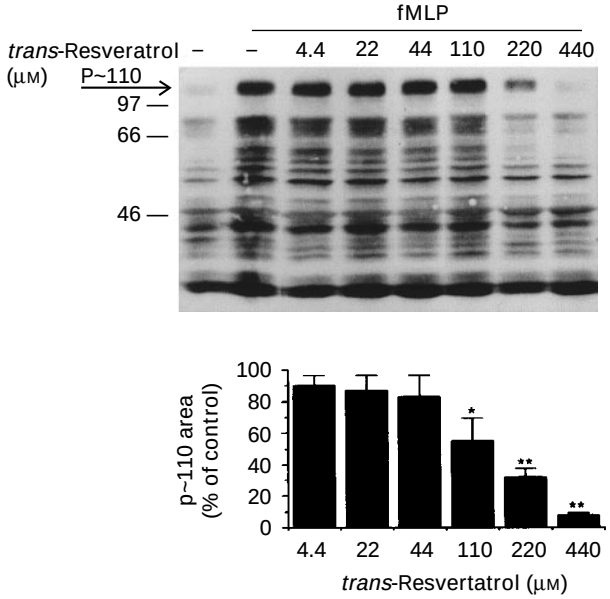

Figure 6 Effect of *trans*-resveratrol on protein tyrosine phosphorylation in fMLP-stimulated PMN. PMN were preincubated with DMSO or the indicated concentrations of *trans*-resveratrol for 2 min at 37°C before stimulation with 1 μM fMLP. After 1 min, one volume of 2× reducing Laemmli sample buffer was added and samples processed for SDS–PAGE and immunoblot as described in Methods. Mobility of standard m.w. is indicated on the left. Representative of 5 experiments. Results of densitometric analysis of P~110 area in selected autoradiograms are presented as % of control (means ±s.e.mean, $n=5$). $^*P<0.05$; $^{**}P<0.01$ significantly different as compared to control, by one way ANOVA-Dunnett's test.

At higher concentrations, *trans*-resveratrol inhibited the release of β-glucuronidase and elastase induced by two receptor agonists, fMLP and C5a, and by the calcium ionophore A23187. Proteolytic enzymes released from neutrophil cytoplasmic granules are responsible for various effects leading to vascular damage. Elastase can cause detachment or lysis of endothelial cells and degradation of subendothelial matrices (Harlan, 1987) and stimulate endothelial cell secretion of growth factors for smooth muscle cells (Totani *et al*., 1994). More recently, a central role of elastase in mediating coronary arteriopathy in rabbits after heterotypic cardiac transplantation (Cowan *et al*., 1996) has been suggested. The increased plasma levels of elastase, found in acute ischaemic heart disease (Mehta *et al*., 1989) and after coronary angioplasty (De Servi *et al*., 1991), strongly support the hypothesis that proteases, released by activated PMN at the level of coronary vessels, may be involved in the progression of vascular disease.

Production of 5-lipoxygenase-derived metabolites, LTB_4 and its isomers 6-*trans*-LTB_4 and 12-*trans*-epi-LTB_4 was also inhibited by *trans*-resveratrol. LTB_4, the main product of neutrophil 5-lipoxygenase, is chemotactic for neutrophils, eosinophils and monocytes, increases PMN adherence to the endothelium and amplifies neutrophil inflammatory responses of aggregation, degranulation and superoxide production (Lewis *et al*., 1990).

Localization of PMN at site of inflammatory reactions or thrombus formation is mediated by adhesion receptors of the β_2 integrin family, identified as CD11a/CD18 (LFA1), CD11b/CD18 (MAC-1) and CD11c/CD18 (p150/95). These molecules are constitutively expressed on PMN surface, and with the exception of LFA1, also stored in secretory granules. On activation with inflammatory stimuli, rapid translocation of these receptors from intracellular pools to the cell surface occurs and, in parallel, conformational changes take place within the molecule to allow competent ligand binding (Arnaout, 1990).

Homotypic PMN aggregation induced by chemotactic peptide stimulation in stirred cell suspensions has been demonstrated to be mediated by MAC-1 (Anderson *et al*., 1986; Arnaout, 1990), while a role has been attributed to LFA1 in shear stress-induced PMN aggregation (Okuyama *et al*., 1996). Homotypic aggregation responses were found to be blocked by MoAbs to the α chain of MAC-1 in experimental models of myocardial reperfusion injury (Simpson *et al*., 1988; Entman *et al*., 1992). PMN and platelets colocalize at sites of vascular damage and atherosclerotic lesions and several *in vitro* studies demonstrated that physical contact between platelets and PMN results in reciprocal functional modulation and metabolic cooperation, possibly playing an important role in inflammation and thrombosis (Cerletti *et al*., 1995). The role of MAC-1 in ensuring stable adhesive interactions, prerequisite for promoting efficient crosstalk between PMN and platelets, has been demonstrated in mixed cell suspensions (Evangelista *et al*., 1996), as well as in PMN transmigrating across adherent platelets (Diacovo *et al*., 1996).

In our system, *trans*-resveratrol significantly inhibited the increase of MAC-1 expression and, more importantly, its activation following fMLP stimulation. As a consequence, PMN homotypic aggregation as well as the formation of PMN/platelet aggregates in mixed cell suspension were also prevented by the compound. *Ex vivo* PMN hyperaggregability (Mehta *et al*., 1989) and increased MAC-1 expression (Mazzone *et al*., 1993), have been observed in clinical manifestations of CHD. Increased PMN/platelet adhesion, associated with increased MAC-1 expression has been found in blood from patients with unstable angina (Ott *et al*., 1996). The extent of PMN/platelet interaction *in vivo*, in patients undergoing coronary angioplasty, correlates with the incidence of reocclusion (Mickelson *et al*., 1996), according to the concept that PMN-platelet interaction might be an additional mechanism by which PMN contribute to the pathogenesis of vascular disease. Prevention of adhesion between PMN and platelets might therefore contribute to limit inflammatory reactions and thrombogenic mechanisms.

Preliminary attempts to elucidate the mechanism(s) by which *trans*-resveratrol affects PMN function are also presented in this study. The observation that *trans*-resveratrol inhibits PMN activation by different receptor agonists, such as fMLP and C5a, suggests that the compound does not act at the level of a specific ligand-receptor interaction. *trans*-Resveratrol inhibited agonist-induced cytoplasmic Ca^{2+} increase, a very early signalling step of the receptor-mediated PMN activation, essential for overall cell function (Sha'afi & Molski, 1990). However, the finding that *trans*-resveratrol inhibited PMN responses also triggered by the calcium ionophore A23187 indicates that it affects signalling steps other than cytoplasmic calcium increase. Tyrosine kinase signalling pathways have been implicated in the activation of selective functional responses of human PMN. In particular, a strong correlation between protein tyrosine phosphorylation and the upregulation of stimulated β_2 integrin surface expression on human PMN has been established (Naccache *et al*., 1994). The observation that *trans*-resveratrol reduced the extent of fMLP-induced tyrosine phosphorylation is therefore of particular interest to understand the mechanism of action of this compound. Interestingly, *trans*-resveratrol, together with a series of hydroxylated *trans*-stilbenes related to the antileukemic natural product piceatannol, has been shown to inhibit the activation of $p56^{lck}$, a cell-lineage specific protein tyrosine kinase which plays an important role in lymphocyte proliferation and immune response (Thakkar *et al*., 1993).

In summary our study offers an experimental contribution to the biological plausibility of recent epidemiological studies, suggesting that acute CHD mortality and morbidity can be decreased by moderate consumption of alcohol in the form of red wine in preference to other alcoholic beverages (Grønbaek *et al*., 1995). The beneficial effect of red wine—or other beverages derived from grapes—might be linked to the biological properties of polyphenols, including *trans*-resveratrol. This compound, found in many plant species and, among food products, particularly in grapes, is thought to be a phytoalexin produced in response to environmental stress or pathogenic attack. Relatively high quantities are therefore found in grapes, possibly as a consequence of the response to fungal infection (Dercks & Creasy, 1989).

The antioxidant as well as the antiplatelet properties of *trans*-resveratrol have already been described (Frankel *et al*., 1993b; Pace-Asciak *et al*., 1995; Rotondo *et al*., 1996). It appears now to reduce significantly another potential mechanism of acute vascular ischaemic disease, such as PMN activation. It is tempting to suggest that down-regulation of PMN function by *trans*-resveratrol might also contribute to the recently observed anti-inflammatory and cancer preventive activities of this intriguing natural compound (Jang *et al*., 1997). Our *in vitro* data are, in contrast, rather difficult to reconcile with the observation that *trans*-resveratrol may accelerate the atherogenic process in some experimental models (Wilson *et al*., 1996).

The concentration of *trans*-resveratrol, as well as that of *cis*-resveratrol, which also has been shown to possess antiplatelet activity (Bertelli *et al*., 1996a), differs widely among wine types, depending on factors such as grape variety and vinification

techniques (Goldberg *et al*., 1995c). *In vivo* studies in man are necessary to establish whether adequate circulating levels of resveratrol are reached following red wine consumption, in order to reproduce the inhibitory effects observed *in vitro*. The levels of resveratrol reached in plasma and in different tissues of experimental animals after short-term as well as prolonged administration of red wine have been recently measured (Bertelli *et al*., 1996b). Even though the amounts of resveratrol were somewhat lower than those required for the inhibitory activities described in this paper, it is conceivable that regular consumption of red wine might increase reservatrol concentrations within the blood circulation and/or relevant tissues, possibly reaching pharmacological relevance. Indirect evidence that *trans*-reservatrol is absorbed after administration of red wine and grapejuice to healthy subjects has been given (Pace-Asciak *et al*., 1996), and preliminary experiments have shown the possibility of analysing the distribution of *trans*-resveratrol in human blood as a prelude to establishing a clinical pharmacological profile for this compound (Goldberg *et al*., 1995b).

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