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Biochimica et Biophysica Acta 1770 (2007) 687–693
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbagen

Inhibition of PTEN and activation of Akt by menadione

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Received 2 October 2006; received in revised form 2 December 2006; accepted 19 December 2006
Available online 24 December 2006

Abstract

Menadione (vitamin K_3) has been shown to activate Erk in several cell lines. This effect has been shown to be due to the activation of EGF receptors (EGFR) as a result of inhibition of some protein tyrosine phosphatases. In the present study, we examined the effects of menadione on Akt in Chinese hamster ovary cells. The phosphorylation of Akt by menadione was not inhibited by AG1478, an inhibitor of EGFR. Menadione inhibited the lipid phosphatase activity of PTEN in a cell-free system. In an intact cell system, menadione inhibited the effect of transfected PTEN on Akt. Thus, one mechanism of its action was considered the accelerated activation of Akt through inhibition of PTEN. This was not the sole mechanism responsible for the EGFR-independent activation of Akt, because menadione attenuated the rate of Akt dephosphorylation even in PTEN-null PC3 cells. The decelerated inactivation of Akt, probably through inhibition of some tyrosine phosphatases, was considered another mechanism of its action.
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Keywords: PTEN; Akt; Naphthoquinone; Menadione (vitamin K_3)

1. Introduction

Vitamin K is a fat-soluble vitamin essential for the post-translational modification of proteins, including the coagulation factors II (prothrombin), VII, IX, X, and proteins C, S, and Z [1]. Two naturally occurring forms of vitamin K, phylloquinone (vitamin K_1) and menaquinone (vitamin K_2), act as coenzymes for γ-glutamyl carboxylase, which produces γ-carboxyglutamic acid (Gla) by carboxylating the side chain of glutamic acid [2]. Vitamin K_3 (menadione, 2-methyl-1,4-naphthoquinone) is a synthetic vitamin K congener that is not a co-factor for γ-glutamyl carboxylase. Menadione has been examined extensively for its inhibitory effect on the proliferation of tumor cells both in vivo and in vitro [3–6]. Similarly to various quinones, menadione can be reduced in cells to produce the semiquinone radical, which may impair the cellular constituents directly or indirectly through production of reactive oxygen species. The 3-position of menadione is also expected to react on the sulfhydryl group of glutathione by the Michael-type addition mechanism [7,8]. Thus, menadione produces oxidative stress on cells through its ability to undergo both redox cycling and conjugate formation. In support of the occurrence of oxidative stress, CHO cells showing resistance to menadione-induced cell death have been reported to exhibit increased concentrations of glutathione and cysteine [9]. On the other hand, some effects of naphthoquinone derivatives are considered to be independent of oxidative stress [10–12].

Menadione has been shown to activate the Erk cascade [13–15]. Abdelmohsen et al. suggested that this action of menadione may be separated from the oxidative stress [12]. They showed that the prior treatment of cells with the antioxidant, N-acetyl cysteine, which prevents the effect of the redox cycler, 1,4-benzoquinone, did not attenuate the effect of menadione. They also showed that menadione had a minor effect on the cellular level of glutathione at the concentration at which it activates Erk. Recent studies have indicated that menadione induces

Abbreviations: CHO-IR cells, Chinese hamster ovary cells expressing insulin receptors; IRβ, the β subunit of insulin receptor; PI 3-kinase, phosphoinositide 3-kinase; pNPP, p-nitrophenyl phosphate; PtdIns-3,4,5-P_3, phosphatidylinositol 3,4,5-triphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10

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doi:10.1016/j.bbagen.2006.12.009

phosphorylation and activation of Erk by activating EGF receptors (EGFR), because pharmacological inhibitors of the receptor tyrosine kinase attenuated menadione-induced activation of Erk [12,15,16]. This conclusion was confirmed by the results of EGFR desensitization [12].

Menadione has been reported to inhibit protein tyrosine phosphatases in cell-free systems [15–17]. An analog of menadione has been shown to be a potent inhibitor of Cdc25 with minor effects on other protein tyrosine phosphatases [18]. The basis of these inhibitory effects is proposed to be the direct interaction of the compounds with the cysteine residue that plays an essential role in the process of tyrosine dephosphorylation [17,19,20]. Based on these observations, menadione is proposed to perturb the balance between the phosphorylation and dephosphorylation of EGFR leading to activation of the Erk cascade [12,16]. The effect of menadione on the PI 3-kinase/Akt cascade has also been observed and thus attributed to the activation of EGFR in rat liver epithelial cells [12].

In the present study, we showed that menadione activates Akt in Chinese hamster ovary cells. This effect was dependent on PI 3-kinase, but was refractory to inhibition of EGFR/tyrosine kinase. Our results indicated that one target of menadione is PTEN (phosphatase and tensin homolog deleted on chromosome 10), which hydrolyzes and inactivates the products of PI 3-kinase [21,22]. We also observed that menadione inhibits the dephosphorylation of both serine and threonine residues of Akt in intact cells. These effects of menadione were considered the basis of the EGFR-independent activation of Akt.

2. Materials and methods

2.1. Materials

Menadione (2-methyl-1,4-naphthoquinone, vitamin K_3), insulin, bovine serum albumin (fatty acid-free), Ni-CAM HC resin, and anti-myc antibody (9E10) were purchased from Sigma (St. Louis, MO). 1,4-Benzoquinone was from Tokyo Kasei (Tokyo, Japan). Wortmannin, LY294002, and AG1478 were from Kyowa Medex (Tokyo, Japan), Cayman Chemical (Ann Arbor, MI), and Merck (Darmstadt, Germany), respectively. [γ-^{32}P]ATP was obtained from Perkin-Elmer (Norwalk, CT). Antibodies against pThr-308 and pSer-473 of Akt were from Cell Signaling Tech (Beverly, MA). Polyclonal anti-Akt antibody, polyclonal anti-IRβ antibody, and monoclonal anti-phosphotyrosine antibody (PY99) were from Santa Cruz (Santa Cruz, CA). Anti-mouse IgG agarose was from American Qualex Antibodies (San Clemente, CA). pGEX-6P-1, precision protease, and PD-10 column were from GE Healthcare (Buckinghamshire, UK). pQE-30 expression vector was from Qiagen (Hilden, Germany).

2.2. Recombinant proteins

cDNA encoding PTEN (GenBank accession number NM000314) was provided by Dr. T. Maehama (National Institute of Infectious Diseases, Tokyo, Japan). cDNAs encoding PTP1B, SHP-1, SHP-2, Cdc25A, and VHR (GenBank accession numbers NM002827, NM002831, NM003834, NM001789, and NM004090, respectively) were obtained by polymerase chain reaction with appropriate cDNA libraries and primers possessing additional nucleotide sequences convenient for subcloning. The cDNA constructs were subcloned into the expression vector pGEX-6P-1, pET28a, or pQE-30. GST (glutathione S-transferase)-fused or 6xHis-tagged proteins were expressed in *Escherichia coli* and adsorbed onto glutathione Sepharose 4B or Ni-CAM HC resin, respectively, in accordance with the manufacturer's instructions. The glutathione beads were incubated with precision protease, and the cleaved proteins were stored at −80 °C in Tris-buffered saline containing 1 mM dithiothreitol (DTT). His-tagged proteins were eluted from the Ni-CAM beads with 250 mM imidazole. The eluate was applied to a PD-10 column to remove imidazole before storage at −80 °C.

2.3. Cell lines

Chinese hamster ovary cells expressing insulin receptors (CHO-IR cells) were kind gifts from Dr. Y. Ebina (Ehime University, Matsuyama, Japan) and were cultured in F12-Ham's medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin. PTEN-null PC3 cells were obtained from Dr. T. Maehama (National Institute of Infectious Diseases, Tokyo, Japan) and maintained in DMEM supplemented with 10% fetal calf serum, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin.

2.4. Immunoblotting

The cells were lysed in lysis buffer consisting of 25 mM Tris–HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 30 mM NaF, 200 μM phenylmethylsulfonyl fluoride (PMSF), 20 μM (4-amidinophenyl)-methylsulfonyl fluoride (pAPMSF), 2 μM leupeptin, 2 μM pepstatin, and 1% Nonidet P-40. After centrifugation (15,000 rpm for 10 min), aliquots of the supernatant were mixed with SDS-PAGE sample buffer and boiled for 5 min. The peptides were separated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking, the membranes were incubated with the indicated antibody, washed, and then incubated with horseradish peroxidase-conjugated second antibody. The second antibody was located using an enhanced chemiluminescence detection system (Perkin-Elmer, Norwalk, CT).

2.5. Lipid phosphatase activity

Recombinant PTEN (50 ng) was incubated in the presence or absence of menadione at 37 °C for 5 min in 10 μl of buffer consisting of 0.1 M Tris–HCl (pH 8.0), 10 mM DTT, 0.25% octyl glucoside, and 50 μg/ml bovine serum albumin. The phosphatase reaction was started by addition of 10 μl of 0.2 mM diC_{16}-PtdIns-3,4,5-P_3 and was stopped after 5 min by addition of 20 μl of 0.1 M *N*-ethylmaleimide. After centrifugation (15,000 rpm for 20 min), aliquots of 20 μl of the supernatant were mixed with 80 μl of GREEN Reagent (BIOMOL, Plymouth Meeting, PA). After development of the color for 15 min, absorbance at 620 nm was measured. The amount of phosphate released was quantified using standard solutions of inorganic phosphate.

2.6. PI 3-kinase activity

CHO-IR cells, cultured on six-well plates, were starved of serum for 15 h. After treatment with insulin and/or menadione, the cells were solubilized in 25 mM Tris–HCl buffer (pH 7.4) containing 50 mM NaCl, 30 mM NaF, 1 mM sodium orthovanadate, 5 mM EDTA, 200 μM PMSF, 20 μM p-APMSF, 2 μM leupeptin, 2 μM pepstatin, and 1% Nonidet P-40. After centrifugation at 15,000 rpm for 20 min, the supernatant was subjected to immunoprecipitation with anti-pTyr and anti-mouse IgG-conjugated agarose beads. The beads were washed twice with the same buffer, twice with 40 mM Tris–HCl buffer (pH 7.4) containing 100 mM NaCl and 1 mM DTT, and once with Tris–HCl buffer containing 0.5 mM EGTA. The beads were suspended in 100 μl of Tris–HCl buffer containing 0.25 mM EGTA, 2.5 mM $MgCl_2$, 0.2 mM phosphatidylserine, 0.2 mM phosphatidylinositol, and 0.2 mM [γ-^{32}P]ATP (50 μCi/ml). The reaction was allowed to proceed at 30 °C for 15 min and stopped by the addition of 20 μl of 8% $HClO_4$. To the mixture was added 450 μl of methanol-$CHCl_3$ (2:1), stirred vigorously, and then 150 μl of each of $CHCl_3$ and methanol were added. The organic phase was washed twice with $CHCl_3$-saturated solution containing 0.5 M NaCl and 1% $HClO_4$. After drying, the extract was spotted onto Silica Gel 60 plates (Merck, Darmstadt, Germany), which was developed in $CHCl_3$/methanol/28% NH_4OH/H_2O (70:100:25:15). The radioactivity in the PtdIns-3-P spot was determined using Fuji BAS2000 analyzer (Fuji, Tokyo, Japan).

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