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Regulation of proliferation and gene expression in cultured human aortic smooth muscle cells by resveratrol and standardized grape extracts

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Abstract

Epidemiologic studies suggest that low to moderate consumption of red wine is inversely associated with the risk of coronary heart disease; the protection is in part attributed to grape-derived polyphenols, notably *trans*-resveratrol, present in red wine. It is not clear whether the cardioprotective effects of resveratrol can be reproduced by standardized grape extracts (SGE). In the present studies, we determined, using cultured human aortic smooth muscle cells (HASMC), growth and specific gene responses to resveratrol and SGE provided by the California Table Grape Commission. Suppression of HASMC proliferation by resveratrol was accompanied by a dose-dependent increase in the expression of tumor suppressor gene p53 and heat shock protein HSP27. Using resveratrol affinity chromatography and biochemical fractionation procedures, we showed by immunoblot analysis that treatment of HASMC with resveratrol increased the expression of quinone reductase I and II, and also altered their subcellular distribution. Growth of HASMC was significantly inhibited by 70% ethanolic SGE; however, gene expression patterns in various cellular compartments elicited in response to SGE were substantially different from those observed in resveratrol-treated cells. Further, SGE also differed from resveratrol in not being able to induce relaxation of rat carotid arterial rings. These results indicate that distinct mechanisms are involved in the regulation of HASMC growth and gene expression by SGE and resveratrol.

Keywords: Resveratrol; Standardized grape extracts; HASMC proliferation; RTP (resveratrol targeting protein); Resveratrol affinity chromatography; Subcellular fractionation

The triphenolic phytochemical resveratrol is present in various plant species and is particularly abundant in red grape skin, red wine, peanuts, and mulberries [1–3]. Since the early 1990s, numerous studies have focused on possible cardiovascular benefits of resveratrol, prompted first by a 1979 report involving an aggregate analysis of data from 17 countries that led to the observation that wine drinking was inversely correlated with the mortality of coronary heart diseases (CHD) [4]. Subsequent *in vivo* studies have shown that the use of red wine, compared to other alcoholic beverages, more effectively protects against CHD [5–9]. These findings have been expanded in the ensuing years, resulting in an intriguing epidemiologic observation known as the French paradox; namely, despite that the population of certain regions of France has the same set of risk factors—marked by increased serum cholesterol, saturated fat intake, blood pressure, and prevalence of cigarette smoking as those of Americans—the reported incidence and mortality rates of CHD are significantly and inexplicably lower [10]. A popular explanation of the French paradox relates to the habitual consumption of red wine at meals in France, which was hypothesized to provide a sustained and possibly bioavailable source of grape-derived polyphenols including resveratrol with cardioprotective

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activities [7,9]. Recent studies have illuminated a number of activities supporting resveratrol as a cardioprotective agent. They include its antioxidant [11–13] and free radical scavenging activities [14,15], suppression of lipid peroxidation [16–19], inhibitory effect on platelet aggregation [20–23], vasorelaxation [24–27], and modulation of various enzymatic activities, e.g., nitric oxide synthase [28,29], lipoxygenase [30], prostaglandin H synthase [30], and quinone reductases [31–34].

In previous studies, resveratrol was shown to suppress proliferation of SMC [35–38]. Oral administration of resveratrol significantly reduced intimal thickening of denuded endothelium in rabbits [39]. Further, the protective effects of resveratrol persisted in rabbits given alcohol-free red wine suggesting that polyphenols present in red wine may suffice to confer the observed cardioprotective effects of resveratrol [40,41]. However, there is a scarcity of data on whether effects of resveratrol are directly comparable to those of SGE. In this report, we have used HASMC to compare the growth and gene regulatory properties of resveratrol with SGE provided by the California Table Grape Commission (CTGC). We found that suppression of HASMC proliferation by resveratrol resulted in a dose-dependent increase in the expression of tumor suppressor gene p53 and HSP27. Analysis of protein elution profiles on resveratrol affinity columns, combined with subcellular biochemical fractionation and immunoblot assays, led to the demonstration that resveratrol increased the expression of quinone reductase I and II, and an alteration in their distribution in various cellular compartments. Although 70% ethanolic SGE also inhibited HASMC growth similar to 25 μM resveratrol, it differed from resveratrol in regard to pattern of gene expression changes in different subcellular compartments. Furthermore, whereas resveratrol dose-dependently induced relaxation of rat carotid arterial rings, a similar exposure to ethanolic extracts of SGE failed to elicit a relaxation response. These results suggest that modulation of HASMC growth by resveratrol involves mechanisms distinct from those elicited by SGE.

Materials and methods

Materials. Human aortic smooth muscle cells and culture media were purchased from Cambrex Corporation (East Rutherford, NJ). Culture media were supplemented with hEGF, insulin, FBS, hFGF-B and, gentamycin/amphotericin-B provided in SingleQuots packages (from Cambrex Corporation). Subcellular Proteome Extraction kit was purchased from Calbiochem (San Diego, CA). Epoxy-activated agarose resin was purchased from Sigma Chemical Co. (St. Louis, MO). Resveratrol was purchased from Sigma Chemical or LKT Laboratories, Inc. (St. Paul, MN).

Resveratrol (12.5 mM) was prepared in dimethyl sulfoxide (DMSO) and kept at −20 °C. SGE was obtained from CTGC (Fresno, CA) and was stored in the laboratory according to the provided instructions. According to CTGC, the SGE was prepared from seeded and seedless variety of red, green, and blue-black California grapes, as freeze-dried powders designed to preserve the integrity of the biologically active compounds found in fresh grapes [42]. The Y2001 preparation used in the present studies contained selected phytochemicals shown below: Total phenol, 6.20 mg/g; flavans, 3.90 mg/g; anthocyanins, 0.77 mg/g; quercetin, 91.0 μg/g; myricetin, 19.0 μg/g; kaempferol, 8.0 μg/g; resveratrol, 4.0 μg/g. Extracts of SGE were prepared by adding 1 g of the dry powder to 10 ml of 70% ethanol, followed by 40 min intermittent mixing at room temperature. The insoluble material was removed by centrifugation and the supernatant was stored at −20 °C in aliquots.

Cell culture and proliferation assay. HASMCs were maintained in complete culture media supplemented with SingleQuots, as recommended by the manufacturer. Cells were maintained in CO_2 incubator and media were changed every 3–4 days. Cultures at >70% confluence were split 1:4 into T-25 flasks containing fresh culture media. Following an overnight incubation, cells were treated with increasing doses of resveratrol or SGE. Cell numbers of control and treated cells were determined at the indicated times by Trypan blue exclusion, as previously described [28,34,43].

Preparation of whole cell lysates. Whole cell extracts were prepared by suspending 10^7 cells in 1 ml of buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. Protease inhibitor cocktail (1%, Sigma) and 1 mM dithiothreitol (DTT) were added to the buffer immediately before use. The mixture was incubated on ice for 20 min with intermittent mixing. The lysate was centrifuged at 14,000 rpm for 10 min, and the supernatant was removed for protein determination and stored in aliquots.

Preparation of subcellular fractions from control and treated HASMC. Cellular fractions were prepared using the Subcellular Proteome extraction kit from Calbiochem. The procedure relied on proprietary extraction buffers and differential centrifugation to stepwisely solubilize four structurally intact subcellular fractions, respectively, F1 (cytosol), F2 (membrane/organelle), F3 (nucleus), and F4 (cytoskeleton). The efficiency of subcellular fractionation was confirmed by gel electrophoresis and immunoblotting of selected marker proteins.

Preparation of resveratrol affinity columns. Resveratrol affinity chromatographic matrix was prepared as described [31]. Control resins consisted of mock-treated beads (identical procedure except that no resveratrol was added).

Fractionation of cytoplasmic extracts on resveratrol affinity columns. Cytoplasmic extracts were prepared from control and treated HASMC by freeze–thaw cycles [31,44,45] and 150 μg of total proteins was fractionated on resveratrol affinity columns by stepwise elution using 0.35 M NaCl, 1 M NaCl, 1 mM ATP, and finally 2 mM resveratrol supplemented buffer. Eluted proteins were concentrated and analyzed by 10% SDS–PAGE, silver staining and Western blot analysis [31]. To determine specificity of binding of distinct proteins to the affinity column, paired extracts were mixed with 1 mM resveratrol prior to binding to the affinity resin. This step effectively competed the binding of some proteins to the affinity column [31].

Western blot analysis. Whole cell extracts and various subcellular fractions containing 10–20 μg of proteins were separated on 10% SDS–PAGE and immunoblot analysis was performed using 1:1000 diluted antibodies purchased from commercial vendors or generated in house (quinone reductase II). Specific immunoreactivity was demonstrated by enhanced chemiluminescence (ECL) or color reaction, as previously described [31,45].

Functional relaxation studies using rat carotid arterial rings. Vascular responses to resveratrol and SGE were assessed as described [46]. In brief, carotid arteries of male Wistar rats were cut into ring segments 2 mm in length and mounted on 40 μm stainless steel wires in the myograph chambers (Danish Myo Technology A/S, Inc., Denmark) for measurement of isometric tension. The vessels were superfused with Krebs buffer solution (118 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 5.6 mM glucose; at 37 °C; gassed with 95% air and 5% CO_2). After an equilibration period of 1 h during which a optimal passive tension of 0.5 g was applied to the rings (as determined from the vascular length–tension relationship), relaxations of pre-contracted (10^{-6} mol/l phenylephrine) vessels to increasing doses of resveratrol (from 10^{-6} to 10^{-4} mol/l) and SGE (2, 10, and 30 μl/ml) were obtained. Vascular responses were normalized to the maximal contraction elicited by phenylephrine.

Results

Effects of resveratrol on growth of cultured HASMC

We first tested the anti-proliferative effects of resveratrol on growth of cultured HASMC. HASMCs split from largely confluent cells were plated, in duplicate or triplicate, in T-25 flasks and treated with increasing concentrations of resveratrol. At 48 h, control and treated cells were trypsinized, and cell number and viability were determined by Trypan blue exclusion using a hemocytometer (Fig. 1A). Growth of HASMC was inhibited by resveratrol in a dose-dependent manner; 50% reduction in proliferation resulted from treatment by 10–25 μM resveratrol (*t*-test, $p < 0.05$), without affecting cell viability (ANOVA, $p > 0.05$). These results are similar to our earlier studies with bovine aortic SMCs showing that resveratrol elicited G1-to-S cell cycle block without inducing apoptosis [35]. To further explore the growth suppressive effects of resveratrol, we analyzed changes in expression of cell cycle regulatory proteins by immunoblot analysis using whole cell extracts prepared from control and 48 h resveratrol-treated HASMC. Representative p53, HSP27, cyclin D1, PCNA, and NFκB responses to resveratrol are depicted in Fig. 1B. Little to no effect of resveratrol was observed on cyclin D1 and PCNA; by contrast, dramatic and dose-dependent increases in the expression of p53 were found, with 10 and 50 μM resveratrol causing a 3- and 6-fold induction (Fig. 1B). Induction of the p53 has been implicated in control of growth of HASMCs *in vitro* [47,48] and *in vivo* [47,49–51]. Moreover, p53 also acts as a transcriptional activator of several genes, including the cell cycle checkpoint regulator p21 [48]. To assess functional significance of induction of p53 by resveratrol, we attempted without success the expression of p21, presumably due to its low level of expression. As an alternative, therefore, we monitored changes in HSP27, another major downstream target of p53 [52] with a presumed role in the attenuation of intimal hyperplasia *in vivo* [53]. HSP27 expression was significantly increased by resveratrol (Fig. 1B). Since HSP27 may interfere with the atherosclerotic inflammatory response by inhibiting NFκB activation [54] and because NFκB activation is also essential for SMC proliferation *in vitro* [55–57], we also determined changes in NFκB in control and treated cells and found a pronounced diminution in NFκB expression in HASMC treated with 50 μM resveratrol (Fig. 1B).

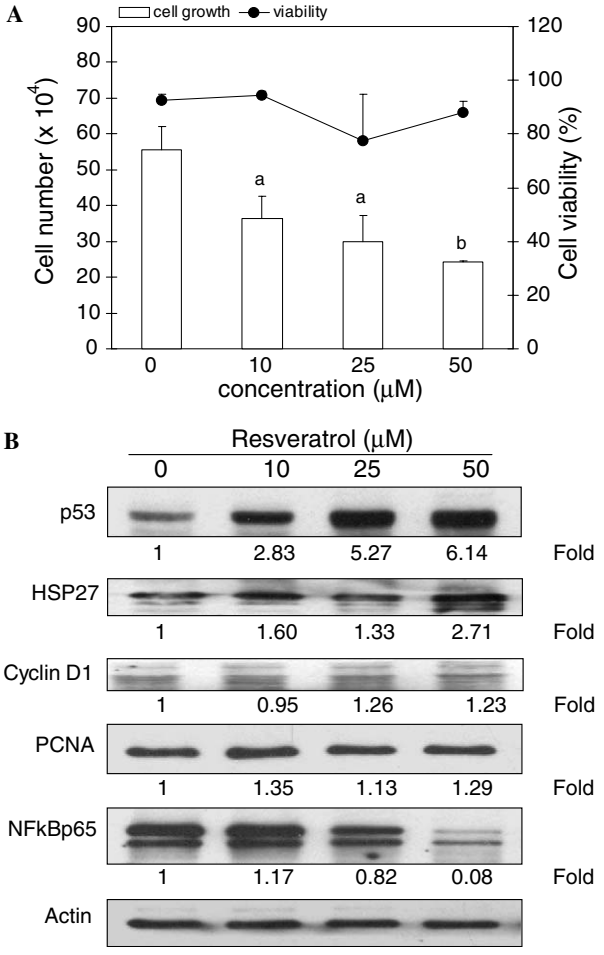

Fig. 1. Growth and cell viability (A) and specific gene expression (B) of HASMC, in response to treatment with varying concentrations of resveratrol, for 48 h. Results in (A) represent means of triplicate experiments ± SD. The significance of cell number decrease was determined by *t*-test: compared to control, results in column a: $p < 0.05$; column b: $p < 0.01$; ANOVA: $p = 0.01$. No significant change in cell viability resulted from treatment by resveratrol (ANOVA: $p > 0.05$). (B) The level of expression of p53, HSP27, cyclin D1, PCNA, and p65 subunit of NFκB was measured by Western blot analyses. The results were normalized using the expression of actin, which was used as a reference for protein loading and efficiency of transfer to nitrocellulose membranes.

Fractionation of cytoplasmic extracts from control and resveratrol-treated HASMC by resveratrol affinity chromatography

To gain additional insights into the anti-mitogenic activities of resveratrol, an affinity chromatography approach was used. The basic tenet of this strategy is that resveratrol might act as a cardioprotective agent by interacting with targets, which we denote resveratrol-targeting proteins (RTPs). Chromatography of control and treated extracts on resveratrol-immobilized solid matrix could therefore provide a panoramic display of RTPs possibly mediating the growth sensitivity and responsiveness of HASMC to resveratrol. Control HASMC cytosolic extracts were fractionated on resveratrol affinity agarose columns, by sequential elution with 0.35 M and 1 M NaCl, followed by 1 mM ATP, and finally 2 mM resveratrol. The variously eluted fractions were concentrated and resolved by 10% SDS–PAGE, followed by silver staining and immunoblot analysis. A typical silver-stained elution profile from control extracts is illustrated in Fig. 2A, showing the following: (1) some differences in silver-stained protein patterns can be observed in 1 M NaCl, 1 mM ATP and resveratrol-eluted fractions, (2) a protein migrating with a molecular weight of 22 kDa, was found in the resveratrol-eluted

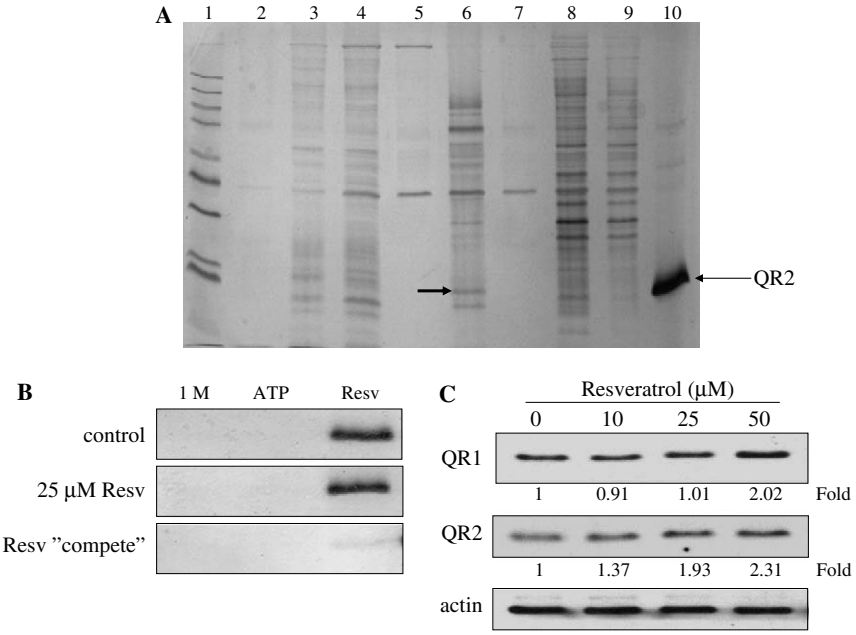

Fig. 2. Analysis of HASMC proteins on resveratrol-immobilized affinity columns. (A) Cytoplasmic extract from untreated HASMC was fractionated on resveratrol affinity columns, as detailed in Materials and methods. Lane 1, molecular weight marker; lane 2, material eluted on seventh wash using the starting buffer; lane 3, 0.35 M KCl wash; lane 4, 1 M NaCl wash; lane 5, 1 mM ATP eluate; lane 6, 2 mM resveratrol eluate; lane 7, 2 mM resveratrol eluate in lysate first incubated with 1 mM resveratrol prior to binding to resveratrol affinity column; lane 8, unfractionated cytoplasmic extract; lane 9, cytoplasmic extract not bound on the affinity column; lane 10, purified recombinant QR2 added as a marker. Fractions were concentrated, separated by SDS–PAGE, and visualized by silver-staining. (B) Western blot analysis of cytoplasmic extract from control and 48 h, 25 μM resveratrol-treated HASMC, with and without prior competition of extracts with 1 mM resveratrol and fractionated on resveratrol affinity columns as described in (A). (C) Western blot analysis of QR1, QR2, and actin from whole cell extracts of control and 48 h, 25 μM resveratrol-treated HASMC.

fraction. Retention of this protein was effectively competed when extracts were first incubated with excess resveratrol prior to fractionation. This protein was identified as quinone reductase II (QR2). When the same approach was used to fractionate 25 μM resveratrol-treated HASMC cytosolic extracts and the fractions were analyzed for the presence of QR2 by Western blot analysis, a small increase in cytosolic QR2 was evident in the fraction eluted using resveratrol (Fig. 2B). To verify induction of QR2 by resveratrol, total cell lysates prepared from 48 h resveratrol-treated cells were also analyzed by immunoblot analysis. Results in Fig. 2C and enzymatic assays of QR2 (data not shown) both showed a substantially greater induction of QR2 by resveratrol, compared to results using the affinity column approach. We also assayed for changes in QR1, a homolog of QR2, in control and resveratrol-treated whole cell extracts. At 50 μM resveratrol, a 2-fold increase in QR1 expression was also observed (Fig. 2C).

Subcellular fractionation of control and resveratrol-treated HASMC extracts

To better understand the disparate smaller increase in QR2 in response to resveratrol as evident by fractionation on resveratrol affinity chromatography, compared to the more robust induction of QR2 in whole cell extract analysis, we tested whether this grape-derived polyphenol might modulate gene expression including QR2 by inducing changes in their subcellular distribution. Control and resveratrol-treated cell extracts were fractionated into cytosol, organelle, nucleus, and cytoskeleton cellular compartments, as described in Materials and methods. Specificity and separation into the different subcellular fractions is monitored by the expression of cytochrome oxidase (OX II) and actin. Aliquots of each fraction were analyzed for expression of QR1, QR2, and several other proteins, notably, nitric oxide synthase (eNOS), ERK, and AIF. Results are illustrated in Fig. 3. Treatment by resveratrol resulted in a significant increase in QR1 and QR2 in F2, concomitant with a decrease in their presence in F1. Treatment by resveratrol also enhanced partitioning of QR2 in F3 and F4, as well eNOS in F4 and AIF in F3 (Fig. 3). In contrast, no change in cellular distribution of ERK was observed. These results suggest that resveratrol may affect HASMC growth by inducing changes in subcellular distribution of proteins.

Effects of ethanolic extracts of SGE on growth of cultured HASMC

To test whether SGE and resveratrol might have overlapping biological activities and cellular mediators, we evaluated the effects of SGE on growth of HASMC. Extracts of SGE (0.05, 0.2, and 1%) were added to the culture media of HASMC. For comparison, cells were also treated with 25 μM resveratrol. Fig. 4 shows that

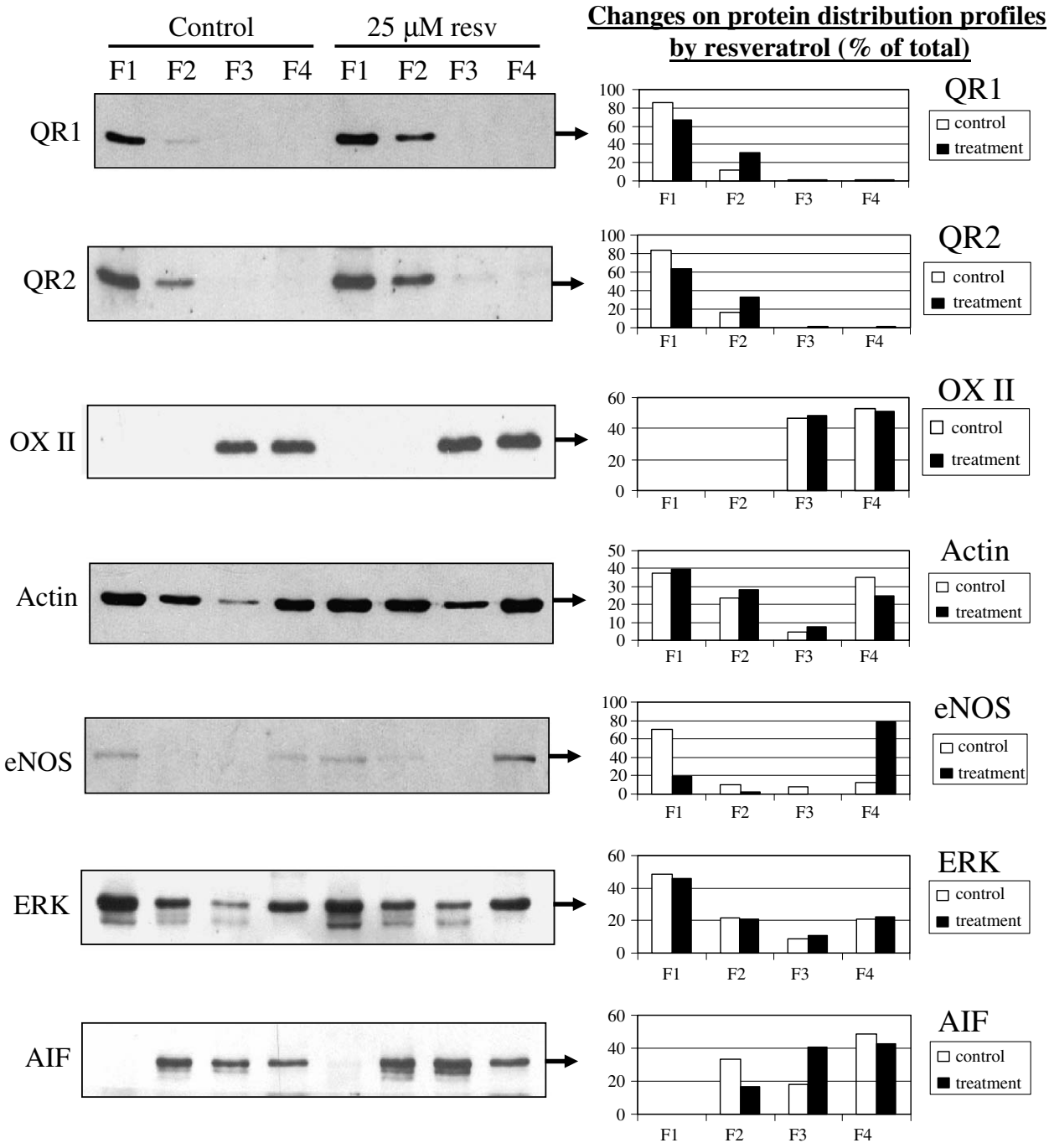

Fig. 3. Subcellular fractionation of extracts from control and 25 μM resveratrol-treated HASMC. Different cellular fractions were obtained as described in Materials and methods and analyzed by Western blot analysis.

proliferation of HASMC at 48 h was inhibited by SGE in a concentration-dependent manner; inhibition by 0.2% SGE was comparable to that of 20–25 μM resveratrol.

Effects of ethanolic extracts of SGE on gene expression patterns in different subcellular fractions of control and treated HASMC extracts

We first used resveratrol affinity chromatography to test whether SGE-treated HASMC elicited the same responses as cells exposed to resveratrol. These experiments confirmed that QR2 was bound to the affinity column but failed to reveal additional insights into cellular effects of SGE. We next explored whether SGE might induce changes in gene expression patterns via alterations in their subcellular distribution. Fig. 5 shows that gene expression patterns in various cellular compartments elicited in response to SGE were substantially different from those observed in resveratrol-treated cells, as demonstrated by AIF, QR1, ERK, and p-ERK. Interestingly, treatment

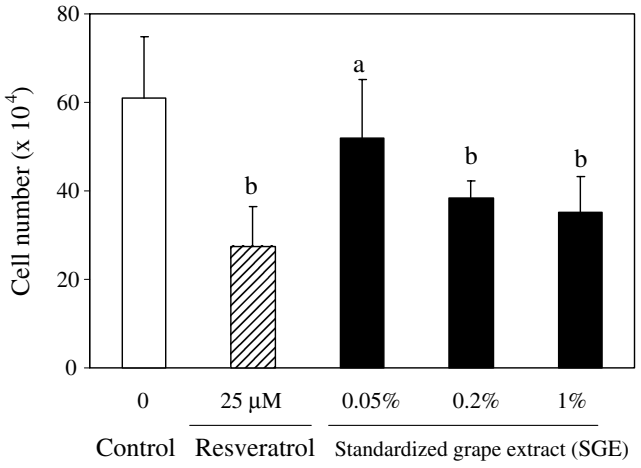

Fig. 4. Inhibition of growth of HASMC by resveratrol and 70% ethanolic extract of SGE. Subconfluent HASMCs were treated for 48 h with the indicated concentration of either agent. Cell proliferation was determined as described in Materials and methods. Results represent means of triplicate experiments ± SD. The significance of inhibition of cell proliferation was determined by *t*-test: compared to control, results in column a: $p > 0.05$; column b: $p < 0.01$; ANOVA: $p = 0.001$. No significant change in cell viability resulted from treatment by resveratrol or SGE (ANOVA: $p > 0.05$).

with SGE also altered the distribution of OX II in F3 and F4 of treated cells.

Effects of ethanolic extracts of SGE on relaxation of rat carotid artery rings

We found that resveratrol ($>10^{-5}$ mol/L) induced relaxation in a dose-dependent manner in rat carotid arteries (Fig. 6A) extending the findings of earlier studies on rat mesenteric arteries [58]. We therefore investigated whether a similar effect can be elicited using SGE. Fig. 6B demonstrates that responses of carotid arteries, in response to addition of increasing amounts of 70% ethanolic extract of SGE, were not significantly different from samples incubated with identical amounts of carrier solvents. Studies performed using aqueous extracts of SGE also yielded negative results (data not shown).

Discussion

The vascular smooth muscle cells (VSMC) play a significant role in the maintenance of vascular tone in the adult blood vessel. Strategically positioned in the medial layer of the artery, VSMCs are usually quiescent under normal conditions; however, they can be induced to undergo proliferation and migration as essential and integral events in the pathological sequelae of CHD. Therefore, agents that restrict VSMC growth may contribute towards a potentially efficacious preventive strategy for CHD. To this end, we have focused on the growth and gene modulatory effects of resveratrol and SGE on HASMC in the context of our overall interest to identify diet-based cardioprotective agents and study their biological mechanisms. Several mechanistic details have emerged from results presented in this report.

Further insights on mechanism of cardioprotection by resveratrol

Resveratrol was found to dose-dependently increase the expression of p53 and HSP27, as inverse correlates of the suppression of HASMC growth (Fig. 1). To obtain information on cellular targets that might mediate the anti-proliferative mechanism of resveratrol, we designed a resveratrol-immobilized affinity column to retain RTPs from control and resveratrol-treated cell extracts. This approach permits, via a single-step concentration, probing of low abundance proteins that might go undetected in analysis using crude extracts and can potentially generate protein profiles distinguishing cells exposed to cardioprotective agents, e.g., resveratrol and SGE, from the untreated controls. Furthermore, with binding to resveratrol as the basis for inclusion in the analysis, there is a greater probability for a functional link to exist between proteins retained and identified with cellular responses to resveratrol. As with our previous studies in prostate cancer cells [31], we found that this biospecific affinity platform selects and captures QR2 as a distinct cellular target of resveratrol. We also observed that induction of QR2 and QR1 by resveratrol was accompanied by a significant shift in their localization to F2 (Figs. 2C and 3). The observed increase in p53, HSP27, and QRs may have several implications regarding cardioprotection by resveratrol. First, QR increases by resveratrol were correlated with the induction of p53 and p53-downstream target HSP27, raising the possibility that QRs, in concert with induction of functional p53, are involved in the suppression of HASMC growth by resveratrol. Conceivably, the QRs might act by binding and stabilizing p53 [59–61]. Second, our observed induction of QR1 (Fig. 2C), a structural analog of QR2, by resveratrol is also significant in that these enzymes reportedly confer protection against oxidative and electrophilic injury and toxicity from menadione [62–64]. Finally, previous studies have shown that HSPs, particularly HSP70, exhibit cardioprotective activities [65–68]; results of the present studies indicate that HSP27 should be added to this list.

Treatment of HASMC by resveratrol also resulted in an alteration in the subcellular distribution of nitric oxide synthase (NOS) and AIF. The significance of these observations remains to be investigated in detail. Several studies have reported that nitric oxide (NO) synthesis is impaired in atherosclerosis [69,70]. Although the primary source of vascular NO is likely to come from endothelial cells, vascular SMCs have been shown to express endothelial NO synthase (eNOS) and produce NO [71,72]. Complex mechanisms, spanning from cell specific expression, co-/post-translational processing, differential cellular compartmentalization, and protein trafficking to plasma

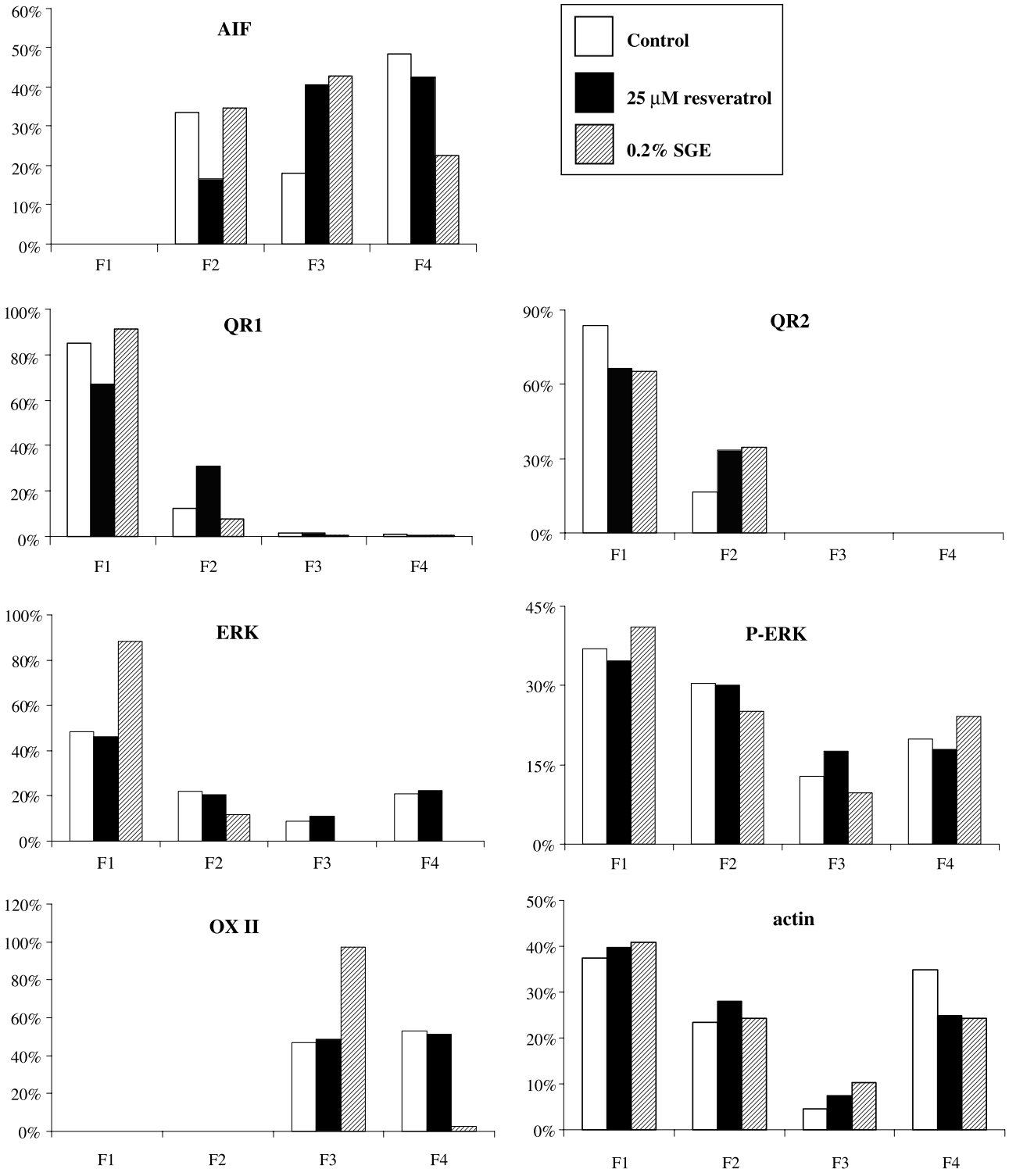

Fig. 5. Subcellular fractionation of extracts from control and resveratrol or 0.2% ethanolic extract SGE-treated HASMC. Different cellular fractions were obtained as described in Materials and methods, and analyzed by Western blot analysis.

membrane caveolae, are involved in eNOS regulation and expression [73]. In line with this overall theme of control of eNOS, our results demonstrate that exposure to cardioprotective cues, such as resveratrol, can induce the cellular redistribution and hence availability in new cellular location of eNOS and bioactive regulatory protein, e.g., AIF.

Distinct mechanisms are involved in HASMC growth and gene control by resveratrol and SGE

Although SGE inhibits HASMC growth to the same degree as 25 μM resveratrol (Fig. 4), the specific gene regulatory effects of resveratrol are not readily reproduced

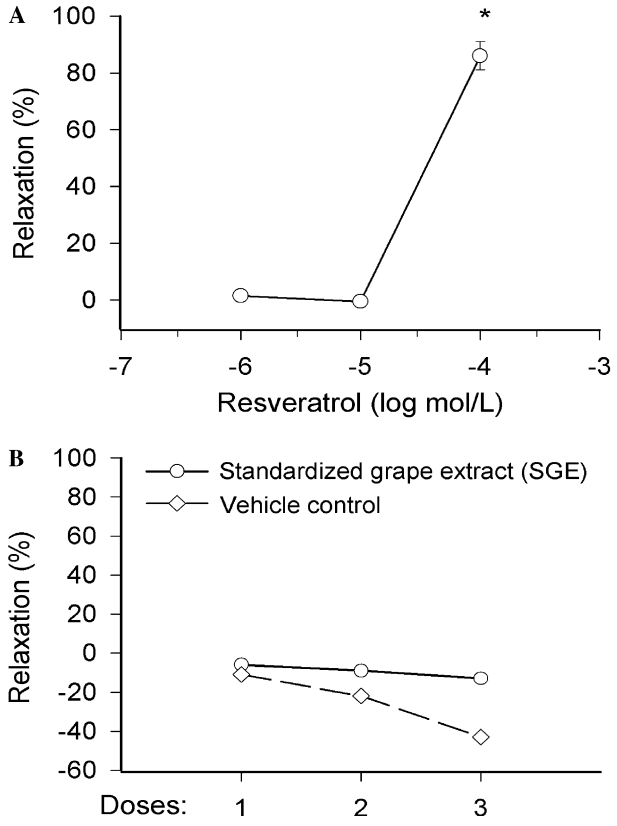

Fig. 6. Relaxation in response to resveratrol (A) or 70% ethanolic extracts of SGE (B) in rat carotid arterial ring preparations. Data are means ± SEM $*p < 0.05$.

using SGE (Fig. 5). Further, SGE also differed from resveratrol with respect to ability to induce relaxation of rat carotid arterial rings (Fig. 6). It is possible that SGE targets proliferation of HASMC through the combined effects of grape-derived polyphenols including flavans and proanthrocyanidin, each of which is present in SGE at sub-optimal bioactive doses and yet when presented together suffice to induce partial growth arrest to a degree similar to that elicited by the much higher concentrations of resveratrol used in the present studies. The same combinatorial mix of bioactive polyphenols present in SGE, however, might not be sufficiently overlapping as to replicate a similar or identical sequence of signaling and gene regulatory events as those elicited by resveratrol.

Acknowledgments

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