Potent Inhibition of Serum-Stimulated Responses in Vascular Smooth Muscle Cell Proliferation by 2-Chloro-3-(4-hexylphenyl)-amino-1,4-naphthoquinone, a Newly Synthesized 1,4-Naphthoquinone Derivative

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Atherosclerosis, a disease of the large arteries, is the primary cause of heart disease and stroke. The abnormal proliferation of vascular smooth muscle cells (VSMCs) in arterial walls is an important pathogenetic factor of vascular disorders like atherosclerosis and restenosis after angioplasty. In the present study, the possible anti-proliferative effect of a synthetic 1,4-naphthoquinone derivative, 2-chloro-3-(4-hexylphenyl)-amino-1,4-naphthoquinone (NQ304) was investigated on rat aortic VSMCs. NQ304 was shown to potently inhibit 5% fetal bovine serum (FBS)-induced growth of VSMCs. Pre-treatment of VSMCs with NQ304 (1—10 μM) for 24 h resulted in significant cell number decreases, i.e., inhibition percentages were 44.75±10.77, 73.85±6.38 and 89.77±6.52% at NQ304 concentrations of 1, 5 and 10 μM, respectively. NQ304 was also found to significantly inhibit 5% FBS-induced DNA synthesis in a concentration-dependent manner. Furthermore, NQ304 elevated p21cip1 and p27kip1 mRNA levels and caused G1/G0 phase arrest in cell cycle progression. However, no evidence of NQ304-induced apoptotic or necrotic cell death was obtained, as determined by flow cytometry analysis and DNA fragmentation assays. To investigate the mechanism underlying the anti-proliferative effect of NQ304, we examined the effects of NQ304 on c-fos mRNA expression, activator protein-1 (AP-1) binding activity and extracellular signal-regulated kinase1/2 (ERK1/2) and Akt activation. Pre-treatment of VSMCs with NQ304 (1—10 μM) was found to significantly inhibit the 5% FBS-induced phosphorylations of ERK1/2 and Akt, the activation of AP-1 and the expression of c-fos. These data suggest that the anti-proliferative and cell cycle arresting effects of NQ304 on serum-induced VSMCs may be mediated by AP-1 activation downregulation via the suppression of phosphatidylinositol 3-kinase 3-kinase (PI3K)/Akt and ERK1/2 signaling pathways, and it may contribute to the prevention of atherosclerosis through inhibition of VSMC proliferation.

Key words 1,4-naphthoquinone; atherosclerosis; cell cycle; proliferation

Several cytokines are involved in the development of atherosclerotic lesions. VSMC proliferation induced by growth factors, such as platelet-derived growth factor (PDGF), is known to be a prerequisite of the intimal thickening that is observed after angioplasty, and mitogen-activated protein kinases (MAPKs) are considered to be a critical step in the proliferation of VSMCs.1,2) Pyles et al. reported that MAPKs are activated in response to balloon stretch injury in porcine carotid arteries.3) Moreover, it has been demonstrated using a carotid artery balloon injury model that MAPK signaling, particularly ERK1/2 signaling, is increased and that medial cell replication following injury is reduced by PD098059.4) The Akt, a serine/threonine kinase is another important pathway triggered by growth factors, and the activity of Akt has been shown to be stimulated by growth factors acting through receptor tyrosine kinases such as PDGF, epidermal growth factor (EGF), and insulin receptors, and the activation of Akt/PKB by these growth factors has been shown to be mediated by PI3K.5—7) The signaling pathway from PI3K to Akt/protein kinase B (PKB) was implicated in some cellular responses of PI3K including protection from apoptosis.8,9) Activation of MAPKs transiently stimulates c-fos mRNA expression, which is responsible for the increased VSMC proliferation associated with the development of atherosclerotic disease.10) AP-1, a transcriptional factor is also required for cell proliferation and tumor progression, and it is also known to regulate the c-fos mRNA expression.11) After vascular injury, VSMCs are stimulated to divide in response to mitogens, and that they exit G1 and enter the S phase.12) Recent studies have implicated p27kip1 and p21cip1 as inhibitors of cell-cycle progression by associating them with the growth inhibitory effects of antiproliferative agents. Cyclin-dependent kinase inhibitors (CKIs), such as p27kip1 and p21cip1, inactivate cyclin-CDK complexes in the G1 phase, and are upregulated during arterial repair whereupon they negatively regulate VSMC growth.13,14)

Compounds with a 1,4-naphthoquinone backbone have many biological and pharmacological effects, i.e., antiviral, antifungal, anticancer, and antiplatelet activities.15—21) In previous study, we reported that NQ304, a newly synthesized 1,4-naphthoquinone derivative, has potent antithrombotic effect in mice in vivo, and antplatelet activities in vitro and ex vivo.22,23) However, a synthetic 1,4-naphthoquinone analogue, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone, was reported to potently inhibit tumor cell growth and to arrest cell cycle progression at G1 and G2/M.24) Therefore, we sought to determine whether NQ304 can also suppress VSMC proliferation. In this study, we provide evidences that NQ304 potently inhibits VSMC proliferation and that it inhibits the ERK1/2 and PI3K/Akt signaling pathways in VSMCs. Our results reveal that NQ304 is a potential agent for the treatment of vascular disorders like atherosclerosis.

MATERIALS AND METHODS

Materials 2-Chloro-3-(4-hexylphenyl)-amino-1,4-naphthoquinone (NQ304) compound was kindly provided by Dr. Chung-Kyu Ryu (Ewha Women’s University, Seoul).25)
NQ304 was dissolved in dimethylsulfoxide (DMSO) and added to Dulbecco’s modified Eagle’s medium (DMEM) with a maximum final DMSO concentration of 0.05%. Cell culture materials were purchased from Gibco-BRL (MD, U.S.A.). The phospho-ERK1/2, ERK1/2, phospho-Akt, and Akt antibodies were purchased from New England Biolabs (MA, U.S.A.). The other chemicals used in the experiments were of the highest analytical grade commercially available.

Cell Culture Rat aortic vascular smooth muscle cells (VSMCs) were isolated by enzymatic dispersion, as previously described according to a modification of the method described by Chamley et al. Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 8 mM HEPES and 2 mM l-glutamine at 37 °C in an humidified 95% air/5% CO₂ incubator. The purity of VSMCs in culture was confirmed immunocytochemically based on α-smooth muscle actin localization. VSMCs at passages 4 to 8 were used in this experiment.

Cell Counts Rat aortic VSMCs were seeded onto 12-well culture plates at 1×10⁵ cells/ml, and then cultured in DMEM containing 10% FBS at 37 °C for 24 h. Under these conditions, a cell confluence of 70% was reached. The medium was then replaced with serum-free medium containing NQ304, and cells were then stimulated with 5% FBS and trypsinized using trypsin-EDTA, and counted using a hemocytometer.

DNA Synthesis by [³H]-Thymidine Incorporation For [³H]-thymidine incorporation experiments, VSMCs were seeded onto 24-well culture plates using conditions identical to those described above, and 2 μCi/ml of [³H]-thymidine was added to medium. Reactions were terminated by aspirating medium and subjecting the cultures to sequential washes with phosphate-buffered saline (PBS) containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v) on ice. The acid-insoluble [³H]-thymidine was extracted into 250 μl of 0.5 M NaOH/well, and this solution was mixed with a 3 ml scintillation cocktail (Ultimagold, Packard Bioscience, CT, U.S.A.). The mixed solution was quantified using a liquid scintillation counter (LS3801, Beckman, Düsseldorf, Germany), and 50 μl of residual solution was measured using a BCA Protein Assay Reagent Kit (Pierce Biotechnology).

Analysis of Cellular DNA Contents by Flow Cytometry Rat aortic VSMCs were trypsinized and cells were centrifuged at 400 × g for 10 min. The centrifuged pellets were then suspended in 70% ethanol and fixed overnight at 4 °C. Fixed cells were briefly vortexed and centrifuged at 400 × g for 5 min. The ethanol was then discarded and cells were stained with 0.5 ml of propidium iodide (PI) solution (50 μg/ml PI in sample buffer containing 100 μg/ml of RNase A), and each sample was then incubated for 30 min at room temperature. The PI-DNA complex in each cell nucleus was measured using a FACSCalibur (Becton & Dickinson Co., U.S.A.). The rate of G₁/G₂, S and G₂/M phases were determined using a computer program ModFitLT (Verity House Soft-ware, Topsham, ME, U.S.A.) and images were captured in Polaroid film.

Flow Cytometry of Annexin V Binding Cells were pre-cultured in serum-free medium in the presence or absence of NQ304 (10 μM) for 24 h, and then stimulated with 5% FBS for 24 h. Apoptosis was determined by staining with Alexa Fluor 488-labeled annexin V (Annexin-V-FLUOS Staining Kit, Roche). After the incubation, floating as well as adherent cells that were later trypsinized were pooled and centrifuged for 5 min at 1000 × g. Pelleted cells were washed in cold phosphate-buffered saline (PBS). Thereafter, cells were centrifuged again for 5 min at 1000 × g and resuspended in 100 μl Annexin-Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) yielding a cell density of 1×10⁶ cells/ml. Five microliters of annexin V conjugate and 1 μl of 100 μg/ml PI working reagent were added to each 100 μl of cell suspension. The cells were incubated at room temperature for 15 min. After the incubation period, 400 μl of Annexin-Binding Buffer was added and mixed gently, and the samples were kept on ice. The stained cells were analyzed by flow cytometry, where the fluorescence emission was measured at 530 nm (Alexa Fluor 488). The rate of cells determined using a computer program ModFitLT (Verity House Soft-ware, Topsham, ME, U.S.A.).

DNA Fragmentation Assays Genomic DNA was extracted using G-Dex™ Genomic DNA extraction kits according to the manufacturer’s instructions (Intron Biotech, Seoul). In brief, cells were trypsinized, collected, rinsed twice with cold PBS, and lysed at 65 °C for 10 min using lysis buffer. The obtained DNA was then incubated with RNase A (100 μg/ml) at 37 °C for 1 h, treated with 100% isopropanol, and precipitated by centrifugation at 12000 × g for 30 min at 4 °C. DNA pellets were then washed with 70% ethanol and dissolved in DNA hydration buffer containing TE buffer. DNA concentrations were determined by spectrophotometry at 260 nm. The 20 μg of DNA was electrophoresed for 3 h at 30 V in a 1.8% agarose gel containing ethidium bromide. DNA fragmentation bands were photographed under UV light (Hoffer, San Francisco, CA, U.S.A.) and images were captured on Polaroid film.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR) Total RNA was isolated by using a single-step procedure using guanidine isothiocyanate–phenol–chloroform extraction and TRIzol reagent (Gibco-BRL). In brief, 1 μg of total RNA was reverse-transcribed to synthesize single strand cDNA using a First-strand synthesis system kit (Gibco-BRL). Subsequently, 2 μl of the RT reaction products were subjected to PCR in order to co-amplify a fragment of c-fos (sense; 5’-CCCCGTGACACACAGGGAC-3’, antisense; 5’-GCAATCTCGGTCTGCA-ACGC-3’), p21cip1 (sense; 5’-TATGGCTGCTGTCCTGTTT-CGG-T3’, antisense; 5’-TCTGTAGGCTGTGCTGCTT-3’), and p27kip1 (sense; 5’-GTCACAGCTGAGCTGTGAC-TA-3’, antisense; 5’-CAAATGCGGTCTCAGAATG-3’). The GAPDH gene was used as an internal control (sense; 5’-CCTGACACACACAGGTCTT-3’, antisense; 5’-GATGCGACTGAGCTCTCCGTG-3’). After an initial denaturation step at 94 °C for 5 min, PCR was performed over 24 cycles (94 °C/30s, 58 °C/30s and 72 °C/1 min) for c-fos, p21cip1, p27kip1 and GAPDH. The images were captured on Polaroid film. The expressions of c-fos, p21cip1 and p27kip1 were normalized versus GAPDH. Band intensities were quantified using Scion-Image software operating in the Windows environment.

Immunoblotting SDS–PAGE was performed on cell
Electrophoretic Mobility Shift Assays (EMSA)

EMSA were performed by using a slight modification of a previously described method.32 Briefly, cells were washed three times with ice-cold phosphate-buffered saline (PBS, pH 7.6) and pelleted. Pellets were resuspended in 400 μl of cold buffer containing 10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and then centrifuged at 15000 g for 6 min to remove everything except nuclei. Nuclear pellets were resuspended in a second buffer containing 20 mM HEPES, 20% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF, centrifuged at 15000×g for 6 min; at this stage the supernatant contained nuclear proteins. Protein levels were determined using a modification of the Bradford method (Bio-Rad Bulletin 1177, Bio-Rad, CA, U.S.A.). The DNA binding activity of AP-1 was assayed according to the manufacturer’s instructions (Promega Co., WI, U.S.A.). In brief, 10 μg of nuclear protein was incubated in 25 μl total volume of incubation buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 4% glycerol, 0.08 mg/ml salmon sperm DNA) at 4 °C for 15 min, and this was followed by another 20 min incubation with 250 μCi [γ-³²P] ATP-labeled oligonucleotide containing AP-1 binding site at room temperature. The DNA-protein binding complexes obtained were run on a 6% non-denatured polyacrylamide gel at 150 V for 1 h. Gels were then dried and autoradiographed using Kodak MR Film at −80 °C overnight.

RESULTS

**NQ304 Inhibited DNA Synthesis and Cell Proliferation**

We assessed the inhibitory effect of NQ304 on serum-induced VSMC proliferation. VSMC numbers in culture were determined after 24, 48, or 72 h of stimulation with 5% FBS. Pre-treatment of VSMCs with 1, 5 or 10 μM of NQ304 for 24 h also resulted in significant cell number decreases, i.e., inhibition percentages were 44.75 ± 2.89, 73.85 ± 6.38 and 89.77 ± 6.52% at NQ304 concentrations of 1, 5 and 10 μM, respectively (Fig. 1A). In addition, stimulation of cells with 5% FBS increased cell numbers in a time-dependent manner, and pre-treatment with 10 μM NQ304 before stimulation with 5% FBS completely inhibited this increase in cell number at 72 h (Fig. 1B). To determine the effect of NQ304 on the proliferation of VSMCs *in vitro*, as an index of cell growth, [³H]-thymidine incorporation into DNA was also measured. Without FBS stimulation, VSMCs did not incorporate [³H]-thymidine into DNA, but the addition of 5% FBS to VSMCs resulted in the incorporation of [³H]-thymidine into DNA. Moreover, pre-treatment of VSMCs with 1, 5 or 10 μM NQ304 for 24 h significantly inhibited [³H]-thymidine incorporation into DNA in a concentration-dependent manner, and this inhibition was significant at 5 and 10 μM (Fig. 1C).

**NQ304 did not Affect Apoptotic and Necrotic Cell Death**

To evaluate whether the reduction in the cell proliferation assay was not only caused by induction of cell death, we assessed the percentage of sub-G₁ in cell cycle phase. VSMCs were pre-treated with NQ304 (10 μM) in serum-free January 2007 123 lysates using 7.5—10% acrylamide gels according to the method devised by Laemmli.31 Proteins were transferred to PVDF membranes (Millipore Corp.) and membranes were blocked overnight at 4 °C in Tris-buffered saline containing 0.1% Tween 20 (TBS/T) and 5% skimmed milk powder. Membranes were then incubated with 1:2000 diluted phospho-ERK1/2, phospho-Akt, ERK1/2, or Akt antibodies. Blots were washed with TBS/T, and then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibodies (New England Biolabs, MA, U.S.A.). Proteins were detected using an enhanced chemiluminescence (ECL) western blotting detect reagent (Amersham Biosciences). Band intensities were quantified using Scion-Image software operating in the Windows environment.

**Statistical Analysis**

The experimental results are expressed as means±S.D. One-way analysis of variance (ANOVA) was used for multiple comparisons, and this was followed by Dunnett’s test. Differences were considered significant at the *p*<0.05 or **p**<0.01 level as indicated.

**RESULTS**

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**Fig. 1.** Effect of NQ304 on Serum-Induced Cell Proliferation

(1B) Effects of NQ304 on serum-induced VSMC cell numbers. Cells were pre-cultured in serum-free medium in the presence or absence of NQ304 (1—10 μM) for 24 h, and then stimulated with 5% FBS for 24—72 h. Cells were trypsinized and then counted using a hemocytometer. (C) Effects of NQ304 on DNA synthesis in serum-in-duded VSMCs. Cells were pre-cultured in serum-free medium in the presence or absence of NQ304 (1—10 μM) for 24 h, and then stimulated with 5% FBS for 20 h. [³H]-Thymidine (2 μCi/ml) was added to the medium and cells were then incubated for 4 h. Radioactivity was measured using a liquid scintillation counter. Values represent means±S.D. of three different assays. *p*<0.05, **p**<0.01 compared with FBS alone.
medium for 24 h, and then were stimulated with 5% FBS for 24 h. Sub-G1 phase was analyzed for cell death effects, but percentages of cell death (sub-G1) were not altered significantly by these treatments (Fig. 2A). Since anti-proliferation is often accompanied by apoptosis, we also measured the apoptotic effects of NQ304 in VSMCs using the same conditions. NQ304 at 10 μM did not induce Annexin-V binding (Fig. 2B) and DNA fragmentation (Fig. 2C) in VSMCs, which is one index of apoptosis. These results indicate that NQ304 blocks VSMC proliferation without inducing apoptotic or necrotic cell death.

NQ304 Induced Cell Cycle Arrest on G0/G1 Phase
Since growth factors regulate cell cycle progression, we assessed whether NQ304 causes cell cycle arrest. As shown in Table 1, after 24 h of stimulation with 5% FBS, we observed an increase in the percentage of cells in the G2/M phase and a reduction in the G0/G1 phase (DMSO; G0/G1-89.17%, S-1.64%, G2/M-9.18%, 5% serum; G0/G1-72.53%, S-10.56%, G2/M-16.89%). Moreover, the pre-incubation of VSMCs with NQ304 (10 μM) caused a significant accumulation of cells in the G0/G1 phase, to the same extent as that observed for serum free media, indicating complete cell cycle arrest (5% serum +NQ304; G0/G1-86.81%, S-3.99%, G2/M-9.18%). This effect strongly correlates with the observed inhibitory effects of NQ304 on VSMC counts and [3H]-thymidine incorporation assays. Cell cycle progression is regulated by the activity of different cyclin-dependent kinases in complex with cyclins and by cell cycle inhibitors such as p21cip1 and p27kip1. To analyze the molecular mechanisms which lead to the observed G1-phase block after NQ304 treatment, we determined the gene expression status of cell cycle inhibitors by western blot analysis.13,33) As shown in Fig 3, pre-incubation of NQ304 was found to lead to marked inductions of p21cip1 and p27kip1 mRNA in a concentration-dependent

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Fig. 2. Effect of NQ304 on Apoptotic or Necrotic Cell Death

Cells were pre-cultured in serum-free medium in the presence or absence of NQ304 (10 μM) for 24 h, and then stimulated with 5% FBS for 24 h. (A) Effect of NQ304 on cell death of serum-stimulated VSMCs. The cells were harvested by trypsinisation, fixed with 80% ethanol and the DNA content was measured using ethidium-iodide staining and flow cytometric analysis. Sub-G1 phase (M1) represents the percentage of cell death and similar sub-G1 phase patterns for assays performed in duplicate. (B) Effect of NQ304 on apoptosis or necrosis of serum-stimulated VSMCs. Apoptotic (R3; Annexin V+/PI-) or necrotic cells (R2; Annexin V+/PI+) were identified by means of Annexin V binding and PI dye detected by flow cytometry. (C) Effect of NQ304 on DNA fragmentation in serum-stimulated VSMCs. DNA fragmentation was analyzed by electrophoresis on 1.8% agarose gel as described in Materials and Methods.
manner during 5% FBS stimulation.

**Table 1. Cell Cycle Analysis of Vascular Smooth Muscle Cells in the Presence of NQ304**

<table>
<thead>
<tr>
<th></th>
<th>G1/G0</th>
<th>S</th>
<th>G2/M</th>
</tr>
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<tbody>
<tr>
<td>DMSO</td>
<td>89.17±0.39</td>
<td>1.64±0.35</td>
<td>9.18±0.64</td>
</tr>
<tr>
<td>NQ304</td>
<td>90.30±0.38</td>
<td>1.48±0.06</td>
<td>8.21±0.43</td>
</tr>
<tr>
<td>Serum+DMSO</td>
<td>72.53±1.82</td>
<td>10.56±2.53</td>
<td>16.89±0.97</td>
</tr>
<tr>
<td>Serum+NQ304</td>
<td>86.81±0.71</td>
<td>3.99±0.61</td>
<td>9.18±0.34</td>
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**Fig. 3. Effect of NQ304 on Serum-Induced Expressions of p21<sup>cip1</sup> and p27<sup>kip1</sup> mRNA**

Confluent cells were pre-cultured in the presence or absence of NQ304 (1—10 μM) in serum-free medium for 24 h, and quantitative RT-PCRs for p21<sup>cip1</sup> and p27<sup>kip1</sup> were performed at 30 min after 5% FBS stimulation. The GAPDH gene was used for normalization. Relative densities were quantified by scanning densitometry, and the values shown represent means±S.D. from three different assays. ∗p<0.05 and ∗∗p<0.01 compared with FBS alone.

**Fig. 4. Effect of NQ304 on Serum-Induced c-fos mRNA Expression and AP-1 DNA-Binding Activity**

(A) Effect of NQ304 on c-fos mRNA expression in serum-stimulated VSMCs. Confluent cells were pre-cultured in the presence or absence of NQ304 (1—10 μM) in serum-free medium for 24 h, and quantitative c-fos RT-PCR was performed at 30 min after 5% FBS stimulation. The GAPDH gene was used for normalization. Relative densities were quantified by scanning densitometry, and the values shown represent means±S.D from three different assays. ∗p<0.01 compared with FBS alone. (B) Effect of NQ304 on AP-1 DNA-binding activity in serum-stimulated VSMCs. Confluent cells were pre-cultured in the presence or absence of NQ304 (10 μM) in serum-free medium for 24 h, and then stimulated with 5% FBS for 40 min. Gel shift assays were performed as described in Materials and Methods. Similar DNA binding activity patterns for assays were performed in duplicate.

**DISCUSSION**

Vascular smooth muscle cell (VSMC) proliferation is an important process for plaque formation in primary atherosclerosis. Moreover, multiple growth factors and cytokines have been found to regulate vascular cell proliferation, platelets, inflammatory cells, damaged vascular cells, and activated VSMC which secrete growth factors and cytokines that trigger signaling pathways.

The aim of this study was to assess the effect of a newly synthesized 1,4-naphthoquinone derivative on the proliferation of serum-induced rat aortic VSMCs. The anti-proliferative effects of 1,4-naphthoquinone derivatives on rat aortic VSMCs were examined by direct cell counting and [³H]-thymidine incorporation assays, and we found that 2-chloro-3-(4-hexylphenyl)-amino-1,4-naphthoquinone (NQ304) is a potent growth inhibitor of VSMCs (Fig. 1). However, the anti-proliferative effect of NQ304 on VSMCs was shown not to be due to apoptotic or necrotic cell death, as demonstrated by flow cytometry and DNA fragmentation analysis (Fig. 2).

After vascular injury, VSMCs are stimulated to divide in response to mitogens, and that they exit the G1 phase and enter the S phase. As shown by cell cycle distribution in our study, the anti-proliferative effect of NQ304 is associated with an accumulation of cells in G0/G1 phase of the cycle (Table 1). We also observed elevated p21<sup>cip1</sup> and p27<sup>kip1</sup> mRNA levels, inhibitors of the cell cycle G1 phase, by...
might be achieved via the down-regulation of the c-fos gene expression and AP-1 activation. NQ304 suppressed serum-induced AP-1 DNA-binding activity (Fig. 4B), and we also observed a decreased c-fos mRNA level (Fig. 4A). Based on these results, we believe that NQ304 may mediate cell cycle progression and proliferation by downregulating c-fos and inhibiting AP-1 binding activity.

ERK 1/2 and Akt, major signal transducing molecules regulate cellular proliferation, differentiation, and apoptosis. These are upstream signaling pathways in the regulation of c-fos and AP-1 binding activation. Our present results showed that NQ304 significantly inhibited the activation of ERK 1/2 induced by 5% FBS in VSMCs (Fig. 5A). We also found that NQ304 caused marked decreases in the serum-induced phosphorylations of Akt (Fig. 5B), and that these were of the same pattern as that noted for the inhibition of ERK 1/2 phosphorylation by NQ304. Although, several studies have indicated that the ERK 1/2 and the Akt pathways appear to grow at distinct effectors, and that signaling pathways are regulated independently by various stimulators and intermediate signal transduction molecules. More recent studies have suggested that these two pathways also cross talk, possibly via an interaction between Raf and Akt. Similarly, Ahn et al. reported that epigallocatechin-3 gallate (EGCG) blocks MAPK and PI3K signal transduction pathways in human smooth muscle cells. Therefore, these results suggest that the inhibitory effect of NQ304 on the serum-induced VSMC proliferation might be attributed, at least in part, to its ability to inhibit ERK 1/2 and Akt activation. However, in our study, the precise mechanism of VSMC proliferation inhibition by NQ304 remains unknown and further studies are needed to determine the target site of NQ304.

In conclusion, our present study showed that the anti-proliferative activity of NQ304, a newly synthesized 1,4-naphthoquinone derivative, is mediated by the downregulations of AP-1 activation and c-fos expression via the suppression of the PI3K/Akt and ERK 1/2 signaling pathways in VSMCs. These findings may offer a new insight into the anti-cardiovascular agent of 1,4-naphthoquinone.

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