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Anti-proliferative actions of 2-decylamino-5,8-dimethoxy-1,4-naphthoquinone in vascular smooth muscle cells

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ABSTRACT

Naphthoquinone derivatives have been reported to possess various pharmacological activities, such as antiplatelet, anticancer, antifungal, and antiviral properties. In this study, we investigated the effects of a newly-synthesized naphthoquinone derivative, 2-decylamino-5,8-dimethoxy-1,4-naphthoquinone (2-decylamino-DMNQ), on VSMC proliferation and examined the molecular basis of the underlying mechanism. In a dose-dependent manner, 2-decylamino-DMNQ inhibited PDGF-stimulated VSMC proliferation with no apparent cytotoxic effect. While 2-decylamino-DMNQ did not affect PDGF-Rβ or Akt, it did inhibit the phosphorylation of Erk1/2 and PLCγ1 induced by PDGF. Moreover, 2-decylamino-DMNQ suppressed DNA synthesis through the arrest of cell cycle progression at the G_0/G_1 phase, including the suppression of pRb phosphorylation and a decrease in PCNA expression, which was related to the downregulation of cell cycle regulatory factors, such as cyclin D1/E and CDK 2/4. It was demonstrated that both U0126, an Erk1/2 inhibitor, and U73122, a PLCγ inhibitor, increased the proportion of cells in the G_0/G_1 phase of the cell cycle. Thus, these results suggest that 2-decylamino DMNQ has an inhibitory effect on PDGF-induced VSMC proliferation and the mechanism of this action is through cell cycle arrest at the G_0/G_1 phase. This may be a useful tool for studying interventions for vascular restenosis in coronary revascularization procedures and stent implantation.

1. Introduction

Cardiovascular diseases are associated with a multitude of pathophysiological conditions, including inflammation, pulmonary hypertension, atherosclerosis, and restenosis following balloon angioplasty [1]. VSMC proliferation in response to vessel injury is an important factor in the pathophysiological courses of atherosclerosis and restenosis [2,3]. The primary cascade in the development of atherosclerosis involves injury to the endothelium, which leads to a wound healing response and causes the migration of VSMCs from the media to the intima, with subsequent proliferation [1,4,5]. Although the proliferation of VSMCs involves various growth factors and cytokines, the principal regulator in the mitogenesis of VSMCs is PDGF [6].

PDGF consists of three isoforms (AA, AB, BB) that can be produced by activated macrophages, VSMCs, and endothelial cells. PDGF-BB, a growth factor secreted by injured endothelial cells, VSMCs, platelets, and macrophages, promotes the proliferation of fibroblasts, glia, and VSMCs [6]. Therefore, PDGF-BB is more important for stimulating the proliferation of VSMCs than PDGF-AA [7]. PDGF-BB propagates mitogenic signals through phosphorylation of its respective PDGF-Rβ on tyrosine residues, thus triggering downstream signal transduction and cell-cycle progression [8–10]. Cellular proliferation is controlled primarily by cell-cycle regulation; the cycle consists of four distinct sequential phases, G_0/G_1, S, G_2, and M. This tightly regulated temporal order is controlled by the sequential activation of serine/threonine protein kinases known as CDKs, which phosphorylate pRb. The cell-cycle transition is controlled by the action of CDKs and their activating subunits,

Abbreviations: 2-decylamino-DMNQ, 2-decylamino-5,8-dimethoxy-1,4-naphthoquinone; VSMCs, vascular smooth muscle cells; PDGF, platelet-derived growth factor; Erk1/2, extracellular regulated kinases 1/2; PLCγ, phospholipase γ; PDGF-Rβ, PDGF receptor β; CDK, cyclin-dependent kinase; PCNA, proliferative cell nuclear antigen; pRb, retinoblastoma protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMSO, dimethylsulfoxide; PI, propidium iodide; MAPK, mitogen-activated protein kinase.

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the cyclins [3,11–13]. After a vascular injury, VSMCs are stimulated to divide in response to mitogens, and they exit the G_1 phase and enter the S phase. Cyclin D_1–CDK4 and cyclin E–CDK2 complexes act predominantly in sequence during the G_1/S transition and are required for cell-cycle progression through this period [14].

Naphthoquinones have been reported to possess various pharmacological activities, including antiviral, antifungal, anticancer, and antiplatelet properties [15,16]. 2-Decylamino-DMNQ is a newly-synthesized naphthoquinone derivative. In the present study, we investigated the molecular mechanisms underlying the effects of 2-decylamino-DMNQ on PDGF-BB-stimulated VSMC proliferation.

2. Materials and methods

2.1. Reagents

The chemical structure of 2-decylamino-DMNQ is shown in Fig. 1A. Cell culture materials were purchased from Invitrogen (Carlsbad, CA, USA). Anti-phospho-PDGF-Rβ, anti-phospho-Erk1/2, anti-phospho-Akt, anti-phospho-PLCγ1, anti-PDGF-Rβ, anti-Erk1/2, anti-Akt, and anti-PLCγ1 antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-phospho-pRb, anti-phospho PCNA, anti-cyclin D1, anti-cyclin E, anti-CDK2, anti-CDK4, and anti-β actin antibodies were from Abfrontier (Geumcheon, Seoul, Republic of Korea). PDGF-BB was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Other chemicals were of analytical grade.

2.2. Cell culture

Rat aortic VSMCs were isolated by enzymatic dispersion as described previously [17]. Cells were cultured in DMEM, supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 8 mM HEPES, and 2 mM L-glutamine at 37 °C in a humidified atmosphere of 95% air/5% CO_2. The purity of the VSMC culture was confirmed by immunocytochemical staining of α-smooth muscle actin. VSMCs at passages 4–8 were used in the experiments.

2.3. Cell viability assay

VSMCs were seeded into 96-well culture plates at 2×10^4 cells/mL and then cultured in DMEM containing 10% FBS at 37 °C for 24 h. When cells reached 70% confluence, the medium was replaced with serum-free medium. After a 24 h incubation, the cells were exposed to 1 μM 2-decylamino-DMNQ or 50 μM digitonin as a cytotoxic control at various times. WST-1 reagent was added to the medium, and the cells were incubated for 2 h. The absorbance was measured at 450 nm using a microplate reader (Packard Instrument Co., Downers Grove, IL, USA).

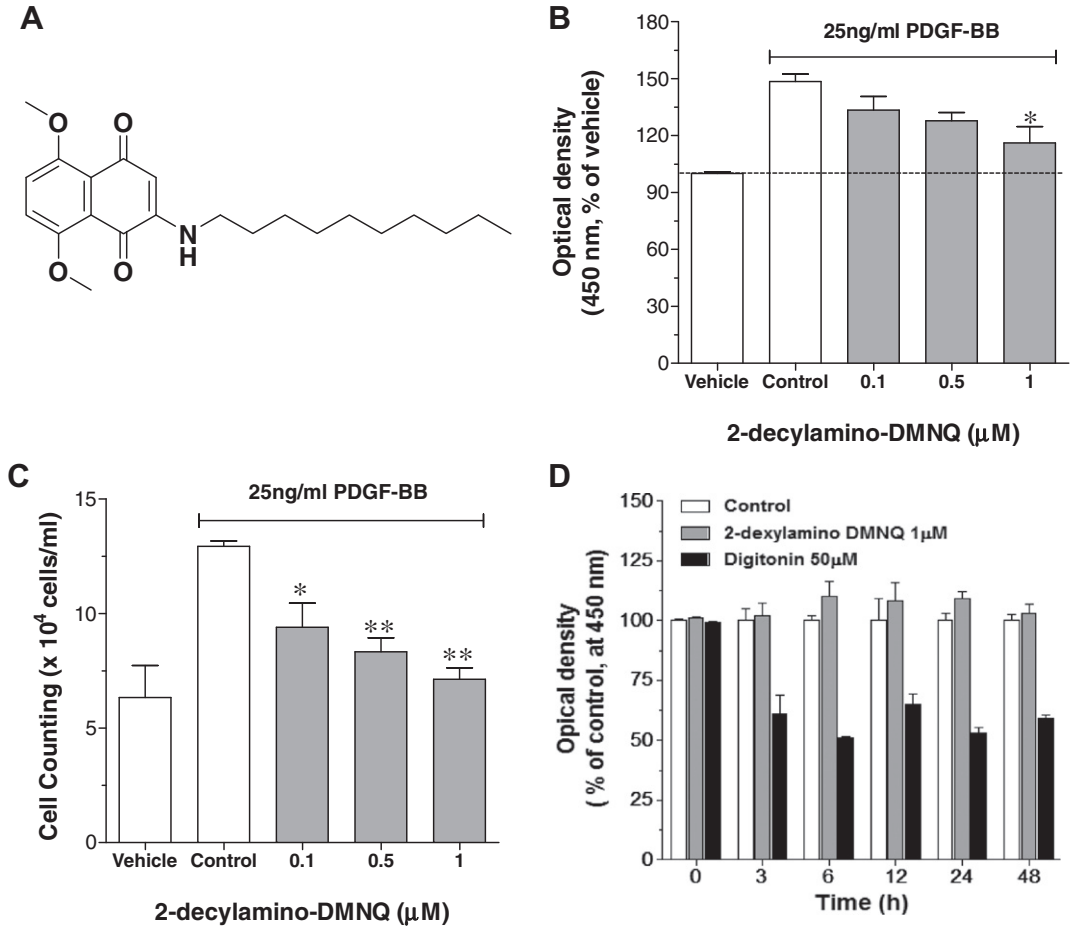

Fig. 1. Chemical structure of 2-decylamino DMNQ and the effects of 2-decylamino DMNQ on VSMC proliferation and viability. VSMCs cultured in serum-free medium were stimulated with 25 ng/mL PDGF-BB for 24 h and the effects of various concentrations of 2-decylamino DMNQ (0.1–1.0 μM) on cell proliferation and viability were measured as described in the Section 2. (A) Chemical structure of 2-decylamino DMNQ; (B) Optimal densities at 450 nm as determined by WST-1 assay ($n = 8$); (C) Cell numbers counted using a hemocytometer ($n = 4$); (D) Viability as determined by WST-1 ($n = 4$) for various incubation times (0–48 h). The values are expressed as means ± SEM. Statistical differences from the PDGF control (PDGF-stimulated, but no 2-decylamino DMNQ) are indicated by *($P < 0.05$) and **($P < 0.01$).

2.4. Cell proliferation assay

VSMC proliferation was measured by both direct counting and a non-radioactive colorimetric WST-1 assay (WST-1 premix, Takara, Japan). For direct cell counting, VSMCs were seeded into 12-well culture plates at 4×10^4 cells/mL and then cultured in DMEM containing 10% FBS at 37 °C for 24 h. After reaching ~70% confluence, the cells were incubated in serum-free medium for 24 h, treated with various concentrations of 2-decylamino-DMNQ for another 24 h in fresh serum-free medium, and stimulated with PDGF-BB (25 ng/mL). 2-Decylamino-DMNQ was dissolved in DMSO, and the final DMSO concentration in the medium did not exceed 0.05%. After 24 h, the cells were treated with trypsin–EDTA and counted using a hemocytometer under a microscope. All experimental procedures for the non-radioactive colorimetric WST-1 assay were performed as recommended by the manufacturer, and the results are expressed as a percentage of the control values.

2.5. DNA synthesis assay

DNA synthesis was determined based on a [^{3}H]-thymidine incorporation assay, as described previously [18,19]. Assay conditions were the same as those described in the cell proliferation assay section. [^{3}H]-thymidine (2 μCi/mL) was added for 4 h before harvesting under 25 ng/mL PDGF-BB-induced stimulatory conditions in serum-free medium. The reaction was terminated by aspirating the medium and subjecting the cultures to sequential washes with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v) on ice. Acid-insoluble [^{3}H]-thymidine was extracted in 250 μL 0.5 M NaOH/well, and this solution was mixed with 3 mL scintillation cocktail (Ultimagold, Packard Bioscience, Meriden, CT, USA) and quantified using a liquid scintillation counter (LS3801, Beckman, Düsseldorf, Germany).

2.6. Cell-cycle progression analysis

Cell-cycle progression was measured as described previously [18,19]. The assay conditions were the same as those described in the cell-proliferation assay section. After being stimulated by PDGF-BB (25 ng/mL) for 24 h, cells were trypsinized and centrifuged (1500*g*, 7 min). The centrifuged pellets were suspended in 1 mL of 1× PBS, washed twice, and fixed with 70% ethanol for 48 h. The fixed cells were briefly vortexed and centrifuged (15,000*g*, 5 min). The ethanol was discarded, and the pellets were stained with 500 μL PI solution (50 μg/mL PI in sample buffer containing 100 μg/mL RNase A). Before the flow cytometry analysis, each sample was incubated at room temperature for 1 h. The PI-DNA complex in each cell nucleus was measured with a FACScalibur flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA). The individual nuclear DNA content was reflected by the fluorescence intensity of the incorporated PI. The proportions of cells in the G_0/G_1, S, and G_2/M phases of the cell cycle were determined using the Modfit LT software.

2.7. Immunoblotting assay

Immunoblotting was performed as described previously [18,19]. VSMCs were stimulated with 25 ng/mL PDGF-BB for 1 min for the PDGF-Rβassay, 5 min for Erk 1/2 and PLCγ1, and 15 min for Akt phosphorylation. For the cyclin D1, cyclin E, CDK2, CDK4, and PCNA expression and pRb phosphorylation assays, VSMCs were stimulated with PDGF-BB (25 ng/mL) for 24 h. The detected proteins were normalized to β-actin or the respective total proteins. Bands intensities were quantified using the Quantity One program (Bio-Rad, Hercules, CA, USA).

2.8. Statistical analysis

Data are expressed as means ± SEM. A one-way analysis of variance (ANOVA) was used for multiple comparisons (GraphPad, San Diego, CA, USA). If a significant difference between treated groups was found, a Dunnett's test was applied. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of 2-decylamino DMNQ on VSMC proliferation

To test the effect of 2-decylamino DMNQ on PDGF-BB-induced VSMC proliferation, a non-radioactive colorimetric WST-1 assay was performed. The data presented in Fig. 1B demonstrate that 2-decylamino DMNQ inhibited PDGF-BB-induced VSMC proliferation in a concentration-dependent manner. The number of cells increased significantly after 25 ng/mL PDGF-BB treatment ($12.9 \pm 0.2 \times 10^4$ cells/well) compared with the unstimulated group ($6.3 \pm 1.4 \times 10^4$ cells/well). As the concentration of 2-decylamino DMNQ increased to 0.1, 0.5, and 1 μM, cell number decreased significantly to 9.4 ± 1.1, 8.3 ± 0.6, and $7.1 \pm 0.5 \times 10^4$ cells/well, respectively (Fig. 1C). Treatment with the highest concentration of 2-decylamino DMNQ (1 μM) for various incubation times did not result in VSMC cytotoxicity in serum-free medium (Fig. 1D), indicating that the antiproliferative effect of 2-decylamino DMNQ on VSMCs was not due simply to cytotoxicity. Digitonin was used as a positive control for cytotoxicity.

3.2. Effect of 2-decylamino DMNQ on PDGF-Rβ, Akt, Erk1/2, and PLCγ1 activation

To assess the early signals that might be involved in the antiproliferative actions of 2-decylamino DMNQ, the activation of PDGF-Rβ, Akt, Erk1/2, and PLCγ1 by 2-decylamino DMNQ was measured in PDGF-BB-stimulated VSMCs. The data presented in Fig. 2A show that 2-decylamino DMNQ did not activate PDGF-Rβ or Akt. However, 2-decylamino DMNQ significantly decreased Erk1/2 phosphorylation at 1 μM (Fig. 2B), and also significantly inhibited PLCγ1 phosphorylation in a concentration-dependent manner (Fig. 2C). These results indicate that the Erk1/2 and PLCγ1-mediated signaling pathway is involved in the antiproliferative actions of 2-decylamino DMNQ.

3.3. Effect of 2-decylamino DMNQ on DNA synthesis and cell-cycle progression

To examine the effects of 2-decylamino DMNQ on DNA synthesis in VSMCs, a [^{3}H]-thymidine incorporation assay was performed. The data in Fig. 3A show that the increased [^{3}H]-thymidine incorporation into DNA (from 488.1 to 3764.1 cpm/well) caused by PDGF-BB was significantly inhibited by 2-decylamino DMNQ in a concentration-dependent manner. As the 2-decylamino DMNQ concentration increased to 0.5 and 1.0 μM, the inhibition decreased to 554.9 ± 9.8 and 932.6 ± 16.5 cpm/well, respectively.

Fig. 3B shows that depriving VSMCs of serum in primary culture for 24 h resulted in an approximately $79.1 \pm 0.8\%$ synchronization of the cell cycle at the G_0 phase, and that adding PDGF-BB increased the percentage of cells in S phase from $5.4 \pm 0.7\%$ to $15.6 \pm 1.0\%$. The percentage of G_0/G_1 phase cells increased to $4.6 \pm 2.0\%$, $7.18 \pm 1.5\%$ ($P < 0.05$, $n = 3$, duplicate), and $13.7 \pm 1.5\%$ ($P < 0.01$, $n = 3$, duplicate) following 2-decylamino DMNQ treatment at concentrations of 0.1, 0.5, and 1.0 M, respectively. This finding suggests that 2-decylamino DMNQ is effective against DNA synthesis during the early events of the cell cycle.

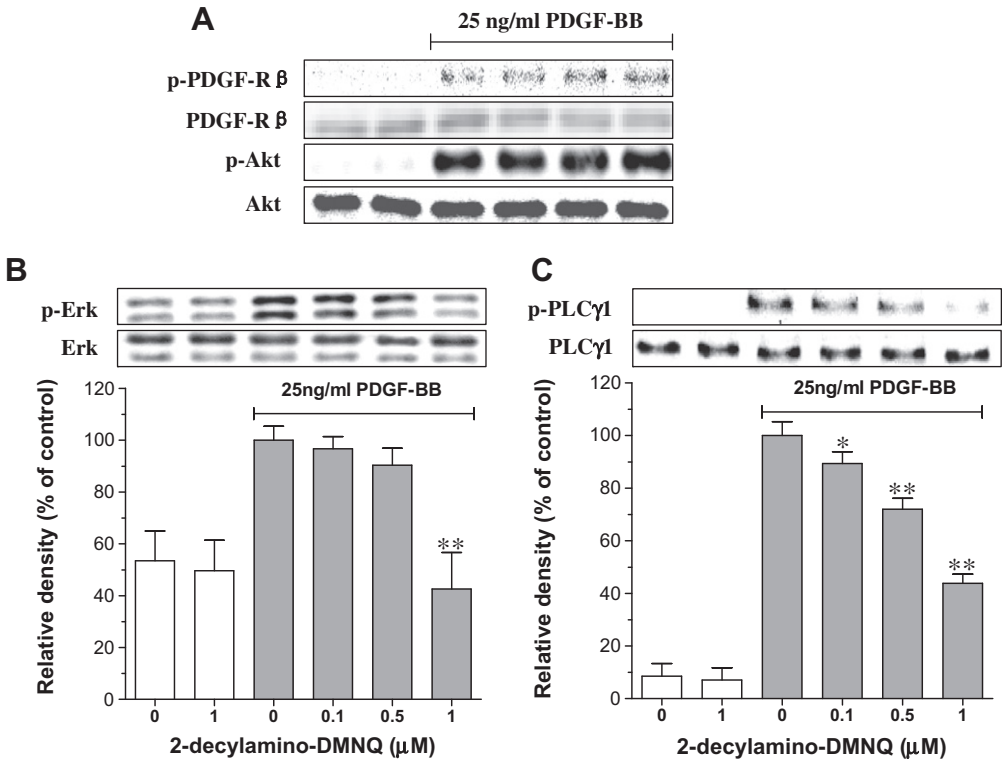

Fig. 2. Effects of 2-decylamino DMNQ on the PDGF-induced activation of PDGF-Rβ, Akt, Erk1/2, and PLCγ1. Quiescent VSMCs cultured in serum-free medium were stimulated with 25 ng/mL PDGF-BB, and the ability of various concentration of 2-decylamino DMNQ (0.1–1.0 μM) to reduce the PDGF-induced phosphorylation of (A) PDGF-Rβ and Akt, (B) Erk1/2, and (C) PLCγ1 were measured by SDS–PAGE and immunoblot using primary antibodies for PDGF-Rβ, phospho-PDGF-Rβ, Akt, phospho-Akt, Erk1/2, phospho-Erk1/2, PLCγ1, and phospho-PLCγ1 as described in the Section 2. Total amounts of PDGF-Rβ, Akt, Erk1/2, or PLCγ1 were used for normalization and immunoblots were analyzed by densitometry; the values are given as a percentage of the control. The results are an average of four similar experiments, expressed as means ± SEM. The insets display representative blots of four similar, independent experiments. Statistical differences from the PDGF control (PDGF-stimulated, but no 2-decylamino DMNQ) are indicated by $^{*}(P < 0.05)$ or $^{**}(P < 0.01)$.

3.4. Effect of 2-decylamino DMNQ on cyclin D/E, CDK2/4, and PCNA expression, and pRb phosphorylation

Cell-cycle progression is tightly regulated through a complex network of positive and negative regulatory molecules, such as cyclins and CDKs. To characterize the mechanism of cell-cycle arrest by 2-decylamino DMNQ, the effects of 2-decylamino DMNQ on cyclin D, cyclin E, CDK2, and CDK4 expression were determined. The data presented in Fig. 3C show that 2-decylamino DMNQ significantly inhibited cyclin D and E expression in a concentration-dependent manner. 2-Decylamino DMNQ significantly inhibited the expression of CDK2 and 4. These results indicate that 2-decylamino DMNQ inhibited the cell cycle at the S phase via G_0/G_1 arrest.

Cells reach the so-called restriction point in late G_1 phase, and pRb is a key component of the molecular network controlling this restriction point. Beyond this point, cells are committed to DNA replication, and further cell-cycle progression proceeds independently of growth factor stimulation. Phosphorylated pRb binds the E2F family of transcription factors, and thus inhibits transcription of E2F-responsive genes necessary for cell cycle progression. 2-Decylamino DMNQ induced a concentration-dependent inhibition of hyperphosphorylation of pRb (Fig. 3C). Furthermore, expression of PCNA, synthesized as a phospho-pRb-mediated gene product during the early G_0/G_1 and S phases, was also inhibited by 2-decylamino DMNQ in the same pattern as pRb phosphorylation was inhibited (Fig. 3C).

3.5. Effects of U0126 and U73122 on cell-cycle progression

To confirm that the Erk- or PLCγ1-mediated signaling pathway was involved in the antiproliferative action of VSMCs via G_0/G_1 cell cycle arrest, cell cycle progression analysis with Erk and PLCγ1 inhibitors was performed. The data presented in Fig. 4 show that both U0126 (Fig. 4A), an Erk inhibitor, and U73122 (Fig. 4B), a PLCγ1 inhibitor, increased the proportion of cells in the G_0/G_1 phase. The percentage of G_0/G_1 phase cells was increased to 6.7 ± 1.9% ($P < 0.05$, $n = 3$, duplicate) by U0126 at 50 μM, and to 2.3 ± 1.0% ($P < 0.05$, $n = 3$, duplicate) by U73122 at 5 μM, respectively. These findings suggest that both Erk- and PLCγ1-mediated signaling pathways are involved in the suppression of VSMC proliferation, and that the antiproliferative effect of 2-decylamino DMNQ via G_0/G_1 phase arrest is due to blockade of both the Erk1/2- and PLCγ1-mediated signaling pathways.

4. Discussion

This study has two major findings: (1) 2-decylamino DMNQ, a newly-synthesized naphthoquinone derivative, had an inhibitory effect on VSMC proliferation, which was associated with inhibition of the Erk1/2 and PLCγ1 signaling pathways, and (2) the inhibitory effect of 2-decylamino DMNQ on Erk1/2- and PLCγ1-mediated signaling pathways induced the suppression of DNA synthesis via G_0/G_1 arrest by regulating cell cycle-mediated proteins, such as cyclin D1/E and CDK2/4. This is the first report of the antiproliferative action of 2-decylamino DMNQ in VSMCs, suggesting that it may be useful as a candidate compound for the management of vascular restenosis and/or atherosclerosis.

The induction of cell proliferation is regulated by early signals, including Erk1/2, PLCγ, Akt, and PDGF-Rβ, which can be promoted by PDGF-BB [20,21]. In the current study, we found that 2-decylamino DMNQ inhibited PDGF-BB-induced VSMC proliferation.

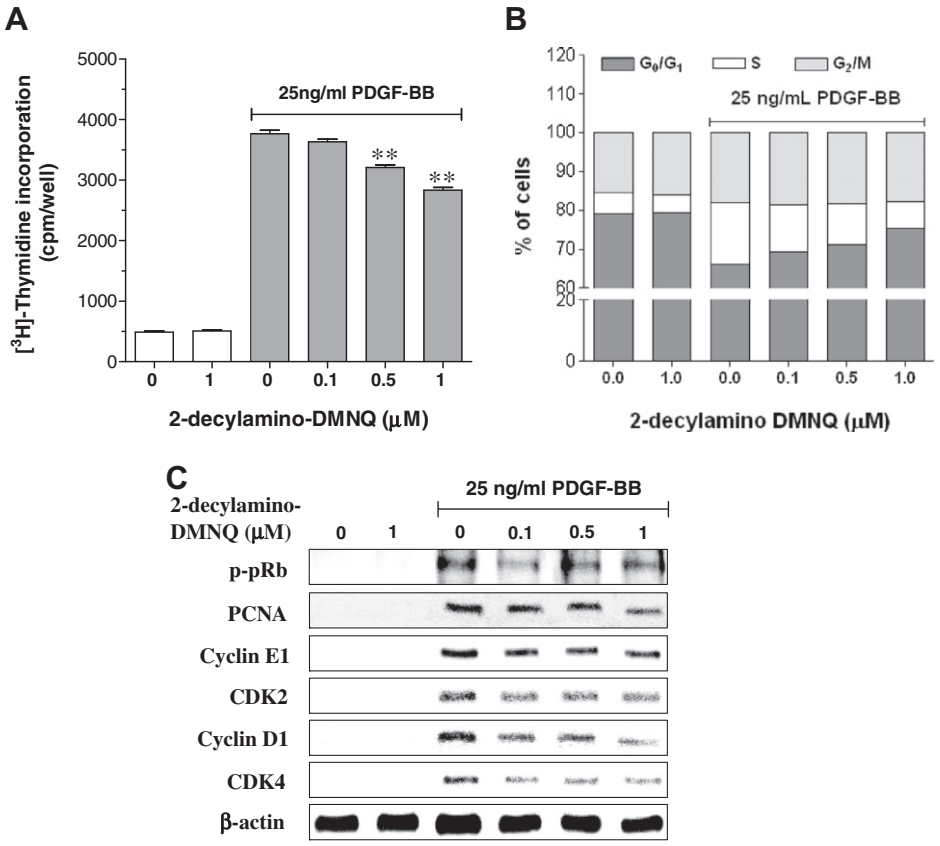

Fig. 3. Effects of 2-decylamino DMNQ on [^{3}H]-thymidine incorporation, cell cycle progression, and the expression or activation of cell cycle regulatory proteins in PDGF-stimulated VSMCs. VSMCs cultured in serum-starved medium were stimulated with 25 ng/mL PDGF-BB, and the effect of various concentrations of 2-decylamino DMNQ (0.1–1.0 μM) on DNA synthesis by the addition of 2 μCi/mL [^{3}H]-thymidine (A), cell cycle progression (B), and cell cycle regulatory proteins by SDS–PAGE followed by immunoblot using antibodies for cyclin D1, cyclin E1, CDK2, CDK4, PCNA and phospho-pRb (C) were measured as described in the Section 2. The values are expressed as means ± SEM (n = 4), and statistical differences from the PDGF control (PDGF-stimulated, but no 2-decylamino DMNQ) are indicated by **(P < 0.01).

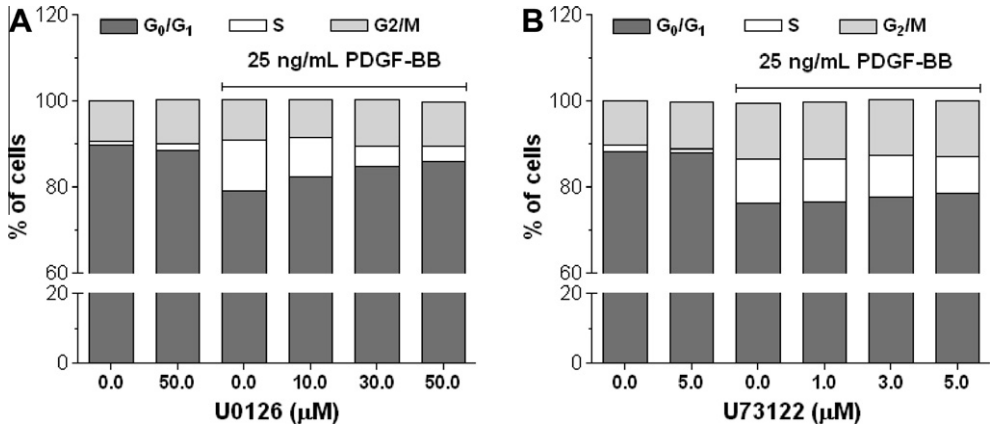

Fig. 4. Effects of U0126 and U73122 on cell cycle progression in PDGF-stimulated VSMCs. VSMCs cultured in serum-free medium were stimulated with 25 ng/mL PDGF-BB, and the effect of various concentrations of U0126, an Erk inhibitor, and U73122, a PLCγ1 inhibitor, on cell cycle progression were measured as described in the Section 2. The values are expressed as means ± SEM (n = 3, duplicate).

Phosphorylation of PDGF-Rβ, Erk1/2, PLCγ, and Akt kinase was induced by PDGF-BB, and treatment with 2-decylamino DMNQ inhibited PDGF-BB-induced phosphorylation of Erk1/2 and PLCγ, but not that of PDGF-Rβ or Akt (Fig. 2). Erk1/2, which is involved in the MAPK pathway, is an important player in the early intracellular mitogenic signal transduction for cell growth and has been implicated in proliferation and migration induced by PDGF-BB [22]. PLCγ signaling mediates a central downstream signal transduction route for various growth factors, including PDGF-BB [10].

Cellular proliferation is regulated primarily by cell cycle progression; the four distinct sequential phases of the cycle are G_0/G_1, S, G_2, and M. In present study, 2-decylamino DMNQ inhibited DNA synthesis through G_0/G_1 arrest of cell cycle progression (Fig. 3A and B). The G_0/G_1 phase is controlled by the sequential activation of CDKs, which are serine/threonine protein kinases that phosphorylate pRb. CDK2 and CDK4 are key mediators during the G_1 to S phase progression of the cell cycle, forming complexes with cyclin E and D [23,24,14]. These complexes phosphorylate many

proteins, resulting in hyperphosphorylation of pRb, which then releases transcription factors that promote DNA synthesis [25,3]. Our results showed that 2-decylamino DMNQ not only inhibited the expression of CDK2, CDK4, cyclin D, and cyclin E, but also suppressed the phosphorylation of pRb in a concentration-dependent manner (Fig. 3C). Although several CDKs are known to phosphorylate pRb, suppression of CDK2 alone may be sufficient to prevent pRb hyperphosphorylation [26,27]. Thus, the inhibition of CDK2, CDK4, cyclin D, and cyclin E expression, as well as pRb phosphorylation, may be sufficient to achieve cell cycle arrest. Moreover, the expression of PCNA, which is synthesized as a phospho-pRb-mediated gene product in the early G_0/G_1 and S phase of the cell cycle [28], was also inhibited (Fig. 3C). This result confirms the arrest of the cell cycle by 2-decylamino DMNQ. Furthermore, because both an Erk inhibitor and a PLCγ inhibitor increased the proportion of cells in the G_0/G_1 phase of the cell cycle (Fig. 4), our results indicate that the antiproliferative effects of 2-decylamino DMNQ in VSMCs are mediated through the blockade of both Erk1/2- and PLCγ1-mediated signaling pathways, leading to G_0/G_1 cell cycle arrest.

In conclusion, the present study provides evidence that 2-decylamino DMNQ inhibits PDGF-BB-induced VSMC proliferation by inhibiting Erk1/2- and PLCγ1-mediated signaling pathways. In addition, the expression of cyclin D, cyclin E, CDK2, CDK4, and PCNA is subsequently downregulated, and the pRb phosphorylation is inhibited, leading to G_0/G_1 cell cycle arrest. Thus, 2-decylamino DMNQ may be useful as a candidate antiproliferative agent for the treatment of vascular restenosis in coronary revascularization procedures and stent implantation.

Acknowledgments

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