

# Activation of Ras signaling pathways by pyrroloquinoline quinone in NIH3T3 mouse fibroblasts

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**Abstract.** Pyrroloquinoline quinone (PQQ) has been implicated in certain physiological activities in mammals such as functioning as a potent growth factor in mice, and promoting DNA synthesis in human fibroblasts. These are clearly important physiological functions, however, the molecular mechanisms involved in PQQ activity are not yet fully understood. In order to address this, in this study we analyzed the effects of PQQ on the proliferation of NIH3T3 mouse fibroblasts and on their intracellular signal transduction mechanism. When activated c-Ha-ras-transformed NIH3T3 cells were treated with PQQ in the presence of 0.5% calf serum in DMEM, the cells showed significantly increased viability. After PQQ addition, flow cytometric analysis revealed a decrease in the population of cells in the G<sub>0</sub>/G<sub>1</sub> phase and a concomitant increase in cells in the S and G<sub>2</sub>/M phases. Although treatment with SNAP, an NO donor, reduced cell viability, this effect was abolished by the addition of PQQ. Activation of ERK and PKC- $\epsilon$  was detected immediately after the addition of PQQ, and subsequent

increases in the phosphorylation of Rb and c-Jun were observed. On the other hand, protein expression levels of growth-inhibitory molecules such as I $\kappa$ B and p27 decreased after PQQ treatment. These results suggest that PQQ stimulates cell proliferation through NO-sensitive Ras-mediated signaling pathways.

## Introduction

Pyrroloquinoline quinone (PQQ, Fig.1) was identified as a novel redox cofactor in several bacterial dehydrogenases in 1979 (1). PQQ is synthesized by a number of microorganisms and is essential for their growth (2-4). Several reports have suggested physiological and pharmacological effects of PQQ such as protection from liver damage by ethanol in rats (5), oxidation of the *N*-methyl-D-aspartate receptor redox site in rat cortical neurons (6), protection from reoxygenation injury in isolated rabbit heart (7), increase in levels of nerve growth factor in mouse astroglial cells (8), inhibition of melanogenesis in murine B16-F10 melanoma (9), and enhancement of rat sciatic nerve regeneration (10). Killgore *et al* (11) reported that mice fed with a PQQ-deficient diet grew poorly, suggesting a nutritional role for PQQ in mammals. Although PQQ is not synthesized in mammals, PQQ has invoked considerable interest because of its presence in human and rat tissues (12,13). Recently, the first eukaryotic PQQ-dependent enzyme, 2-aminoadipic 6-semialdehyde dehydrogenase (EC 1.2.1.31), was identified, indicating that PQQ may function as a vitamin in mammals (14). However, this potential role is currently under debate (15-17).

Various quinones are involved in a number of biochemical reactions (18) including redox reactions such as the transport of electrons in the mitochondrial respiratory chain, the carboxylation of blood coagulation factors, and a variety of alkylation reactions. The exposure of rat liver epithelial cells to menadione (2-methyl-1,4-naphthoquinone, vitamin K<sub>3</sub>), a quinone derivative, was shown to lead to the activation of extracellular signal-regulated protein kinase (ERK) (19). ERK is well known as one of the mitogen-activated protein kinases (MAPKs) (20) and for its prominent role in controlling proliferation, differentiation and cell survival

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**Abbreviations:** CDK, cyclin/cyclin-dependent kinase; DFMO, difluoromethylornithine; ECs, endothelial cells; E2F, E2 factor; ERK, extracellular signal-regulated protein kinase; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; I $\kappa$ B, inhibitory  $\kappa$ B; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NO, nitric oxide; NOS, nitric oxide synthase; ODC, ornithine decarboxylase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PQQ, pyrroloquinoline quinone; Rb, retinoblastoma protein; ROS, reactive oxygen species; SNAP, *S*-nitroso-*N*-acetylpenicillamine

**Key words:** pyrroloquinoline quinone, cell proliferation, signal transduction

(21). In a previous study, we demonstrated that PQQ was effective in increasing [ $^3\text{H}$ ]thymidine incorporation into DNA in cultured human fibroblasts (22). Understanding the mechanism by which PQQ modulates DNA synthesis could provide critical information pertinent to cell growth and other physiological activities in mammals; however, details of the molecular mechanisms by which PQQ exerts its effects, such as the MAPK cascade, remain unclear. In the present study, we investigated the interrelationship between the effects of PQQ on cell growth and the process of cellular signaling transduction by PQQ, using activated c-Ha-ras-transformed NIH3T3 cells.

## Materials and methods

**Chemicals.** PQQ disodium salt was obtained from Wako Pure Chemical Industries (Osaka, Japan). Difluoromethylornithine (DFMO) hydrochloride, erbstatin, genistein, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) dihydrochloride and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were purchased from Sigma Chemicals (St. Louis, MO, USA). Other common chemicals were of the highest purity commercially available.

**Cells and culture conditions.** Activated c-Ha-ras-transformed NIH3T3 (Ras-NIH3T3) cells were kindly provided by Dr Takao Sekiya (National Cancer Center Research Institute, Tokyo, Japan) (23). The Ras-NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and maintained in a 37°C humidified atmosphere containing 5% CO<sub>2</sub> in air.

**Flow cytometry analysis.** Ras-NIH3T3 cells were washed three times with PBS and treated with staining solution (50 µg/ml propidium iodide, 4 mM sodium citrate, 0.5 mg/ml RNase A and 0.1% Triton X-100) on ice for 10 min. NaCl was then added to the solution to a final concentration of 0.15 M. The cells were examined by FACScan, and cell cycle distribution was analyzed by ModFit LT Software (Becton, Dickinson and Co., Franklin Lakes, NJ, USA).

**Method of assessing growth effects.** Logarithmically growing cells ( $1 \times 10^4$ ) were plated in each well of 96-well plates containing various concentrations of the test compounds and then cultured for 72 h according to the method of Mosmann (24) as described previously (25). The culture was incubated for 4 h in the presence of 0.5 mg/ml MTT, and then the absorbance at 570 nm (using a reference wavelength of 655 nm) was measured. The absorbance reflected the viable cell number and was reported as a percentage of the viability of the control cells.

**Preparation of cell extract and Western blot analysis.** Ras-NIH3T3 cells were grown in 6-cm tissue culture dishes and treated with the test compounds. The cells were then washed with phosphate-buffered saline (PBS) 3 times and incubated in lysis buffer (0.5% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM leupeptin, 50 mM antipain, 50 mM pepstatin A and 50 mM acetyl-Leu-Leu-norleucinal) for 10 min at 4°C.

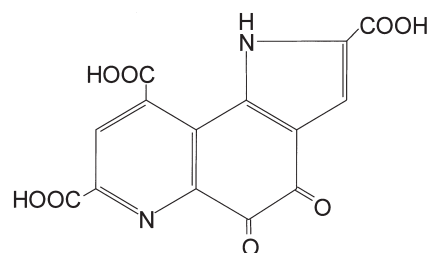


Figure 1. Chemical structure of PQQ.

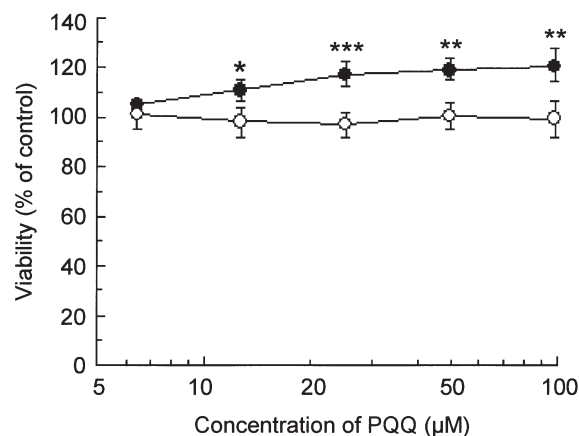


Figure 2. Effects of PQQ on proliferation of Ras-NIH3T3 cells. Cells were cultured in DMEM containing 0.5% (●) or 5% (○) calf serum in the presence of various concentrations of PQQ for 72 h. After incubation with MTT for 4 h, the absorbance at 570 nm was measured and expressed as a percentage of that in the absence of PQQ. Data are means  $\pm$  SD (bars) of the values of 4 experiments. Asterisks indicate significant differences between 0.5 and 5% calf serum; \* $p < 0.05$ , \*\* $p < 0.005$  and \*\*\* $p < 0.001$  as determined by the Student's *t*-test.

The cell lysate was centrifuged at 13,000  $\times$  g for 10 min, and then the supernatant was lyophilized and used as 'cytoplasmic extract'. The pellet following centrifugation was washed once with lysis buffer, directly dissolved in SDS-sample buffer and used as 'nuclear extract'. Immunoblot analysis was carried out using Immuno-Star (Wako Pure Chemical Industries). The antibodies used were anti-phospho-specific ERK, anti-phospho-specific c-Jun, anti-phospho-specific PKC- $\epsilon$ , anti-phospho-specific Rb, anti-I $\kappa$ B and anti-p27.

## Results

**Effect of PQQ on cell viability.** Ras-NIH3T3 cells were cultured with 0.5% or 5% calf serum in the presence of various concentrations of PQQ for 72 h, and then the relative cell viability was measured by the MTT assay. The cells cultured with 0.5% calf serum showed a significant increase in cell viability at concentrations  $> 10$  µM PQQ (Fig. 2); the maximum increase in cell viability was 124% at 100 µM PQQ. However, Ras-NIH3T3 cells cultured with 5% calf serum showed no effect of PQQ on cell viability. These data suggest that the growth-promoting effects of PQQ were already active in the presence of 5% serum but not in the presence of 0.5% serum. In preliminary experiments, normal

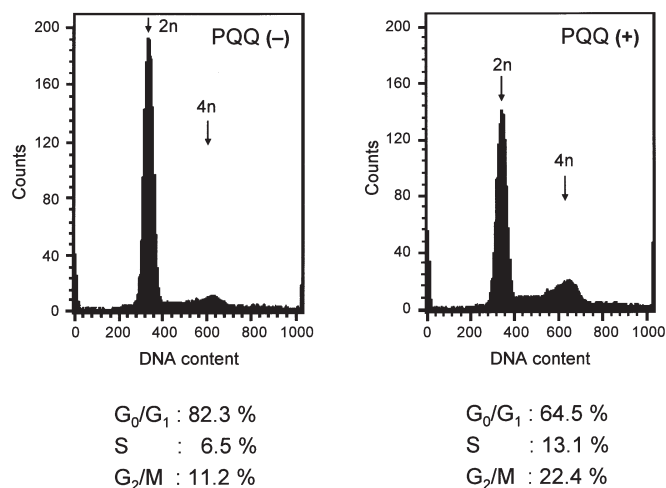


Figure 3. Flow cytometry analysis of Ras-NIH3T3 cells. Cells were cultured in DMEM containing 0.5% calf serum and 100  $\mu$ M PQQ for 72 h, and analyzed using a Becton Dickinson FACScan flow cytometer.

NIH3T3 cells were cultured with 0.5 or 5% calf serum in the presence of various concentrations of PQQ for 72 h, and then the relative viability was measured as described above; however, no significant increase in cell viability was observed (data not shown). Therefore, Ras-NIH3T3 cells were treated with 100  $\mu$ M PQQ in the presence of 0.5% calf serum in the following experiments.

**Effects of PQQ on cell cycle progression.** To determine the effects of PQQ on cell cycle progression, flow cytometric analysis was conducted on Ras-NIH3T3 cells. After 72 h of culture in the absence of PQQ, the cells were arrested in the G<sub>0</sub>/G<sub>1</sub> phase. In contrast, in Ras-NIH3T3 cells treated with PQQ for 72 h, the population in the S and G<sub>2</sub>/M phases increased from 6.5 to 13.1% and 11.2 to 22.4%, respectively, with a concomitant decrease in the number of G<sub>0</sub>/G<sub>1</sub> cells from 82.3 to 64.5% (Fig. 3). These results suggest that PQQ reduced G<sub>1</sub> arrest in Ras-NIH3T3 cells.

**Western blot analysis.** To investigate the molecular events initiated by PQQ, cell extracts were analyzed by Western blotting with specific antibodies to a number of key signaling molecules. ERK is a typical growth-promoting molecule and is activated through phosphorylation by MAPK/ERK kinase (MEK) (19,20,26). It is presently believed that protein kinase C (PKC)- $\epsilon$  participates in serum-induced cell growth of NIH3T3 fibroblasts (27). Therefore, we first investigated the effects of PQQ on the activation of ERK and PKC- $\epsilon$  enzymes in Ras-NIH3T3 cells using phospho-specific antibodies. The cells were incubated with PQQ for various periods. Maximal ERK activation through phosphorylation was observed after 1 h of PQQ treatment (Fig. 4A), and PKC- $\epsilon$  activation was also markedly increased after 1 h of treatment with PQQ (Fig. 4B). Since the activation of ERK and PKC- $\epsilon$  occurred shortly after PQQ addition, the primary target of PQQ may be closely related to these molecules. These results suggest that PQQ can promote both ERK and PKC- $\epsilon$  activities as early events in the progression of Ras-NIH3T3 cells.

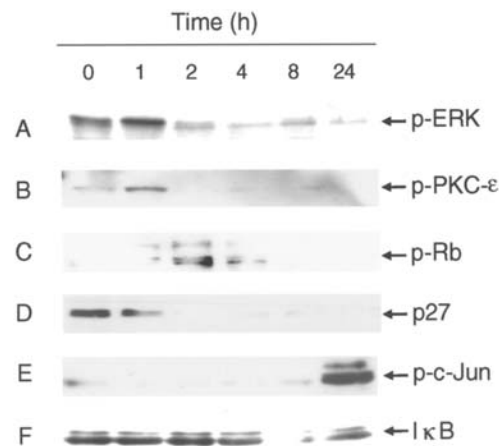


Figure 4. Effects of PQQ on signaling molecules in Ras-NIH3T3 cells. Cells were cultured in DMEM containing 0.5% calf serum for 1, 2, 4, 8 and 24 h in the presence of 100  $\mu$ M PQQ and then subjected to immunoblot assay with the indicated antibodies.

Retinoblastoma protein (Rb) is a major regulator of the G<sub>1</sub>/S transition in the cell cycle (28). Phosphorylation of Rb results in a change in its molecular configuration, the release of bound transcription factors such as E2F, and, ultimately, progression through the cell cycle. The proteins p27 and p21 represent a family of molecules which block the activity of cyclin/cyclin-dependent kinase (CDK) complexes responsible for phosphorylation of Rb (29). As shown in Fig. 4C, Rb phosphorylation was induced by incubation with PQQ, reaching its highest level after 2 h. In addition, PQQ reduced protein expression levels of p27 after 1-2 h (Fig. 4D), but had no effect on the levels of p21 (data not shown).

To assess the participation of transcription factors on the effects of PQQ, we confirmed the presence of phosphorylated c-Jun protein in Ras-NIH3T3 cells. Time-course studies of PQQ effects showed a gradual activation of c-Jun, but a dramatic increase in c-Jun phosphorylation after 24 h of PQQ treatment (Fig. 4E). The activity of transcription factor NF- $\kappa$ B is suppressed in the cytoplasm in quiescent cells via association with I $\kappa$ B protein (30). However, under certain conditions during cell growth and cell-cycle progression, phosphorylation of I $\kappa$ B by I $\kappa$ B kinase allows nuclear translocation and DNA binding of NF- $\kappa$ B, and proteolytic degradation of I $\kappa$ B by the proteasome (31,32). We therefore investigated the expression level of I $\kappa$ B and found that an 8-h treatment with PQQ reduced I $\kappa$ B levels, although levels recovered completely 24 h after the addition of PQQ (Fig. 4F). These data suggest that PQQ affects the transcriptional activities of c-Jun and NF- $\kappa$ B in Ras-NIH3T3 cells.

**Effects of SNAP, DFMO, H-7, genistein and erbstatin on PQQ-induced cell proliferation.** We next examined the growth-promoting effects of PQQ in the presence of various drugs. More than 50 drugs were examined using the MTT method (33) and several were found to affect the activity of PQQ. Treatment of Ras-NIH3T3 cells with SNAP, an NO donor, for 24 h decreased cell viability to 62% of that of the control cells (Fig. 5). This SNAP-induced decrease in

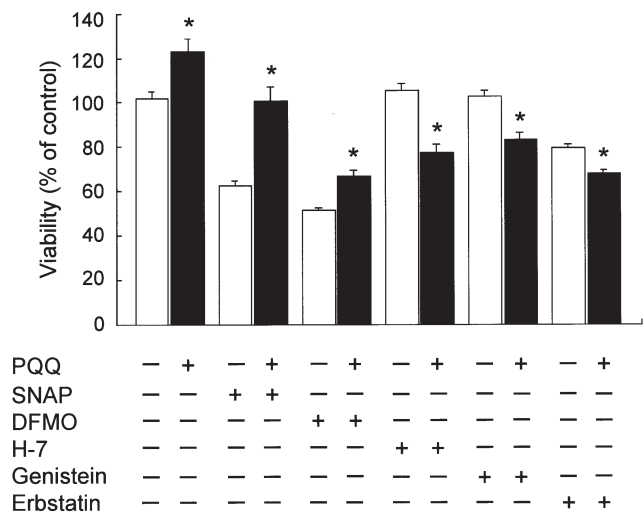


Figure 5. Effects of PQQ on cell viability of SNAP-, DFMO-, H-7-, genistein- and erbstatin-treated Ras-NIH3T3 cells. Cells were cultured in DMEM containing 0.5% calf serum in combination with 5 test compounds (125  $\mu$ M SNAP, 100  $\mu$ M DFMO, 6  $\mu$ M H-7, 3  $\mu$ M genistein or 6  $\mu$ M erbstatin) for 72 h in the absence or presence of 100  $\mu$ M PQQ. The relative cell viability was examined by the MTT assay, as described in Materials and methods. PQQ was added to the cells at the same time as each of the test compounds. Data are means  $\pm$  SD (bars) of the values of 4 experiments. Asterisks indicate significant differences between treatment with and without PQQ; \* $p$ <0.001 as determined by the Student's *t*-test.

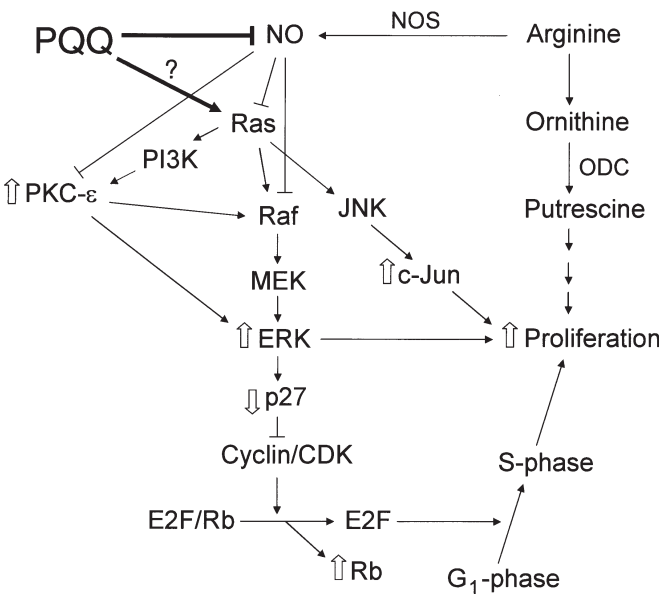


Figure 6. Schematic illustration showing the involvement of NO-sensitive Ras-mediated signaling pathways in the proliferation of Ras-NIH3T3 cells induced by PQQ. The symbols  $\uparrow$ ,  $\downarrow$ ,  $\perp$  and  $\nearrow$  indicate upregulation, downregulation, inhibition and activation of signaling molecules, respectively.

viability was almost completely recovered by the addition of PQQ. Similar but incomplete recovery of viability was also observed in the presence of DFMO, an irreversible inhibitor of ornithine decarboxylase (ODC) (34). In contrast, PQQ suppressed cell proliferation in the presence of a serine/threonine protein kinase inhibitor, H-7 (35), and in the presence of two tyrosine kinase inhibitors, genistein and

erbstatin (36) (Fig. 5). Although PQQ decreased cell viability by ~28, 20 and 11%, respectively, H-7 or genistein alone did not apparently affect cell viability. These results suggest that NO and protein phosphorylation are involved in the PQQ-induced signaling pathways.

Discussion

The major findings of the present study are that PQQ can induce cell proliferation in Ras-NIH3T3 cells, that this effect occurs via Rb-, p27-, ERK- and PKC- $\epsilon$ -dependent mechanisms, and that PQQ protects the cells against NO-induced inhibition of cell proliferation.

The growth inhibitory protein, p27, plays a critical role in cell cycle regulation by virtue of its ability to respond to changes in the growth environment of a cell (37). This protein induces G<sub>1</sub> arrest by inhibiting specifically CDK2-mediated Rb phosphorylation. A decrease in p27 levels was observed at the early phase after addition of PQQ (Fig. 4), which is consistent not only with an increase in phosphorylated Rb protein but also with a decrease in the population of G<sub>1</sub> phase cells (Fig. 3). These observations are supported by our previous study in which PQQ enhanced the incorporation of [<sup>3</sup>H]thymidine into DNA in human fibroblast cells, and in which a significant increase in [<sup>3</sup>H]thymidine incorporation was observed 24 h after incubation with various concentrations (<10 mM) of PQQ. In particular, a large increase in [<sup>3</sup>H]thymidine incorporation (202-243% of control cells) was observed at a concentration of 15-30  $\mu$ M PQQ (12). In oncogenic Ras-transformed cells, p27 has been shown to be under the control of Ras-Raf-MEK-ERK signaling pathways (38). The activation of ERK leads to the downregulation of p27 production and cell cycle regulation (39). The Ras-PI3K-PKC- $\epsilon$  signaling pathway is also necessary for maintenance of the Ras-transformed state (40), and PKC- $\epsilon$  acts as a Raf activator (41). The present study revealed that prominent phosphorylation of ERK was accompanied by PKC- $\epsilon$  phosphorylation in Ras-NIH3T3 cells exposed to PQQ for 1 h (Fig. 4A and B). Based on these previous observations and our current findings, it is strongly suggested that PQQ contributes to the stimulation of DNA synthesis in cell proliferation via simultaneous phosphorylation of ERK and PKC- $\epsilon$ , followed by degradation of p27 and phosphorylation of Rb.

The downstream effectors of the MAPKs include several transcription factors, such as c-Jun, c-Fos and Elk-1 (38,42). The activation of the MAPK cascade is succeeded by an increase in the expression of c-Jun and c-Fos, which form the AP-1 complex. The transcriptional activity of AP-1 is also dependent on the degree of phosphorylation of c-Jun and c-Fos, as well as their abundance. In addition, phosphorylation of c-Jun by c-Jun N-terminal kinase (JNK) is known to stimulate AP-1 transactivation activity, resulting in an enhancement of the transcription-stimulating activity of c-Jun. In the present study, we showed that PQQ induced c-Jun phosphorylation in Ras-NIH3T3 cells (Fig. 4E), suggesting that PQQ can regulate c-Jun expression through the MAPK cascade. Involvement of protein phosphorylation in PQQ-induced signaling pathways was also supported by the observation that the growth-promoting effects of PQQ were abolished by the protein kinase inhibitors H-7, genistein and erbstatin (Fig. 5).



Reactive oxygen species (ROS) including superoxide anion, hydroxyl radical and hydrogen peroxide, and reactive nitrogen species such as NO and peroxynitrite, are biologically important oxygen derivatives that are increasingly recognized as being necessary in numerous cellular and organ functions, including inhibition of cell proliferation (43-45). It was previously reported that PQQ scavenges superoxide anion and hydroxyl radicals in cell-free *in vitro* experiments (46). A recent study has indicated that NO donors, such as 3-morpholinonydnonimine, cause neuronal cell death in cultured rat forebrain neurons, and that PQQ can suppress peroxynitrite formation and block neurotoxicity (47). However, the detailed molecular mechanisms involved in the interaction of ROS and PQQ have not been elucidated.

Raines *et al* (48) reported that the production of NO inactivates Ha-Ras protein and inhibits downstream ERK; NO can directly modify the Ras protein at Cys<sup>118</sup> by S-nitrosylation, which subsequently leads to inhibition of signaling through the ERK pathway. In this study, SNAP (125  $\mu$ M), an NO donor, inhibited the proliferation of Ras-NIH3T3 cells, but the effect was abolished by the addition of 100  $\mu$ M PQQ (Fig. 5). Thus the decrease in viability of Ras-NIH3T3 cells by SNAP might be due to inhibition of the ERK-dependent MAPK cascade, and PQQ may prevent or alleviate NO-induced inhibition of the ERK-dependent MAPK cascade. In contrast, several studies have reported that PKC- $\epsilon$  is required for fluid shear stress-mediated activation of ERK in endothelial cells (ECs) (49), and PKC- $\epsilon$  overexpression was shown to induce sustained phosphorylation of MAPK in epidermal growth factor-treated PC12 cells (50). Similarly, PKC- $\epsilon$  acts as a Raf activator that leads to a prolonged effect on the MAPK cascade and gene induction in ECs (41). NO and PKC- $\epsilon$  have recently been implicated in the proliferative response in ECs, whereby the generation of NO reduces the activation of PKC- $\epsilon$  (51). These observations predict that S-nitrosylation of the Ha-Ras protein in Ras-NIH3T3 cells by NO may lead to the inhibition of PKC- $\epsilon$  and its downstream signaling molecules such as Raf and ERK, and that PQQ may then prevent their inhibition.

ODC catalyzes the conversion of ornithine to putrescine in mammalian cells, and putrescine is further converted to spermidine and spermine. It is well known that these three polyamines (putrescine, spermidine and spermine) are required for mammalian cell growth (52), and are depleted by up to 60-70% after exposure to DFMO for 24 h (53). The present study revealed that DFMO reduced Ras-NIH3T3 cell viability to 51% of that of the control cells (Fig. 5), presumably due to the depletion of intercellular polyamines. It should be noted that ornithine is a substrate for the production of NO by NO synthase (NOS) (54). Thus, DFMO might also affect NO levels. Because the cytostatic effects of DFMO as well as SNAP were suppressed by PQQ (Fig. 5), it is possible that PQQ regulates intracellular NO levels.

In conclusion, this study provides the first evidence that PQQ increases cell proliferation in Ras-NIH3T3 cells via Ras-related signaling pathways such as Ras-Raf-MEK-ERK and Ras-PI3K-PCK- $\epsilon$ -Raf/ERK (Fig. 6). This effect of PQQ might be derived from its regulation of NO levels. The present study not only expands our knowledge of mechano-transduction in Ras-NIH3T3 cells, but also contributes to the

understanding of cellular mechanisms, cell signaling and gene expression relevant to PQQ in mammals.

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