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Resveratrol attenuates hyperproliferation of vascular smooth muscle cells from spontaneously hypertensive rats: Role of ROS and ROS-mediated cell signaling

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ABSTRACT

Resveratrol, a natural polyphenolic compound has been reported to attenuate angiotensin II -induced vascular smooth muscle cell (VSMC) hypertrophy; however, whether resveratrol could also inhibit hyperproliferation of VSMC from spontaneously hypertensive rats (SHR) is unexplored. The present study investigates the effect of resveratrol on hyperproliferation of VSMC from SHR and the underlying molecular mechanisms responsible for this response. For these studies, aortic VSMC from SHR and Wistar-Kyoto (WKY) rats were used. The proliferation of VSMC was determined by [^{3}H] thymidine incorporation and the levels of proteins were determined by Western blotting. The enhanced proliferation exhibited by VSMC from SHR was attenuated by resveratrol. In addition, resveratrol attenuated the overexpression of cyclin D1, cyclin E, cyclin dependent kinase 4 (Cdk4), Cdk2, phosphorylated retinoblastoma protein (pRb), Giα proteins and enhanced phosphorylation of ERK1/2 and AKT in VSMC from SHR. Furthermore, the overproduction of superoxide anion, increased NADPH oxidase activity, overexpression of Nox2, Nox4 and p47^{phox} proteins, increased phosphorylation of EGFR, IGF-IR, and c-Src were all abrogated by resveratrol. These results suggest that resveratrol attenuates the hyperproliferation of VSMC from SHR through the inhibition of ROS, c-Src, growth factor receptor activation, MAPK/PI3K, Giα and cell cycle proteins that are implicated in VSMC hyperproliferation.

1. Introduction

Hypertension is a chronic systemic disease, affects approximately 25% of the adult population [1]. Uncontrolled high blood pressure can lead to serious complications including heart attack, heart failure, and atherosclerosis [2]. Despite hypertension has been focus of intense research, 20 to 30% of patients with hypertension are resistant to currently available antihypertensive treatment [3]. One of the pathophysiological mechanisms involved in both development and complications of hypertension is vascular remodeling including abnormal VSMC growth, proliferation, migration, etc. [4,5]. Hyperproliferation of VSMCs not only plays pivotal role in the development of essential hypertension, but also atherosclerosis and restenosis [6]. Small mesenteric arteries of spontaneously hypertensive rats (SHR) (animal model for genetic hypertension) exhibited typical hallmarks of vascular remodeling including smaller lumen, a greater media thickness, and augmented media-to-lumen ratios than Wistar–Kyoto (WKY) rat [7]. VSMC cultured from the aorta of SHR have been reported to exhibit enhanced proliferation compared with normotensive WKY [8,9].

The cellular signaling pathways mediating hyperproliferation in VSMCs are complex. Several distinct signal transduction pathways including reactive oxygen species (ROS) and ROS mediated signaling pathway are implicated in vascular remodeling by promoting VSMC proliferation [10]. One of the most important sources of ROS in the VSMC are membrane bound NADPH oxidases which are responsible for the formation of superoxide anion (O_2^-) [11] [12]. The levels of ROS, have been shown to be augmented in VSMC from SHR due to the increased levels of superoxide anion (O_2^-), NADPH oxidase activity and increased expression of the NADPH oxidase subunits Nox1/Nox2/Nox4 and p47^{phox} [13].

The enhanced oxidative stress in VSMC from SHR was attributed to increased level of endogenous vasoactive peptides such as angiotensin II (Ang II), augmented expression of Giα proteins, and the decreased levels of cAMP [14]. It was reported that endogenous vasoactive peptides, through increased oxidative stress and resultant activation of c-Src, transactivate EGFR, which through mitogen-activated protein (MAP) kinase signaling and resultant overexpression of Giα proteins, contributes to the hyperproliferation of VSMC from SHR [9] [14,15].

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Hyperproliferation of VSMC from SHR is associated with accelerating entry of cells from G_0/G_1 phase of cell cycle to the synthetic phase [6]. In addition, the cell cycle proteins from G_1-phase were reported to be over expressed in VSMC from SHR and implicated in the hyper-proliferation [16,17]. We previously demonstrated that the over expression of Giα proteins and enhanced MAP kinase/PI3 kinase activation contribute to the enhanced proliferation and expression of cell cycle proteins in VSMC from SHR, because PD98059, wortmannin and pertussis toxin, the inhibitors of MAP kinase, PI3 kinase and Giα proteins respectively, attenuated the hyperproliferation of VSMC from SHR and overexpression of cell cycle proteins to control levels [17].

Resveratrol (RV) is polyphenolic molecule found in many natural sources including skin of grapes, berries and peanut [18]. Polyphenols including RV have been proposed to contribute to the “French paradox” phenomenon which consists of lower incidence of coronary heart disease (CHD) in the French population [19]. Therefore, RV is receiving recently huge attention for its beneficial effects against different cardiovascular diseases like hypertension [20]. In addition, RV has also been shown to exert antimitogenic effect on hyperproliferation of VSMC induced by hyperglycemia [21] serum [22], and tumor necrosis factor alpha (TNF-α) [23]. However, whether RV exerts antiproliferative effect in VSMC from SHR is not completely understood. Therefore, the present study was undertaken to examine the effect of RV on the proliferation of VSMC from SHR and to explore the underlying molecular mechanisms mediating this effect. We showed that RV inhibits hyperproliferation of VSMC from SHR that involves the attenuation of enhanced expression of cell cycle proteins, Giα proteins, enhanced activation of MAP kinase/PI3 kinase, transactivation and over expression of growth factor receptors, enhanced c-Src activation and oxidative stress.

2. Materials and methods

2.1. Materials

Resveratrol (3, 4′, 5-Trihydroxy-trans-stilbene) and β-Actin (AC-15) antibody were purchased from Sigma (St-Louis, MO, USA). Antibodies against cyclin D1 (sc-20,044), Cdk4 (sc-23,896), cyclin E (sc-481), Cdk2 (sc-6248), phospho-specific-Ser^{249}/Thr^{252} Rb (sc-377528), Rb (sc-102), Giα-2 (sc-13534), Giα-3 (sc-262), ERK1/2 (sc-135900), phospho-specific-Tyr^{204} ERK1/2 (sc-7383), phospho-specific-Ser^{473} AKT (sc-7985-R), AKT (sc-8312), phosphospecific-Tyr^{530} c-Src (sc-16,846), c-Src (sc-19), phospho-specific-Tyr^{1173} EGFR (sc-12,351), EGFR (sc-03), phosphor-specific-$Tyr^{1165/1166}$ IGF-IR (sc-101704), IGF-IRβ (sc-713), dynein IC1/2 (sc-13524), and goat anti-mouse IgG-HRP (sc-2005) from Santa Cruz Biotechnology Inc. (Santa Cruz, DA, USA). Nox2/gp91 antibody was purchased from Abcam Inc. (Toronto, ON, Canada). Polyclonal Nox4 antibody was from protein tech. (Manchester, United Kingdom). Polyclonal $p47^{phox}$ antibody was purchased from Bioss Inc. (Massachusetts, USA). Western blotting reagents were from St. Cruz Biotech (Santa Cruz, CA). The L-[4,5-^{3}H]-Thymidine was from PerkinElmer Inc. (Waltham, Massachusetts, USA).

2.2. Cell culture

VSMC from 14-week-old SHR and their age-matched WKY rats were cultured from aortas, as described previously [24]. The purity of the cells was checked by immunofluorescence technique using α-actin, as described previously [25]. These cells were found to contain high levels of smooth-muscle-specific actin [26]. The cells were plated in 75 cm^2 flasks and incubated at 37 °C in 95% air and 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (with glucose, L-glutamine, and sodium bicarbonate) containing 1% antibiotics and 10% heat-inactivated fetal bovine serum (FBS). The cells were passaged upon reaching confluence with 0.5% trypsin containing 0.2% EDTA and utilized between passages 3 and 10. Confluent cells were then starved by incubation for 4 h in DMEM without FBS at 37 °C to reduce the interference by growth factors present in the serum. The cells were then incubated in the absence or presence of various concentrations of RV (10 to 100 μM) or as otherwise indicated for 16 h. After incubation, the cells were washed three times with PBS and lysed in 200 μl of buffer containing 25 mM Tris·HCl (pH 7.5), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1%Triton X-100, 0.1% sodium dodecyl sulfate, (SDS), and 0.5 μg/ml leupeptin on ice. The cell lysates were centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentration was measured by Bradford assay [27]. All the animal procedures used in the present study were approved by the Comité de Déontologie de l'Expérimentation sur les Animaux (CDEA) of the University of Montreal (protocol #99050). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Guide, NRC 2011).

2.3. Western blot analysis

The levels of proteins were determined by Western blotting using specific antibodies as described previously [28]. Equal amounts of protein (30 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and incubated with the primary antibodies against cyclin D1, Cdk4, cyclin E, Cdk2, p-Rb, Rb, Giα-2, Giα-3, ERK1/2, p-ERK1/2, p-AKT, AKT, p-c-Src, c-Src, p-EGFR, EGFR, p-IGF-IR, IGF-IRβ, Nox2/gp91, Nox4, $p47^{phox}$, β-actin and dynein. The antigen-antibody complexes were detected by incubating the blots with respective secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature and then the blots were then washed three times with PBS before reacting with enhanced-chemiluminescence Western-blotting detection reagents. Quantitative analysis of specific bands was performed by densitometric scanning of the autoradiographs with the enhanced laser densitometer LKB Ultroscan XL and quantified using the gel-scan XL evaluation software (version 2.1) from Pharmacia (Baie d'Urfé, QC, Canada).

2.4. Measurement of [^{3}H] thymidine incorporation

Cell proliferation was quantified by DNA synthesis which was evaluated by incorporation of [^{3}H] thymidine into cells as described previously [29]. Subconfluent VSMC from SHR and WKY rats were plated in 6-well plates for 24 h and were serum deprived for 4 h to induce cell quiescence. The cells were then incubated in the absence or presence of different concentration of RV (10–100 μM) for 16 h. [^{3}H] thymidine (1 μCi) was added and further incubated for 4 h before the cells were harvested. The cells were rinsed twice with ice-cold PBS and incubated with 5% trichloroacetic acid for 1 h at 4 °C. After being washed twice with ice-cold water, the cells were incubated with 0.4 N sodium hydroxide solution for 30 min at room temperature, and radioactivity was determined by liquid scintillation counter.

2.5. Superoxide anion (O_2^-) measurements

Basal superoxide anion production in VSMC was measured using the lucigenin-enhanced chemiluminescence method with low concentration (5 μM) of lucigenin as described previously [30]. The cells after treatment with RV (50 μM), were washed in oxygenated Kreb–Hepes buffer, scraped and placed in scintillation vials containing lucigenin solution, and the emitted luminescence was measured with a liquid scintillation counter (Wallac 1409; Perkin Elmer Life Science, St Laurent, Quebec, Canada) for 5 min. The average luminescence value was estimated, the background value subtracted and the result was divided by the total weight of proteins in each sample.