Brazilian Journal of Medical and Biological Research (2015) 48(1): 51-56, http://dx.doi.org/10.1590/1414-431X20144032
ISSN 1414-431X

Resveratrol inhibits the intracellular calcium increase and angiotensin/endothelin system activation induced by soluble uric acid in mesangial cells

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Abstract

Resveratrol (Resv) is natural polyphenol found in grapes. This study evaluated the protective effect of Resv against the effects of uric acid (UA) in immortalized human mesangial cells (ihMCs). ihMCs were preincubated with Resv (12.5 μM) for 1 h and treated with UA (10 mg/dL) for 6 or 12 h. The intracellular calcium concentration $[Ca^{2+}]i$ was quantified by fluorescence using flow cytometry. Angiotensinogen (AGT) and pre-pro endothelin-1 (ppET-1) mRNA were assayed by quantitative real-time RT-PCR. Angiotensin II (AII) and endothelin-1 (ET-1) were assayed by ELISA. UA significantly increased $[Ca^{2+}]i$. Pre-incubation with Resv significantly reduced the change in $[Ca^{2+}]i$ induced by UA. Incubation with UA for 6 or 12 h also increased AGT mRNA expression and AII protein synthesis. Resv blunted these increases in AGT mRNA expression and AII protein. Incubation with UA in the ihMCs increased ppET-1 expression and ET-1 protein synthesis at 6 and 12 h. When ihMCs were pre-incubated with Resv, UA had a significantly diminished effect on ppET-1 mRNA expression and ET-1 protein synthesis at 6 and 12 h, respectively. Our results suggested that UA triggers reactions including AII and ET-1 production in mesangial cells. The renin-angiotensin system may contribute to the pathogenesis of renal function and chronic kidney disease. Resv can minimize the impact of UA on AII, ET-1 and the increase of $[Ca^{2+}]i$ in mesangial cells, suggesting that, at least in part, Resv can prevent the effects of soluble UA in mesangial cells.

Key words: Resveratrol; Intracellular calcium; Angiotensin; Endothelin; Mesangial cells; Uric acid

Introduction

Resveratrol (Resv), a phenolic compound with significant biological activity, is of great interest to several research groups worldwide (1,2). Dietary intake of Resv in grapes, red wine, peanuts, purple grape juice, and berries may have beneficial effects on human heath (1-7), and may have health-promoting antinephrolithic, antidiabetes, anticancer, antioxidation, anti-inflammation, cardioprotective, chemopreventive, and neuroprotective properties (5-9). In addition, Resv may prevent renal lipotoxicity and have antihyperuricemic activity (10,11).

Several pathways are thought to be related to the protective effect of Resv. In a nephrolithic model, about 70% of kidney stones were composed of calcium oxalate (CaOx), which causes renal cell injury through the production of reactive oxygen species (ROS) and nicotinamide adenine dinucleotide phosphate (NADPH) when deposited in kidney tissue (12). Resv exerts its antinephrolithic potential via the inhibition of ROS, monocyte chemoattractant protein-1 (MCP-1), hyaluronan, and osteopontin (OPN) signaling (13). Resv has been shown to suppress the migration of oxalate-treated human renal epithelial cells (HRCs), It also can attenuate the expression of NADPH oxidase subunit, MCP-1, and OPN mRNAs, and downregulate the expression of transforming growth factor-β (TGF-β1), TGF-β receptor and hyaluronan proteins in oxalate-treated HRCs (13).

Diabetic nephropathy is a serious complication of type 1 and type 2 diabetes. It is characterized by an expansion of the glomerular mesangium caused by mesangial cell proliferation and an excess of extracellular matrix (ECM) proteins synthesized by mesangial cells (14). There is increasing evidence that overproduction of ROS is involved in the development of diabetic nephropathy (15,16) by activating protein kinase C, mitogen-activated protein (MAP) kinases, and transcription factors nuclear factor kappa beta (NF-κB) and activated protein-1. The

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Received April 12, 2014. Accepted September 10, 2014. First published online October 24, 2014.

resulting altered expression of genes and ECM proteins leads to diabetic nephropathy (17). Mesangial cell proliferation and fibronectin expression are induced by hyperglycemia through the janus kinase (JNK)/NF-κB/NADPH oxidase/ROS signaling pathway (14). Resv has been shown to inhibit hyperglycemia-induced mesangial cell expansion and fibronectin expression by blocking this signaling pathway (14).

Hyperuricemia, present as a metabolic disorder, is usually associated with gout, kidney disease, hypertension, cardiovascular diseases, inflammation, diabetes and metabolic syndrome. There is increasing evidence that hyperuricemia is an independent risk factor for the occurrence and development of kidney disease, including damage to mesangial cells. At least one study reported that Resv has antihyperuricemic and nephroprotective activity in oxonate-induced mice (18). These effects, mediated by changes in renal expression of mGLUT9, mABCG2, mOAT1, and mOCT1, are evidence of the possible preventive efficacy of Resv on hyperuricemia (11,18). The present study analyzed the effects of Resv on uric acid (UA)-treated mesangial cells as well as effects on the renin-angiotensin system (RAS) and the endothelin system.

Material and Methods

Cell culture

Immortalized human mesangial cells (ihMCs), kindly provided by Dr. Richard Banas (Munich, Germany), were grown in Dulbecco's modified eagle medium (DMEM; Gibco, USA) with the addition of 10% fetal bovine serum (FBS; Gibco), 24 mM $NaHCO_3$ (Merck, USA), 10 mM HEPES (Sigma, USA) and 10,000 U/L penicillin/streptomycin (Gibco). The cultures were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. When semiconfluent, the cells were detached from the plastic flasks with trypsin (0.5%; Cultilab, Brazil), centrifuged for 5 min at 1000 *g*, and then resuspended in DMEM and subcultured in 22-cm^2 plastic culture flasks for further experimental procedures.

Resv preparation

Resv of 99% purity (Sigma) was dissolved in DMSO (Merck), and stored at –20°C in the dark.

Uric acid preparation

UA (Sigma) was sterilized in a steam autoclave and added to culture medium without FBS. The UA crystals were suspended in DMEM containing 1% FBS, incubated at 37°C, and sonicated for 15 min to solubilize the crystals.

Exposure of the ihMCs to uric acid

Prior to the experiments, ihMCs were transferred to a plastic plate (1×10^6 cells/plate) and maintained under culture conditions for 3 days. At confluence, the cells were exposed for 6 or 12 h to either DMEM, without FBS, for the control or DMEM containing 10 mg/dL UA.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was isolated and purified from ihMCs by a phenol and guanidine isothiocyanate-cesium chloride method, using the appropriate kit (Trizol; Life Technologies, USA). Two micrograms of total RNA were treated with DNase (RQ1 RNase-Free DNase; Promega, USA) to prevent genomic DNA contamination. The RNA pellet was resuspended in RNase-free water and was reverse transcribed into cDNA by the addition of 0.5 mg/mL oligo d(T), 10 mM dithiothreitol (DTT), 0.5 mM dNTPs (GE Healthcare, UK), and 200 U reverse transcriptase enzyme (SuperScript RT, Gibco, USA).

Real-time amplification was carried out with a 7500 Real-Time Sequence Detection System (SDS; ABI Prism 7500; Applied Biosystems, USA). The real-time PCR product was quantified using an intercalating dye (SYBR Green I; Molecular Probes Inc., USA) that exhibits increased fluorescence upon binding double-stranded DNA.

The relative gene expression was calculated using the conditions at the early stages of the PCR. At this point, the amplification is logarithmic and can thus be correlated to the initial copy number of the gene transcripts. The reactions were cycled 40 times under the conditions previously determined by conventional PCR. The fluorescence for each cycle was quantitatively analyzed using an ABI Prism 7500 SDS (Applied Biosystems). At the end of the PCR reaction, the temperature was increased from 60°C to 95°C at a rate of 2°C/min. During that time, fluorescence was measured every 15 s to construct a melting curve. A nontemplate control was run with each assay.

The PCR was performed with primers specific for *pre-pro endothelin-1* (*ppET-1*), *angiotensinogen (AGT)*, and *β-actin*. The primer sequences were: *ppET-1* sense (5′-GAGAAACCCACTCCCAGTCC-3′) and antisense (5′-GATGTCCAGGTGGCAGAAGT-3′); *β-actin*, sense (5′-TCACCCACACTGTGCCCATCTACGA-3′) and antisense (5′-CAGCGGAACCGCTCATTGCCAATGG-3′); and *AGT* sense (5′-ACTTCTCGGTGACTCAAGTGCC-3′) and antisense (5′-GAAAGTGAGACCCTCCACCTTGT-3′). The results of these experiments are reported in each group as the relative expression normalized to the *β-actin* housekeeping gene. This gene was used as an internal control and is expressed here as a percentage of the control reaction.

Real-time data analysis

The cycle threshold (Ct) values were subtracted from the Ct value for each gene to yield the ΔCt values. These values were used for statistical comparisons. For the graphic representations, the fold variation was determined using the $2^{-(\Delta\Delta Ct)}$ method according to published protocols (19) and manufacturer recommendations. The fold variations were calculated by determining the difference in

the ΔCt values between the chosen reference and the test samples (ΔΔCt value), with subsequent application of the $2^{-(\Delta\Delta Ct)}$ formula.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of angiotensin II (AII) and endothelin (ET)-1 were assayed in cell cultures treated with UA using a commercially available competitive ELISA (Cayman Chemical, USA). All assays were performed according to the manufacturer's protocols. The absorbance of each sample was determined using an Ultra Microplate (Biotek, USA) and reported as ng/mL and pg/mL.

Measurement of intracellular calcium by flow cytometry

For the cytometric analyses, the cells were cultured to a density of 2×10^6 cells/well, were pretreated with Resv (12.5 uM) and stimulated with UA (10 mg/dL). The cells were then trypsinized, washed twice with PBS, and resuspended in 0.5 mL isotonic solution. The osmolarity of the isotonic solution was 323 ± 6 mOsm and consisted of 80 nM D-mannitol with 120 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 5.4 mM HEPES. The cells were loaded with 2 μM fluo-4/AM (Invitrogen, USA) for 30 min and were analyzed by flow cytometry (BD FACSCanto II, BD Biosciences, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The fluorescence intensity of approximately 1×10^6 labeled cells was measured in each assay, and the data are reported as the median fluorescence intensity in arbitrary units (AU) obtained by averaging at least 3 separate experiments (20).

Statistical analysis

The data are reported as means ± SE. The experimental and control groups were compared via the Student *t*-test. The significance level for the null hypothesis was set at 5% ($P<0.05$).

Results

Resv inhibited the increase in $[Ca^{2+}]i$ induced by UA

To study the effect of Resv on mesangial cells, the effect of UA was first analyzed. Treatment of ihMCs with 10 mg/dL UA increased $[Ca^{2+}]i$ (80.60 ± 0.20) compared with the fluorescence at baseline, but the increase in AII was greater than that of $[Ca^{2+}]i$ after UA administration. The effect of UA was inhibited by pre-exposure of mesangial cells to Resv. When the ihMCs were pre-incubated with 12.5 μM Resv for 1 h, $[Ca^{2+}]i$, as estimated by fluorescence intensity, was 38.34 ± 0.01 compared to 80.60 ± 0.20 in cells that had not been pretreated ($P<0.005$; Figure 1).

Resv inhibited the RAS system in the mesangial cells

One of the main regulators of contraction in mesangial cells is the peptide AII; therefore, we evaluated the effect of Resv on UA-induced AII synthesis. There was a significant increase in UA-induced AGT mRNA expression in the ihMCs in a time-dependent manner. Incubation of the ihMCs with 10 mg/dL UA significantly increased AGT mRNA expression at 6 h (12.40 ± 0.92 *vs* 1.24 ± 0.18 AU for the control, $P<0.001$) and at 12 h (22.04 ± 0.79 *vs* 1.24 ± 0.18 AU for the control, $P<0.001$). When ihMCs were pre-incubated with 12.5 μM Resv for 1 h, the increase in AGT mRNA was not as great at 6 h (12.40 ± 0.92 *vs* 3.03 ± 1.08 AU for the UA condition) and at 12 h (22.04 ± 0.79 *vs* 5.36 ± 1.06 AU for the UA condition) (both $P<0.001$, Figure 2A).

UA increased AII protein synthesis at 6 h (0.07 ± 0.01 *vs* 0.05 ± 0.01 ng/mL for the control, $P<0.05$) and 12 h (0.10 ± 0.01 *vs* 0.05 ± 0.01 ng/mL for the control, $P<0.05$) Pre-incubation with 12.5 μM Resv for 1 h attenuated the increase in AII protein synthesis at 6 h (0.05 ± 0.01 *vs* 0.07 ± 0.01 ng/mL for the UA group) and at 12 h (0.04 ± 0.003 *vs* 0.10 ± 0.01 ng/mL for the UA group) (both $P<0.05$, Figure 2B).

Resv inhibited the endothelin system in mesangial cells

ET-1 is also a peptide involved in smooth muscle cell contraction (21). Nevertheless, the effect of UA on ET-1 synthesis in mesangial cells is not known. UA significantly increased ppET-1 mRNA expression in cultured ihMCs in a time-dependent manner. Incubation of the ihMCs with UA increased ppET-1 expression at 6 h (1.52 ± 0.23 *vs* 1.02 ± 0.10 AU for the control group) and 12 h (2.32 ± 0.18 *vs* 1.02 ± 0.10 AU for the control group) (both $P<0.05$, Figure 3A). When the ihMCs were pre-incubated with 12.5 μM Resv for 1 h, the increases were significantly

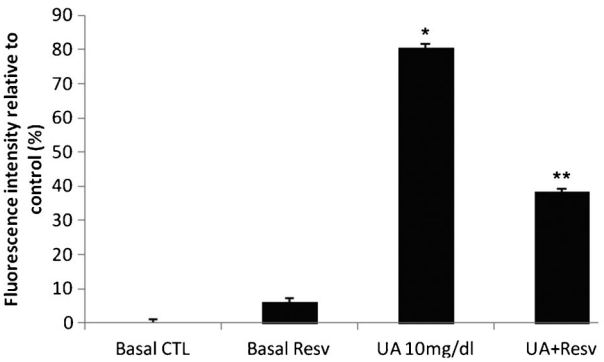

Figure 1. Intracellular calcium concentration in immortalized human mesangial cells (ihMCs) treated with uric acid (UA) and resveratrol (Resv). Briefly, semi-confluent ihMC cells were detached with trypsin and the fluorescence intensity of intracellular calcium was determined by Fluo-4/AM in the presence of 10 mg/dL UA and 12.5 μM Resv. Data are reported as the mean percentage (± SE). *$P<0.001$ *vs* control (CTL); **$P<0.005$ *vs* UA (Student *t*-test).

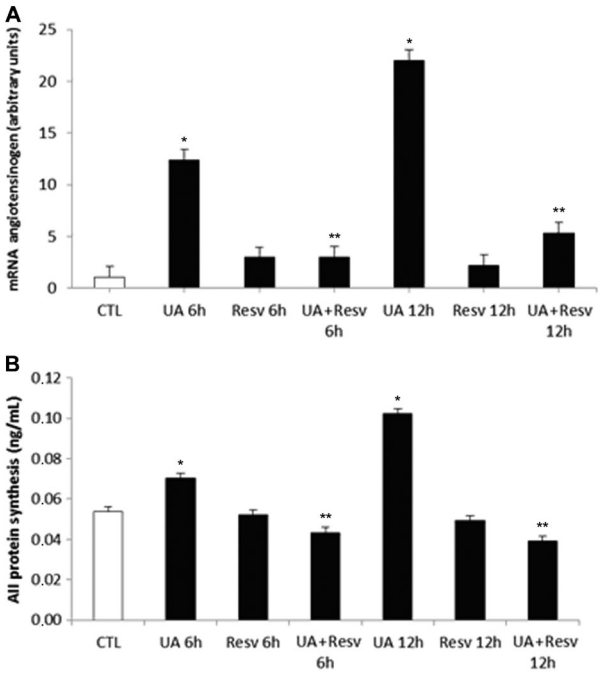

Figure 2. Effect of resveratrol (Resv) treatment on the angiotensinogen mRNA expression (*A*) and AII protein synthesis (*B*) in immortalized human mesangial cells (ihMCs) stimulated with uric acid (UA). Cells were seeded onto plates and after 6 and 12 h were exposed to DMEM with 1% FBS containing 10 mg/dL UA, and 12.5 μM Resv. Data are reported as means ± SE. *P<0.001 *vs* control (CTL); **P<0.001 *vs* UA (Student *t*-test).

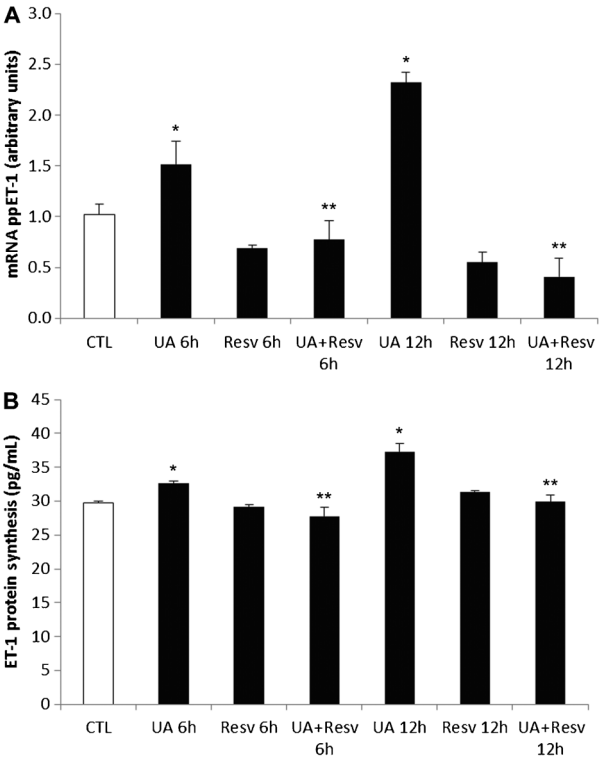

Figure 3. Effect of resveratrol (Resv) treatment (12.5 μM) on mRNA pre-pro endothelin-1 (ppET-1) expression (*A*) and ET-1 protein synthesis (*B*) in immortalized human mesangial cells (ihMCs) stimulated with uric acid (UA). Cells were seeded on plates and after 6 and 12 h were exposed to DMEM with 1% FBS containing 10 mg/dL UA and 12.5 μM Resv. Data are reported as means ± SE. *A*, *P<0.05 *vs* control (CTL); **P<0.05 *vs* UA (Student *t*-test). *B*, *P<0.05 *vs* CTL; **P<0.001 *vs* UA (Student *t*-test).

lower at both 6 h (0.77 ± 0.18 *vs* 1.52 ± 0.23 arbitrary units for the UA group) and 12 h (0.41 ± 0.06 *vs* 2.32 ± 0.18 arbitrary units for the UA group (both P<0.05, Figure 3A).

UA increased ET-1 protein synthesis at 6 h (32.69 ± 0.22 *vs* 29.68 ± 0.35 pg/mL for the control group) and 12 h (37.29 ± 1.23 *vs* 29.68 ± 0.35 pg/mL for the control group (both P<0.05, Figure 3B).

Again, when the ihMCs were pre-incubated with 12.5 μM Resv for 1 h, the increases were significantly lower in ET-1 protein synthesis at 6 h (27.78 ± 1.38 *vs* 32.69 ± 0.22 pg/mL for the UA group) and at 12 h (29.92 ± 1.02 *vs* 37.29 ± 1.23 pg/mL for the UA group (all P<0.05, Figure 3B).

Discussion

Mesangial cells play a major role in glomerular contraction and regulate filtration surface area and ultrafiltration coefficient (Kf) through the release of hormones such as AII. Mesangial cells express all components of RAS, and can regulate the Kf via contraction or the release of vasoactive hormones (22). ETs are also powerful vasoconstrictor peptides, and act as modulators of vasomotor tone, cell proliferation, and hormone production. Mesangial cells are the main site of ET production in the kidney (23).

UA has recently been recognized as a key factor in multifactorial renal disorders, including chronic kidney disease, hypertension and acute kidney injury (24,25). Recent studies have confirmed that RAS is strongly related to elevated serum UA levels in hypertensive patients. We have previously demonstrated that UA stimulates RAS and ET-1 in mesangial cells after 24 h (26). Experimental studies *in vitro* have also demonstrated that UA has direct effects on rat vascular smooth cell, proliferation (27), human vascular endothelial cell dysfunction (28), and ihMC proliferation (26) via local tissue RAS activation. This evidence indicates that the activation of systemic and local tissue RAS by UA may be partially responsible for the pathogenetic effects of UA in hypertension.

The experimental choice of 10 mg/d UA was determined by the saturation concentration of monosodium urate in human plasma of approximately 7 mg/dL. Thus, the definition of hyperuricemia is usually a plasma UA concentration >7 mg/dL (29). In addition, Aida et al. (30)

reported that the plasma concentration of UA in the general population has a wide range, extending from 1 to 12 mg/dL. Observations in an American cohort were confirmed in a community-based study with participants in Vienna that reported mild hyperuricemia in patients with uric acid concentrations of 7.0-8.9 mg/dL (31). High UA concentrations (5 or 15 mg/dL) upregulated both RAS mRNA expression and AII II protein secretion in 3T3-L1 adipocytes. In addition, UA caused a significant increase in ROS production in differentiated adipocytes (32).

Evidence from studies indicates that the generation of ROS is, at least in part, involved in endothelial dysfunction (33,34), and the production of ROS, particularly the superoxide anion (O_2^-), is closely related to calcium concentration (35).

The concentration of Resv (12.5 μM) was chosen from among various concentrations that are known to have relevant cardiovascular effects on isolated tissues or organs (36). The first evidence of the specific benefits of red wine was shown in 1993 when Frankel et al. (37) demonstrated that red wine diluted 1000-fold and containing 10 μmol of total phenols inhibited the oxidation of LDL. This is approximately the Resv concentration found in red wines, and suggested that half a bottle of such wine should contain an active concentration of resveratrol (37).

In a study similar to ours, Chao et al. (38) found that Resv (10 μM) inhibited the formation of AII-induced ROS, extracellular signal-regulated kinase phosphorylation, ET-1 gene expression, and cell proliferation in smooth muscle cells.

In this study, UA induced an increase in ppET-1 mRNA expression and protein synthesis after 6 and 12 h and increased AGT mRNA expression and AII protein synthesis after 6 and 12 h. In addition, Resv reduced UA-induced pre-proET-1 gene expression and the production of AII and ET-1 in mesangial cells, suggesting that it can minimize the impact of these hormones on glomerular function. In addition, Resv inhibited the increase in [Ca^{2+}]i. These results are the first direct evidence that UA induces an increase in [Ca^{2+}]i that is minimized by Resv. Our results suggested that UA triggers reactions including AII and ET-1 production in mesangial cells. RAS may contribute to the pathogenesis of renal function and chronic kidney disease. Resv can minimize the impact of UA on the increases of AII, ET-1 and [Ca^{2+}]i in mesangial cells, suggesting that it can prevent the effects of soluble UA on mesangial cells.

Lastly, multidisciplinary approaches are recommended for future investigations because of the wide range of polyphenol actions on body fat reduction, mitigation of liver disease, improvements in muscle function, and cardiovascular and renal protection.

Acknowledgments

Research supported by CNPq, FINEP, FAPESP, CAPES, and Fundação Oswaldo Ramos (FOR).

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