Resveratrol inhibits MAPK activity and nuclear translocation in coronary artery smooth muscle: reversal of endothelin-1 stimulatory effects

Abdalla M. El-Mowafy*, Richard E. White

Department of Physiology and Biophysics, Wright State University School of Medicine, Room 158 Biological Sciences Bldg., Dayton, OH 45435, USA

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Abstract In porcine coronary arteries, short-term treatment with resveratrol (RSVL) substantially inhibited MAPK activity (IC_{50} = 37 μM); and immunoblot analyses revealed consistent reduction in the phosphorylation of ERK-1/-2, JNK-1 and p38, at active sites. Endothelin-1 (ET-1), a primary antecedent in coronary heart diseases, enhanced MAPK activity, phosphorylation and nuclear translocation in a concentration-responsive but RSVL-sensitive manner. RSVL had no effect on basal or forskolin-stimulated cAMP levels, a known downregulator of the MAPK cascade. Likewise, inhibition of MAPK by RSVL was not reversed by the estrogen receptor blockers tamoxifen and ICI-182,780. Conversely, RSVL remarkably attenuated basal and ET-1-evoked protein tyrosine phosphorylation. Because MAPKs promote smooth muscle proliferation and contraction, their current inhibition may contribute to the putative protection by RSVL against coronary heart diseases. These effects apparently do not involve interaction with estrogen receptors.

Key words: Vascular smooth muscle; Coronary artery; Resveratrol; Endothelin-1; Mitogen-activated protein kinase; Nuclear translocation

1. Introduction

Evidence from epidemiological and experimental studies that natural edible compounds can protect against diseases is currently best exemplified by resveratrol [1]. Resveratrol (RSVL), a phytoalexin in grapes, berries and peanuts, has been shown to interfere with tumor initiation, promotion and progression in different systems [2,3]. At the molecular level, these effects were related to inhibition of ribonucleotide reductase with cellular arrest in the S-phase or the S/G2 phase transition [4,5]. Likewise, stimulation of adenylyl cyclase and inhibition of hydroperoxidase or cyclooxygenase have been reported in various systems [2,6].

In contrast to other systems, little is known about the cellular/molecular mechanisms whereby RSVL could protect against coronary heart diseases [1]. Resveratrol and related stilbenes such as quercetin relaxed rat aortic rings through an endothelium-mediated enhancement of the NO-cGMP cascade [7]. Subsequent pharmacological studies indicated a direct relaxant component exerted on vascular smooth muscle, though of unknown nature [8]. Indeed, whether resveratrol's antiproliferative effects could be mimicked in smooth muscles remains an intriguing possibility.

Among the endogenous mediators of cardiovascular disorders, endothelin-1 (ET-1), a 21 amino acid peptide, is a primary antecedent in coronary heart diseases [9–12]. Such effects are mediated by extremely potent vasopressor, and mitogenic responses for ET-1 in the vasculature [10,11]. Results from preclinical studies on humans, as well as from studies on animals, showed that plasma ET-1 levels are consistently elevated in many spasm-related cardiovascular diseases [11,13], and that blockers for ET receptors can substantially alleviate complications of such diseases [13,14]. In eliciting its biological responses, ET-1 targets a variety of molecular effectors including activation of mitogen-activated protein kinases (MAPKs), a superfamily of proline-directed serine/threonine protein kinases [15]. MAPKs, in addition to growth factors, are also upregulated with environmental stress signals such as heat shock, irradiation, osmotic stress and ischemia [16]. This superfamily of enzymes includes the extracellular signal-regulated kinases (ERKs) and the stress-activated protein kinases (SAPKs). The latter subfamily encompasses c-Jun N-terminal kinases (JNKs) and p38 MAP kinases [16–18]. Downstream targets for the action of MAPKs comprise mitogenic/proinflammatory enzymes and nuclear transcription factors [19,20]. Moreover, multiple evidence now suggests a positive impact for MAPK on mechanisms culminating in smooth muscle contraction [21,22].

The present study was undertaken to identify potential signaling components underlying resveratrol's protective actions in coronary artery. Because of endothelin's pathophysiological influence on the cardiovascular system, we investigated the capacity of this peptide to activate the MAPK pathways, ERK-1/-2, JNK-1 and p38, and to upregulate tyrosine kinase activity in coronary artery. Further, we examined whether RSVL can inhibit these enzymes in the presence and absence of ET-1. To our knowledge, this is the first report to demonstrate downregulation of MAPK/JNK/p38 by RSVL in the vasculature, a plausible mechanism that could account, at least in part, for its cardiovascular benefits.

2. Materials and methods

2.1. Materials

The MAPK peptide substrate (APRTGGRR) was purchased from Calbiochem. ET-1 was obtained from Phoenix Pharmaceuticals. P-81 phosphocellulose paper discs were from Gibco. cAMP enzyme immunoassay kit was from Biomol. The anti-MAPK, anti-P-ERK-1/-2, anti-P-JNK-1, anti-P-p38 anti-phosphotyrosine (PY-99), the horseradish peroxidase-linked secondary antibodies and protein agarose A were from Santa Cruz. Anti-histone H1 was from Upstate Biotechnology. ICI-182,780 was from Tocris, and tamoxifen was from Calbiochem. Various proteinase and phosphatase inhibitors were obtained from Sigma Chemical Co.

*Corresponding author. Fax: (1) (937) 775-3769.
E-mail: aelmowaf@wright.edu

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2.2. Subcellular fractionation

Left anterior descending coronary arteries of pig hearts were dissected out and their endothelia were mechanically removed. Following equilibration and initial RSVL/ET-1 treatments, subcellular nuclear fraction was prepared according to the method of Mizukami et al. [23]. Thus, arterial smooth muscle layers were isolated on ice and then homogenized using a Polytron homogenizer in 2 volumes of STE buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 5 mM NaN_3, 10 mM β-mercaptoethanol, 20 μM leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM sodium orthovanadate and 50 mM β-glycerophosphate). The homogenates were mixed with two volumes of STE buffer and centrifuged ($1000\times g$, 10 min, 4°C). The pellet (nuclear fraction) was washed once by suspension in STE and centrifugation then solubilized in Triton buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 20 μM leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 0.2 mM sodium orthovanadate and 30 mM β-glycerophosphate). This nuclear extract was centrifuged ($15000\times g$, 30 min, 4°C) and the supernatant was separated, aliquoted and stored at −80°C.

2.3. Determination of MAPK activity

Arteries, prepared as outlined above, were cut into strips to satisfy the needs of different treatments, and equilibrated in Krebs-Ringer bicarbonate buffer at 37°C for 60 min with the buffer replaced with fresh one at 20 min intervals. Muscle strips were treated with RSVL (20 min) and/or ET-1 (5 min) versus control from the same tissues. Reactions were terminated by ice cooling. Smooth muscle strips were finely chopped on ice and suspended in 4 volumes ice-cold homogenization buffer (Tris-HCl, pH 7.4, 20 mM; dithiothreitol, 1 mM; EGTA, 1 mM; EDTA, 1 mM; phenylmethylsulfonyl fluoride, 1 mM; leupeptin, 10 μg/ml; aprotinin, 2 μg/ml and Triton X-100, 0.1%. After homogenization with a Polytron homogenizer, the homogenate was centrifuged at $13\,000\times g$ (4°C). Protein concentration of extracts was determined by the method of Lowry et al. [24]. MAPK was immunoprecipitated by treatment with mammalian anti-ERK antibody (1:500) for 2 h (4°C), as determined by preliminary experiments. Protein agarose A (20 μl/ml) was then added with rocking for 2 h (4°C) and the immune complexes were pelletted by centrifugation at 2500 rpm for 5 min (4°C). Pellets were washed three times, by suspension and centrifugation, in homogenization buffer and once in assay buffer (Tris-HCl, 50 mM (pH 7.5); $MgCl_2$, 10 mM; β-glycerophosphate, 50 mM; sodium pyrophosphate, 1 mM; and sodium vanadate, 0.1 mM). ERK activity was determined in a total volume of 25 μl that contained ∼30 μg protein equivalent of immune complex, 5 μg of the specific MAPK substrate APRTPGGRR and 100 μM cold ATP/1.0 μCi [γ-^{32}P]ATP (4 mCi/μmol). Reactions were carried out at 30°C for 30 min, and were terminated by spotting (12 μl) onto p-81-phosphocellulose paper discs; which bind the phosphorylated substrate by ion-exchange chromatography. Discs were then washed (2×10 min) with 1% phosphoric acid and (2×5 min) with distilled water. The ^{32}P incorporation into the MAPK substrate was determined by liquid scintillation counting.

2.4. Determination of cAMP

Smooth muscle strips were equilibrated in Krebs-Ringer bicarbonate buffer at 37°C for 60 min with the buffer replaced with fresh one at 20 min intervals. 3-Isobutyl-1-methylxanthine (IBMX; 0.1 mM) was then added for 15 min prior to treatment with RSVL, forskolin or their combination (20 min). cAMP was extracted by 0.1 M HCl, determined by enzyme immunoassay and its levels were normalized to smooth muscle wet weight.

2.5. Immunoblot analysis

Arterial strips were treated with RSVL (20 min) and/or ET-1 (5 min) versus control. The estrogen receptor blockers tamoxifen or ICI-182,780 were added 15 min before RSVL treatment. Smooth muscle lysates were prepared as described above using the ice-cold homogenization buffer (Tris-HCl, pH 7.4, 20 mM; dithiothreitol, 1 mM; EGTA, 1 mM; EDTA, 1 mM; phenylmethylsulphonyl fluoride, 1 mM; leupeptin, 10 μg/ml; aprotinin, 2 μg/ml; Triton X-100, 1%; NP-40, 1%; sodium deoxycholate, 0.5%) and the phosphatase inhibitors, β-glycerophosphate (50 mM), sodium pyrophosphate (1 mM) and sodium vanadate (1 mM). The $13\,000\times g$ supernatant was-isolated, aliquoted and stored at −80°C. Protein content of the cellular or nuclear lysates was determined by the method of Lowry et al. Samples (30–60 μg) were separated by discontinuous SDS-PAGE on a 10–12% gel, according to the method of Laemmli [25], and proteins were electrotransferred to PVDF membranes. Membranes were reversibly stained with Ponceau-S red to ensure the homogeneity of protein blotting. Membrane proteins were then probed with primary antibodies, as follows: a rabbit anti-ERK (1:3000) in 5% non-fat dry milk, mouse anti-P-ERK1/ERK2 (1:2000), mouse anti-P-JNK (1:2000), mouse anti-P-p38 (1:2000) in 2% BSA or mouse anti-phosphotyrosine antibody (PY-99, 1:3000) in 4% BSA. Primary antibodies were then detected with appropriate secondary antibodies linked to horseradish peroxidase (1:2000–1:3000) and signals were visualized by the enhanced chemiluminescence system using a kit from New England Biolabs.

2.6. Statistical analysis

All data are expressed as mean ± S.E.M. Comparisons between two groups were made by Student's *t*-test and among multiple groups by the one-way ANOVA test. A probability less than 0.05 was considered to indicate a significant difference.

3. Results

3.1. Inhibition of MAPK activity, phosphorylation and nuclear translocation

Treatment of coronary arteries with resveratrol for 20 min, as guided by preliminary studies, inhibited MAPK activity in a concentration-dependent fashion (IC_{50} 37 ± 4.2 μM) (Fig. 1A). Western blotting under similar experimental conditions, with antibodies against the phosphorylated (activated) ERK-1/-2 (anti-P-ERKs) or against protein tyrosyl phosphate (PY-99), revealed considerable reductions in the phosphorylation of p-44 and p-42 bands (MAPK isoforms), implying that RSVL possibly interferes with the mechanisms underlying MAPK activation (Fig. 1A). ET-1, as an extracellular activator, increased MAPK activity by 2–3-fold in a concentration-responsive manner. Pretreatment with RSVL (50 μM) attenuated ET-1-enhanced MAPK activity (Fig. 1B). As can be seen in Fig. 2, modulation of ERK-1/-2 phosphorylation seems to be the target for actions of both ET-1 and RSVL. Probing total ERK protein levels with anti-ERK antibody, as indicated by densitometry, showed no difference in the total amount of enzyme present among different treatments. To further determine whether these mechanisms are operational for cognate MAPK family members, such as JNK-1 and p38, antibodies against the active (phosphorylated) forms of these enzymes were employed. Fig. 2 indicates that ET-1 augmented the phosphorylation of JNK-1 (p-46) and p38-MAPK, whereas RSVL counteracted such effects.

In addition to cytosolic targets, MAPK translocates to the nucleus where it phosphorylates transcription factors. Therefore, it was of interest to monitor levels of 'P-ERK' in the nucleus and the effects of ET-1 and/or RSVL thereon. To this end, we prepared a nuclear subcellular fraction from coronary arteries. Purity of this fraction was verified by immunodetection/densitometry of the nuclear marker protein (histone H1). Histone H1 also served as an internal reference for protein homogeneity among groups. Results indicated that histone H1 chiefly existed (∼88–91%) in the nuclear – rather than in the postnuclear – fraction (Fig. 3). Results in Fig. 3 demonstrate that P-ERK translocation to the nucleus is appreciably enhanced by ET-1, a response that was markedly reduced by RSVL.

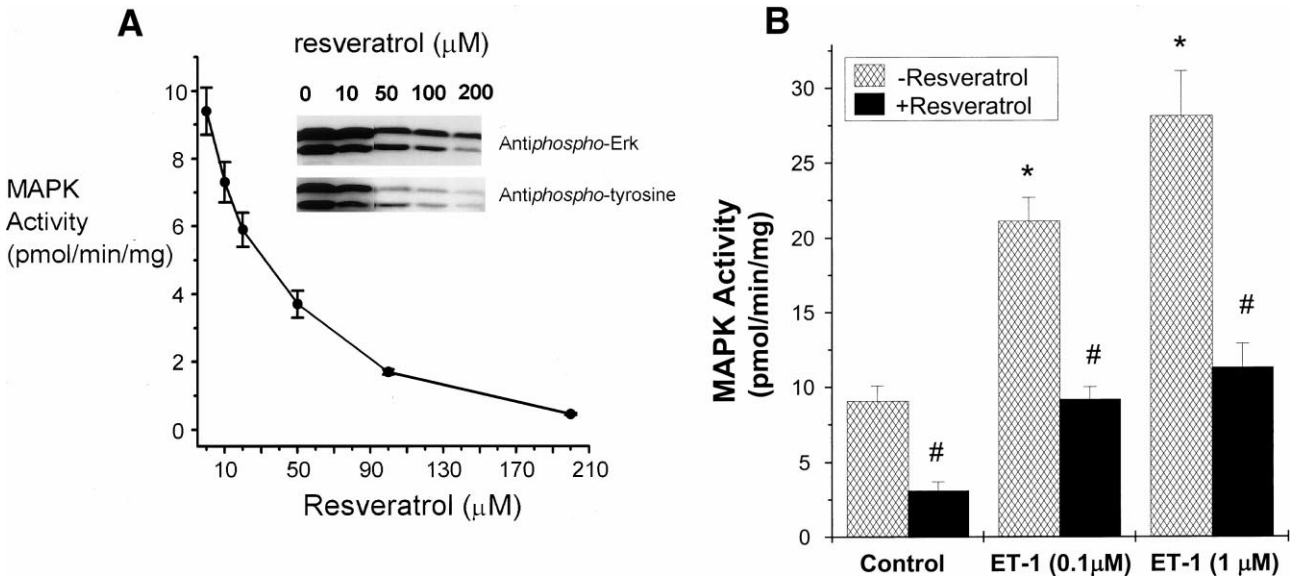

Fig. 1. RSVL inhibits MAPK activity in porcine coronary arteries in a concentration-responsive manner (A, $n=4$); inset shows corresponding phosphorylation levels of MAPK as determined by Western blotting from similar treatments (a representative of three similar blots). B: Reversal of ET-1-induced MAPK activation by resveratrol (50 μM). *Significantly different from control and #significantly different from ET-1 alone ($n=4$–5).

3.2. Effect on cAMP/protein kinase A (PKA)

The cAMP/PKA system, a known inhibitor of the MAPK cascade, is upregulated by RSVL in polymorphonuclear (PMN) leukocytes. However, similar stimulation by RSVL in vasculature has not been demonstrated. Measurements of cAMP levels (Fig. 4A) suggested that RSVL (10–1000 μM) had no effect on adenylyl cyclase (AC) activity in coronary smooth muscle. Similar results were obtained when the phosphodiesterase inhibitor, IBMX, was omitted from incubations, when AC was stimulated with forskolin or isoproterenol or when PKA activity was measured in the presence of RSVL (data not shown).

Previous studies have suggested a potential action of RSVL on receptors for estradiol (E_2), a known activator of AC and inhibitor of MAPKs in vascular smooth muscle [1]. Therefore, it was of concern to determine whether RSVL-induced inhibition of MAPK activity involved activation of E_2 receptor. As illustrated in Fig. 4B, pretreatment with blockers for E_2 receptor, tamoxifen or ICI-182,780 (1 μM), did not reverse the inhibition of ERK-1/-2 phosphorylation by RSVL.

3.3. Inhibition of cellular protein tyrosine phosphorylation

Tyrosine phosphorylation is an established upstream promoter of MAPK activity. Because RSVL is a polyphenolic compound, we considered that it could potentially affect protein tyrosine phosphorylation. Fig. 5 shows that RSVL suppressed basal as well as ET-1-evoked protein tyrosine phosphorylation.

4. Discussion

The putative cardiovascular benefits of RSVL have been related to inhibition of platelet aggregation, perturbation of prostanoid synthesis and regulation of lipoprotein metabolism [26]; however, the cellular and molecular targets of RSVL action remained largely ambiguous. We currently demonstrate

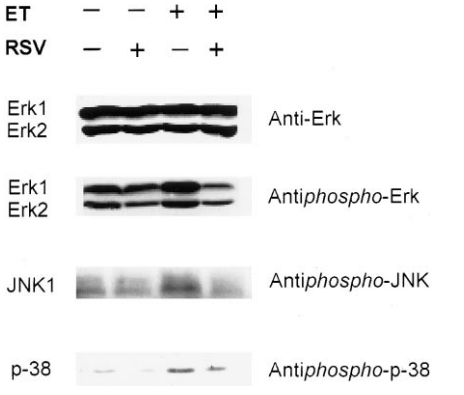

Fig. 2. ET-1 (0.1 μM)-induced phosphorylation of ERKs, JNK-1 and p38 and the effect of RSVL (50 μM) thereon; a representative of three similar experiments.

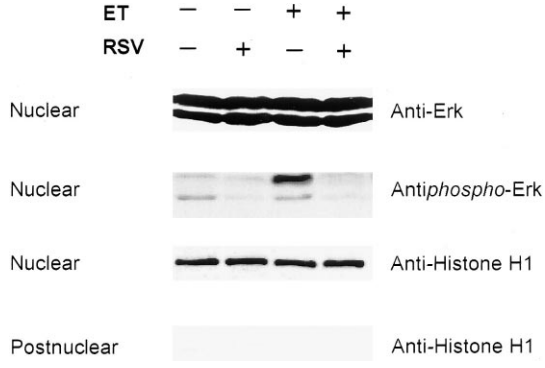

Fig. 3. Western blotting for detection of P-ERK-1/-2 levels relative to the total enzymes (ERK-1/-2) in a nuclear subcellular fraction in response to ET-1 and/or RSVL treatments. Densities of the nuclear marker histone H1 in nuclear and postnuclear fractions are also shown; a representative of three similar experiments.

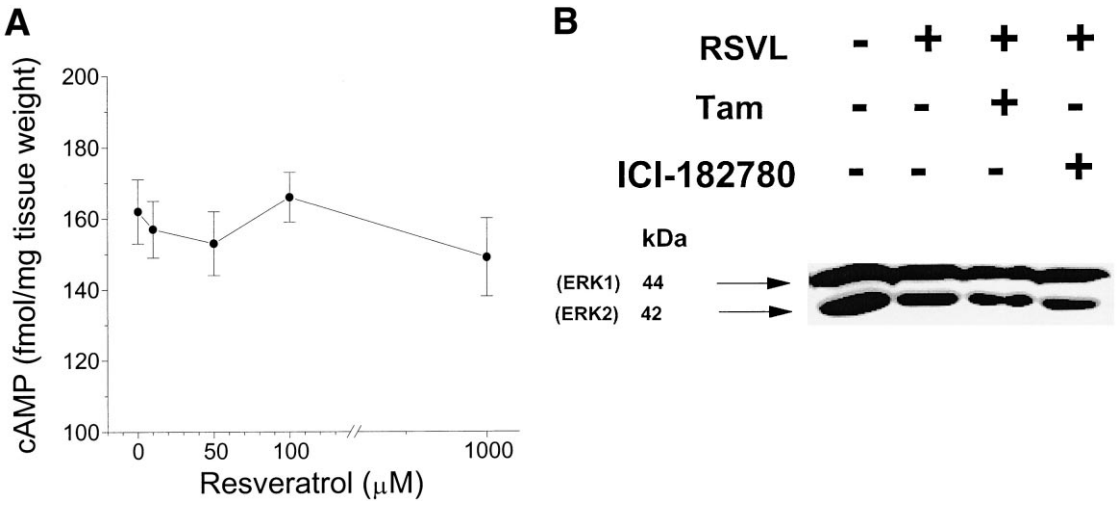

Fig. 4. Effect of RSVL (10–1000 μM) on cAMP levels in porcine coronary artery (A, $n=4$–6), and on ERK-1/-2 phosphorylation in the presence and absence of the estrogen receptor blockers, tamoxifen (TAM) or ICI-182,780, 1 μM each (B; a representative of three similar blots).

the upregulation/nuclear translocation of MAPK family of enzymes by ET-1 and their inhibition by RSVL in coronary arteries even in the absence of ET-1. These effects for RSVL involved hypophosphorylation of MAPKs at active sites. The IC_{50} value found for MAPK inhibition (37 μM) was comparable to those reported for inhibition of other enzymes such as hydroperoxidase, cyclooxygenase and ribonucleotide reductase (15–100 μM) in various systems [2,4].

Given its chemical structure and biological profiles in E_2-responsive systems, RSVL was considered a phytoestrogen [1]. Although RSVL was shown to be a 'superagonist' in the E_2 receptor-dependent breast cancer cells MCF-7 [27], it elicited E_2 receptor-independent effects in cognate cell lines [3]. In the vasculature, E_2 is known to lower the incidence of cardiovascular diseases among premenopausal women [28,29]. In light of similar protective effects, RSVL was speculated to be an 'estrogen agonist' in the vasculature [1]. The E_2-evoked vasodilatory and antiproliferative effects in vascular smooth muscles involve activation of AC and the nitric oxide/cGMP

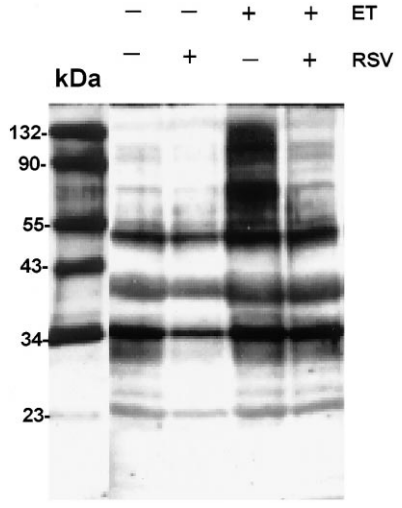

Fig. 5. Promotion by ET-1 (0.1 μM), but inhibition by RSVL (50 μM), of cellular protein tyrosine phosphorylation; a representative of three similar blots.

systems, as well as inhibition of MAPK signal [28–31]. On the other hand, in rat aorta, RSVL augmented NO and cGMP production evidently via an endothelium-dependent mechanism [7,8], and stimulated cAMP formation in PMN leukocytes [6]. Because stimulation of the cAMP/PKA pathway inhibits the MAPK cascade [32,33], we investigated the effects of RSVL on cAMP formation/PKA activity in coronary artery. Our data indicated that RSVL had no effect on this cascade. Moreover, RSVL-induced inhibition of MAPK phosphorylation was insensitive to the E_2 receptor blockers tamoxifen and ICI-182,780l, thereby arguing against a role for estrogen receptors. However, exclusion of 'estrogen-like' effects in our system may be premature in light of recent reports suggesting 'direct' receptor-independent responses for estradiol [34]. In support, recent studies in E_2-responsive pituitary tumor cell lines demonstrated the inability of RSVL to bind E_2 receptors, although it mimicked some of the E_2 responses in this system [35]. Clearly, RSVL is not merely an 'E_2-like compound', but exhibits unique pharmacological characteristics.

The cGMP/PKG system is another MAPK downregulator in vascular smooth muscle [36]. Because RSVL's agonistic effects on cGMP are both NO- and endothelium-dependent [7,8], this mechanism may not be operational in our endothelium-denuded arteries. Further, in such endothelium-free arteries, the observed inhibition of MAPKs by RSVL may substantiate its vascular benefits in diseases associated with damaged endothelium such as atherosclerosis [37] or conditions associated with elevated plasma levels of ET-1 and/or growth factors [38].

Signal triggers mediating the spasmogenic/mitogenic effects of ET-1 in vasculature include activation of PLC, PKC, non-receptor protein tyrosine kinases (n-PTK) and the MAPK superfamily of enzymes [15,39]. Causal relationships in the activation of these signals have also been established [40]. Not surprisingly, therefore, we found that the specific MEK/MAPK inhibitor, PD-98059, significantly attenuated ET-1-induced contraction of porcine coronary artery (data not shown), which is consistent with previous reports [21,22]. Tyrosine phosphorylation enhances 'ras' association with 'c-raf-1' and is also an essential mechanism in the activation of MAPK by MEK [36]. Moreover, PTKs are known to activate

mitogenic and spasmogenic signals in smooth muscles [41,42]. The polyphenolic nature of RSVL motivated the initial finding that it inhibits the activity of purified PTKs [43]. Because we had shown previously that ET-1 activates n-PTKs in coronary smooth muscle [12], it was reasonable to challenge RSVL in such an 'upregulated' system. The present inhibition of cellular tyrosine phosphorylation by RSVL is likely to contribute to the downstream effects on MAPKs.

Although RSVL is an edible compound, detailed pharmacokinetic information concerning its therapeutic benefits has been lacking. A recent elegant 'population study' now provides evidence that beverage intake of RSVL results in sufficient absorption/plasma levels to account for its beneficial health effects [44]. Collectively, the present study suggests the MAPK and PTK pathways are important 'targets' for the pathophysiological impact of ET-1 in the etiology of coronary heart diseases as well as for the putative cardiovascular protective effects of RSVL. Inhibition of these enzymes could help minimize the risk of smooth muscle cell proliferation and spasm underlying coronary stenosis.

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