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Meso-dihydroguaiaretic acid inhibits rat aortic vascular smooth muscle cell proliferation by suppressing phosphorylation of platelet-derived growth factor receptor beta

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ABSTRACT

Abnormal proliferation of vascular smooth muscle cells (VSMCs) plays an essential functional role in the pathogenesis of vascular disorders, such as atherosclerosis, restenosis, and neointimal hyperplasia. In this study, we examined the effects of meso-dihydroguaiaretic acid (MDGA) on platelet-derived growth factor (PDGF)-BB-induced proliferation and the molecular basis of its underlying mechanism of action in rat aortic VSMCs. Incubation of resting VSMCs with MDGA for 24 h significantly diminished PDGF-BB-induced DNA synthesis in a dose-dependent manner. We also examined the effects of MDGA on PDGF-BB signal transduction. Pre-treatment of VSMCs with MDGA inhibited PDGF-BB-induced phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), p38, and C-Jun N-terminal kinase (JNK). MDGA also effectively inhibited phosphorylation of Akt, phospholipase C gamma 1 (PLCγ1), and PDGF receptor beta (PDGFRβ). These results indicate that MDGA may inhibit proliferation of VSMCs by suppressing autophosphorylation of PDGFRβ, and may be useful in the treatment of VSMC-associated vascular disease such as atherosclerosis, restenosis, and neointimal hyperplasia after angioplasty.

1. Introduction

Proliferation of vascular smooth muscle cells (VSMCs) is a key feature of diverse vascular diseases such as atherosclerosis, hypertension, restenosis, and cardiovascular disease (Dzau et al., 2002; Rosamond et al., 2008). The proliferation and migration of VSMCs are involved in intimal lesion formation, which can be induced by various growth factors, such as platelet-derived growth factor (PDGF) (Kim et al., 2002; Raines, 2004). Among the isomers of PDGF, PDGF-BB is a potent inducer of VSMC proliferation and migration compared with PDGF-AA and PDGF-AB (Jiang et al., 1996; Koyama et al., 1994). Indeed, the PDGF-BB-induced mitogenesis signaling pathway has already been relatively well characterized. Specifically, binding of PDGF-BB to the PDGF receptor (PDGFR) leads to dimerization and autophosphorylation of PDGFRβ tyrosine residues, resulting in activation of a downstream signal transduction pathway including Akt, PLCγ1, and mitogen-activated protein (MAP) kinase (Millette et al., 2006; Pearson et al., 2001; Kondo et al., 1993; Claesson-Welsh, 1994; Heldin et al., 1998; Cospedal et al., 1999).

MDGA has antioxidant (Yu et al., 2000; Ma et al., 2005), anti-aging (Moon and Chung, 2005), and anti-inflammatory effects (Moon et al., 2008). In addition, MDGA displays significant neuroprotective activities against glutamate-induced neurotoxicity in primary cultures of rat cortical cells (Ma et al., 2004), and has a suppressive effects on *umu* gene expression during the SOS response in *Salmonella typhimurium* against the mutagen 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole, which requires liver metabolizing enzymes (Miyazawa et al., 1998). MDGA also directly inhibits activation of hematopoietic stem cells and down-regulates transforming growth factor beta 1 gene expression by inhibiting the activity of activator protein1 (Park et al., 2005). However, there are no reports describing the anti-proliferative effects nor the underlying mechanism of MDGA isolated from *Machilus thunbergii*.

In this study, we showed for the first time that MDGA inhibits PDGF-BB-induced VSMC proliferation. We also investigated the signaling pathways responsible for the effects of MDGA on PDGF-BB-induced VSMC proliferation.

2. Materials and methods

2.1. Reagents and antibodies

PDGF-BB was purchased from Koma Biotechnology (Seoul, Korea). Anti-phospho-ERK 1/2, anti-ERK 1/2, anti-phospho-p38, anti-p38, anti-phospho-Akt, anti-Akt, anti-phospho-JNK, anti-JNK, anti-phospho-PLCγ1, anti-PLCγ1, and anti-β-actin antibodies were

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purchased from Cell Signaling Technology (Danvers, MA, USA). An anti-phospho-PDGFRβ polyclonal antibody was obtained from Upstate Biotechnology (NY, USA). Cell culture agents were obtained from Gibco-BRL (MD, USA). All other biochemical reagents were purchased from Sigma-Aldrich (MO, USA). MDGA was obtained from Dr. Sang-Hyun Sung (Seoul National University, Seoul, Korea). MDGA was dissolved by dimethyl sulfoxide (DMSO).

2.2. Cell culture

Rat aortic VSMCs were purchased from BioBud Co. Ltd. (Seoul, Korea). VSMCs were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. VSMCs between passages 5 and 12 were used for all experiments.

2.3. [^{3}H]-thymidine incorporation assay

[^{3}H]-thymidine incorporation was analyzed as described previously (Kim et al., 2007). Briefly, VSMCs were seeded in 24-well plates at 1×10^4 cells/well and cultured in DMEM containing 10% FBS at 37 °C. After 48 h, the medium was removed and replaced with serum-free medium containing MDGA (1–20 μM), and the cells were incubated for 24 h. VSMCs were then stimulated with 50 ng/ml PDGF-BB for 20 h, after which 5 μCi/ml of [^{3}H]-thymidine was added to each well, and the cells were placed in an incubator for 4 h. The labeling reaction was terminated by aspirating the medium and washing cultures with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v). Acid-insoluble [^{3}H]-thymidine was extracted into 500 μl of 0.5N NaOH/well, mixed with 3 ml of scintillation cocktail (Ultimagold, Packard Bioscience, Meriden, CT, USA), and quantified using a scintillation counter (LS3801, Beckman, Düsseldorf, Germany).

2.4. Cytotoxicity assay.

VSMCs were seeded at 1×10^6 cells/ml in 96-well plates and incubated with MDGA at various concentrations for 23 h at 37 °C. After incubation, the cells were exposed to 10% of the volume of an EZ-Cytox kit (Daeil Lab Service, Korea) for 1 h at 37 °C. Cytotoxicity was measured using a microplate reader with absorbance at 450 nm.

2.5. Western blot analysis

Western blot analysis was performed as described previously (Kim et al., 2009). Briefly, VSMCs were seeded in 6-well plates at 1×10^5 cells/well, and cultured in DMEM containing 10% FBS at 37 °C. After 48 h, the medium was removed and replaced with serum-free medium containing MDGA (1–20 μM), and the cells were incubated for a further 24 h. VSMCs were then stimulated with 50 ng/ml PDGF-BB. Cell lysates were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 5–10% polyacrylamide gels, and the proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 (TBS/T) and 5% skim milk powder. Membranes were then incubated with primary antibodies (1:1000 or 1:2000 dilution) overnight at 4 °C. Blots were washed with TBS/T and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibodies for 1 h at room temperature. Membranes were detected using an enhanced chemiluminescent detection reagent for Western blots (GE Healthcare, Buckinghamshire, UK).

2.6. Immunofluorescence analysis

VSMCs were seeded in coverglass at 1×10^4 cells/well, and cultured in DMEM containing 10% FBS at 37 °C. After 48 h, the medium was removed and replaced with serum-free medium containing MDGA (20 μM), and the cells were incubated for a further 4 h. VSMCs were then stimulated with 50 ng/ml PDGF-BB for 1 min. Cells were then rinsed with PBS, fixed in fresh 4% paraformaldehyde for 5 min at room temperature, and permeabilized in 0.2% Triton X-100. Nonspecific sites were blocked by incubation with PBS containing 1% goat serum before incubating the cells with an anti-phospho-PDGFRβ antibody. After washing, cells were incubated with a goat anti-mouse secondary AlexaFluor 488 for 2 h, washed, counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (CA, USA), and mounted. Slide images were acquired by Cal Zeiss LSM710 confocal microscopy (Oberkochem, Germany).

2.7. Statistics analysis

Experimental results are expressed as mean ± standard error of the mean (S.E.M.). One-way ANOVA followed by Dunnett's test was used for multiple comparisons. We considered P values of < 0.05 and < 0.01 statistically significant differences.

3. Results

3.1. Effect of MDGA on PDGF-BB-induced DNA synthesis in VSMCs

We examined the effect of MDGA on cell proliferation in PDGF-BB-induced VSMCs by [^{3}H]-thymidine incorporation assay. As shown in Fig. 1, stimulation of VSMCs with 50 ng/ml PDGF-BB potently increased [^{3}H]-thymidine incorporation from 418.1 ± 163.2 to 4640.2 ± 89.5 cpm/well. MDGA significantly inhibited the PDGF-BB-induced DNA synthesis in a concentration-dependent manner by 3864.1 ± 346.5, 2656.6 ± 310.0, 1308.4 ± 381.5, 716.3 ± 253.0 and 186.0 ± 111.7 cpm/well at 1, 5, 10, 15 and 20 μM, respectively. Also, MDGA did not show any cytotoxic effects on cell viability as assessed by EZ-Cytox assays at those used in these experiments (Fig. 2), suggesting that the inhibitory effects of MDGA on cell proliferation and DNA synthesis were not due to cytotoxicity.

3.2. Effect of MDGA on PDGF-BB-induced ERK1/2, p38, and JNK phosphorylation in VSMCs

We next examined the signaling pathways involved in the inhibitory effect of MDGA on VSMC proliferation in response to

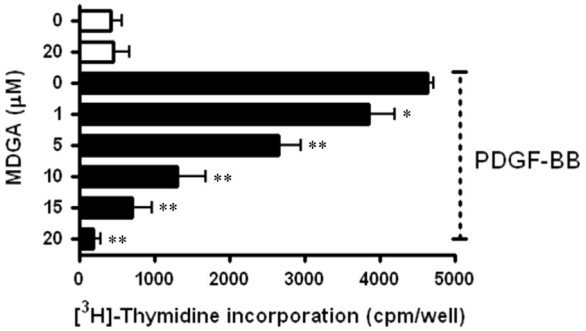

Fig. 1. Effect of MDGA on [^{3}H]-thymidine incorporation in PDGF-BB-induced VSMCs. Cells were seeded in 24-well culture plates at 1×10^4 cells/well and cultured in 10% DMEM at 37 °C in a 5% CO_2 incubator for 48 h. Cultured cells were then incubated in serum-free media containing MDGA (1–20 μM) for 24 h and stimulated by PDGF-BB (50 ng/ml) for 20 h. 5 μCi/ml of [^{3}H]-thymidine was added to each well, and the cells were incubated for 4 h. Radioactivity was quantified using a scintillation counter. Data are expressed as mean ± S.E.M. ($n=4$). *$P < 0.05$, **$P < 0.01$.