Mechanism of Cell Growth Inhibition by Menadione

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Received April 23, 2002

Key Words : Menadione, Phosphatase

Vitamin K_3 (menadione) and synthetic vitamin K analogues have attracted attention because of their significant anti-cancer.[1] Recent study has shown that menadione treatment causes cells to arrest in G_1, and its mechanism of in cell growth inhibition has been suggested by the generation of superoxide.[2] However, it was reported that superoxide dismutase did not antagonize the growth inhibitory effects of menadione[3] and the toxic oxygen species, inducing most of the DNA breakages in menadione-treated cells were not responsible for menadione's toxicity.[4] In the previous study, we have proposed alternative mechanism of G_1 arrest where menadione inactivates cdc25A phosphatase,[5] sulfhydryl-dependent protein, which is presumed to promote entry into S phase by acting on cdk.[6] Recently, the binding of menadione to the active site of the enzyme was also proven by incubating [methyl-^{3}H]-menadione with the catalytic domain of cdc25A phosphatase.[7] However, it was found that menadione also inactivated cdc25B and -C,[8] which are mostly expressed in G_2-M.[9] These observations make it unclear whether the action of cell growth inhibition at G_1 phase simply arises from the inhibition of cdc25A phosphatase or oxidative stress.

In previous study, we showed that MKP-1, dual-specificity phosphatase, which mediates dephosphorylation of MAP, was inactivated by menadione.[10] Since MAP kinase is capable of phosphorylating p53 at threonine 73 and 83[11] and activation of p53 through phosphorylation can lead to the transcriptional upregulation of the cyclin-dependent kinase inhibitor, p21, it was proposed that the inhibitory action of p21 on cdk by MAP kinase activation might result in cell cycle arrest at G_1 phase.[12]

To demonstrate this hypothesis, logarithmically growing human hepatocarcinoma SK-Hep-1 cells were first incubated with 100 μM of menadione for a period of 6, 12, or 24 h. Cells were then harvested and soluble extracts were assayed for the expression levels of p53 using p53 monoclonal antibody. Interestingly, as shown in Figure 1 (lane 1), Western blotting showed that similar amounts of p53 were recovered in immunoprecipitates from menadione-treated and non-treated cells. In conjunction with the earlier observation that the amount of p53 protein increases in response to a variety of signals including DNA-damaging agents through the production of reactive oxygen species (ROS).[13]

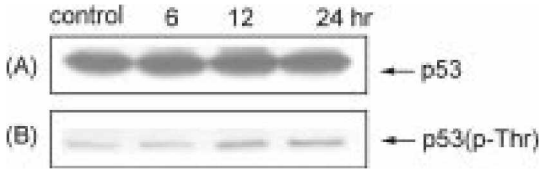

Figure 1. Effects of menadione on expression and phosphorylation level of p53 in SK-hep-1 cells. Human hepatocarcinoma SK-Hep-1 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin) at 37 °C in 5% CO_2 humidified incubator. Cells were plated at a density of 3×10^5/mL on a culture dish. Cells with or without 100 μM water soluble menadione (Sigma) were washed with PBS and lysed in TENS buffer (50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, pH 7.5). For Western blot, protein extracts (100 μg) were separated on 12% SDS-PAGE, and electrotransferred to the PVDF transfer membrane (Schleicher and Schuell). (A) The blot was incubated with anti-p53 monoclonal (Santa Cruz Biotechnology) for 15 h, followed by horseradish peroxidase labeled secondary antibody (Amersham) for 2 h, and then developed by the enhanced-chemiluminescence (ECL) detection kit (Amersham). (B) The lysates were immunoprecipited with anti-p53 antibody followed by Western blotting with phosphothreonine antibody (Zymed).

our results show that enzymatic redox cycling does not play a critical role in menadione-induced cell cycle arrest.

To determine whether MAP kinase was activated *via* hyperphosphorylation, the extracts of menadione-treated cells were also immunoprecipitated with anti-MAP kinase antibody and anti-phospho-MAP kinase antibody. The results shown in Figure 2 (lane 1 and 2) demonstrate that the intensity of phosphorylarion of the MAP kinase was increased. The activities of MAP kinase were also assayed using myelin basic protein (MBP) as a substrate. When proteins were resolved by SDS polyacrylamide gel electrophoresis, the results presented in Figure 2 (lane 3) show that MAP kinase was activated by treatment of the cell with menadione.

We next evaluated whether activation of MAP kinase was necessary for the activation of p53, although menadione was proved to have no effect on p53 induction. To detect the phosphorylation level of threonine, p53 was immunoprecipitated with anti-p53 antibody from the cell extracts, and immunoblotted with anti-phosphothreonine antibody. As shown in Figure 1 (lane 2), the results correlated with the hyperphosphorylation status of p53 in menadione-treated

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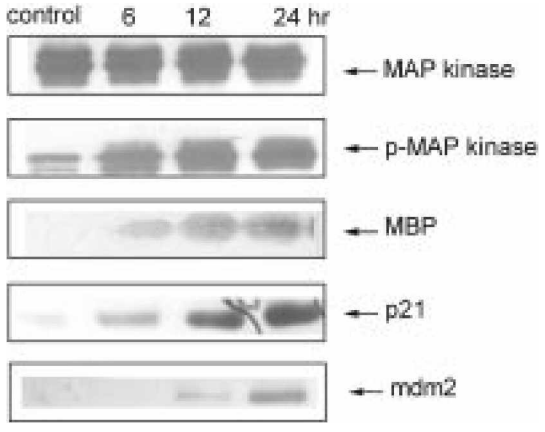

Figure 2. Effects of menadione on MAP kinase activity and expression level of p21 and mdm2 in SK-hep-1 cells. The blot was incubated with monoclonal antibodies such as anti-MAP kinase, -phospho-MAP kinase, -p21, and -mdm2 (Santa Cruz Biotechnology). For MAP kinase assay, the lystes (10 μg total proteins) were suspended in kinase buffer (50 mM Tris-HCl, 25 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, pH 7.4) with 20 mM ATP, 1 mCi [γ-^{32}P]ATP, and 0.5 mg/mL myelin basic protein (Sigma). After 30 min at 30 °C, the reaction was terminated by 10 mL of 5 × Laminis sample buffer. The proteins were resolved by electrophoresis on 12% SDS-PAGE, followed by autoradiography to visualize the phosphorylated myelin basic protein.

cells. Since active p53 turns on the transcription of one of its downstream genes, p21[11]. p21 was also immunoblotted with p21 monoclonal antibody. Figure 2 (lane 4) showed that prolonged incubation with menadione increased the level of p21, suggesting that menadione can activate p53 *in vivo*. However, it has also been reported that oxidative stress can lead to activate p21 expression in the p53-independent pathway. In order to rule out this pathway and further confirm that p53 activity was increased in the presence of menadione, the expression of other p53 downstream effector, mdm2, was also examined. In Figure 2 (lane 5), we showed that p53 stimulated mdm2 expression with treatment of menadione. Taking all of these results together, we could conclude that the activity of p53 might be increased by hyperphosphorylation of MAP kinase.

p53 is also dephosphorylated by multiple protein-serine/threonine phosphatases such as PP1 and PP2A.[14] Therefore, we tested whether or not menadione affected these enzymes and found that they were not inactivated by it up to 50 μM (data not shown). Moreover, menadione showed no inactivation of the protein tyrosine phosphatases, such as LAR, PTP1B, and Yersinia PTP (data not shown).

The results reported here show that enzymatic redox cycling does not play a critical role in menadione-induced cell cycle arrest in cells, while inactivation of dual-specificity phosphatases is likely to be menadione's primary mechanism of action.

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