In Vivo Anti-estrogenic Effects of Menadione on Hepatic Estrogen-responsive Gene Expression in Male Medaka (*Oryzias latipes*)

Akemi Yamaguchi,[a, b] Shinya Kohra,[b, c] Hiroshi Ishibashi,[d] Koji Arizono,[d] and Nobuaki Tominaga*[, a]

[a]*Department of Chemical and Biological Engineering, Ariake National College of Technology, 150 Higashihagio-machi, Omuta, Fukuoka 836–8585, Japan,* [b]*Graduate School of Science and Technology, Nagasaki University,* [c]*Faculty of Environmental Studies, Nagasaki University, 1–14 Bunkyo-machi, Nagasaki 852–8521, Japan, and* [d]*Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3–1–10 Tsukide, Kumamoto 862–8502, Japan*

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Menadione, a synthetic vitamin K3, exhibits anti-estrogenic activity on *in vitro* assay. However, the *in vivo* anti-estrogenic effects of menadione have not been determined, while correlations between biological effects and structural changes are unclear. Thus, we investigated the *in vivo* anti-estrogenic activity of menadione under fluorescent light and dark conditions. Suppression of the hepatic estrogen response genes vitellogenin1 (VTG1), VTG2 and estrogen receptor-α (ER-α) was used as an index of anti-estrogenic activity. Male medaka (*Oryzias latipes*) were treated with nominal concentrations of menadione in the presence or absence of 17β-estradiol (E2), and hepatic VTG1, VTG2 and ER-α mRNA levels were determined by quantitative real-time PCR. In the presence of E2 under dark conditions, expression of hepatic VTG2 and ER-α genes was suppressed by menadione treatment. On the other hand, menadione activity was lost under fluorescent light conditions. These results suggest that menadione has anti-estrogenic activity *in vivo*, and that this activity is diminished under fluorescent light, probably due to a structural change in menadione.

*To whom correspondence should be addressed: Department of Chemical and Biological Engineering, Ariake National College of Technology, 150 Higashihagio-machi, Omuta, Fukuoka 836–8585, Japan. Tel.: +81-944-53-8747; Fax: +81-944-53-8747; E-mail: tominaga@ariake-nct.ac.jp

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INTRODUCTION

Since endocrine-disrupting chemicals (EDCs) have been discovered, numerous studies on the hormone agonistic or antagonistic effects of these EDCs have been performed.[1, 2] Vitellogenin (VTG), an estrogen-inducible phosphoprotein and complex precursor protein in egg yolk, is a useful biomarker for evaluating the (anti)estrogenic potential of certain EDCs.[3] Estrogenic compounds, including natural estrogens, regulate expression levels of the estrogen-responsive VTG gene by binding to a specific estrogen receptor (ER). Our previous studies have demonstrated the short-term effects of estrogenic compounds, such as E2, nonylphenol, bisphenol A and fluorotelomer alcohols, on the expression of estrogen-responsive genes, such as VTG and ER, in male medaka.[4, 5]

Menadione (2-methyl-1,4-naphthoquinone) is a synthetic vitamin K3. Vitamin K is reported to essential for blood coagulation and bone metabolism in vertebrates.[6] Menadione has been used extensively in the research of oxidant stress in mammalian hepatic cells. In hepatocyte systems, menadione reportedly increases consumption of molecular oxygen and production of superoxide radical anions, depletion of glutathione pools, and oxidation of protein sulfhydryl groups.[7, 8] Menadione has also been shown to induce single-strand (ss) and double-strand (ds) DNA breaks in human MCF-7 cells, and to possess significant anticancer activity *in vivo* and *in vitro*.[9] In addition, menadione is toxic in invertebrates; Kim *et al*. assessed the genotoxicity of menadione in grass shrimp (*Palaemonetes pugio*) embryos using single cell gel electrophoresis, and reported that menadione induced DNA strand breaks during embryonic development.[10]

Ship ballast water and sediment contain diverse invertebrate species, and the release of these species facilitates biological invasions that negatively affect the ecology of recipient ecosystems.[11] As menadione shows broad spectrum toxicity for invertebrates, is simple to transport and is safe to handle, Wright *et al*. investigated the toxicity of naphtoquinones, including menadione, against phytoplankton and bacteria, and examined their usage as biocides for ships ballast water treatment.[12] Fur-

thermore, Raikow *et al*. demonstrated that the toxicity of menadione is affected by sunlight in their assessment of the biocide potency of menadione against zooplankton and their resting eggs.[13)]

Mee *et al*. investigated the structural changes of menadione under sunlight conditions by gas chromatography and mass spectrometry. They suggested the possibility that menadione is converted to an oxide or epoxide on exposure to sunlight.[14)] However, little is known about the biological effects of menadione, such as the correlation between the endocrine system and menadione molecular structure *in vivo*. Moreover, a previous study demonstrated the anti-estrogenic potential of menadione in an *in vitro* yeast two-hybrid assay.[15)] They suggested that menadione inhibits the transcriptional activity of the E2 reporter gene, and directly binds to ER-α on competitive binding assay. However, there is no data on the anti-estrogenic potential of menadione *in vivo*.

The aim of this study is to investigate the *in vivo* anti-estrogenic activity of menadione in the presence or absence of 17β-estradiol (E2) on expression of hepatic VTG1, VTG2 and ER-α in male medaka under fluorescent light or dark conditions. The anti-estrogenic potential of menadione has been compared with that of tamoxifen, an ER antagonist. Furthermore, we assess the variations in anti-estrogenic activity of menadione after fluorescent light treatment.

MATERIALS AND METHODS

Test Chemicals —— Menadione (purity > 98.5%, Wako Pure Chemical Industries, Ltd., Osaka, Japan), E2 (purity > 98%, Sigma, St. Louis, MO, U.S.A.), and tamoxifen ([Z]-1-[*p*-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene, purity > 99%, Sigma), were used in this study. Reagents were dissolved in dimethyl sulfoxide (DMSO, purity > 99%, Wako Pure Chemical Industries) for preparation of test solutions. Menadione stock solution was stored under dark conditions.

Chemical Exposure Design —— d-rR strain medaka were kept for several years in glass tanks at 25 ± 1°C under a 16 : 8 light : dark photoperiod and fed *Altemia naupli* once daily. Male medaka (approximately 4 months after hatching) were exposed to test compounds as reported previously.[4)]

In order to investigate the anti-estrogenic effects of menadione or tamoxifen (as a positive control), three male medaka were exposed to nominal concentrations of 0.1, 1, 10 and 100 μM menadione or 0.1 and 1 μM tamoxifen in the presence or absence of 3.7 nM E2 for 8 hr. These treatments were performed in 200-ml glass beakers at 25 ± 1°C under light or dark conditions. In exposure experiments, the control group was only exposed to the solvent carrier 0.01% DMSO for 8 hr. None of the exposure groups, including controls, were fed during the exposure period. At the end of the exposure period, livers were collected, rapidly frozen in liquid nitrogen, and stored at −80°C until analysis.

Quantitative Real-time PCR —— Total RNA preparation and reverse transcription were performed as described previously.[4)] Quantification of hepatic VTG1, VTG2, ER-α and β-actin mRNA was performed by real-time PCR using FullVelocity SYBR Green Master Mix (Stratagene, La Jolla, CA, U.S.A.) and STRATAGENE Mx3000P™ (Stratagene). The specific primers for estrogen-responsive and reference genes were as follows: VTG1 (DDBJ accession no. AB064320) forward, 5′-TGGAAAGGCTGATGGGGAAG-3′; reverse, 5′-AACTGCAGGCATGGTGAGCC-3′; VTG2 (AB074891) forward, 5′-GTCTTCAGGAGGTCTTCTTC-3′; reverse, 5′-GGTAGACAATGGTATCCGAC-3′; ER-α (AB033491) forward, 5′-GTCAGTCGGGTTACTTGGCC-3′; reverse, 5′-CATCACCTTGTCCCAACCTG-3′; β-actin (S74868) forward, 5′-AGACCACCTACAGCATC-3′; reverse, 5′-TCTCCTTCTGCATTCTGTCT-3′. Reaction mixtures were incubated at 94°C for 5 min, followed by 35 PCR cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min. All experiments were performed in duplicate. Expression levels of VTG1, VTG2 and ER-α mRNA were normalized against those of β-actin mRNA. Statistical analysis for VTG1, VTG2 and ER-α mRNA expression was performed by one-way analysis of variance (ANOVA) using Excel NAG statistical analysis (Numerical Algorithms Group Ltd., Oxford, UK).

Spectrum Measurement of Menadione Exposed to Fluorescence Light —— A 1-mM menadione solution (200 ml) was prepared from menadione stock solution, and was left to stand under an 18-W fluorescent light. Samples were collected every 0, 2, 8 and 12 days after exposure, and were stored at 4°C under dark conditions. Each sample was subjected to measurement using a U-3000 spectrophotometer (Hitachi High-Technologies Co., Ltd., Tokyo, Japan). The absorptions at 260 nm (reflect menadion) and 230 nm (reflected the decomposed prod-

uct) were measured. Then the rate of decomposition under this condition was estimated from alteration of absorbance ratio of 260 nm/230 nm.

RESULTS

Effects of Tamoxifen on Expression of Estrogen-responsive Genes

Zhang and Hu reported that β-actin was down regulated in the estrogenic chemical treated medaka liver.[16] However we used β-actin to normalize gene expression in this study, since statistically difference was not observed in each treatment group (data not shown).

Treatment with 1 μM tamoxifen in the presence of 3.7 nM E2 significantly suppressed the expression levels of VTG1 and ER-α mRNA in the livers of male medaka ($p < 0.05$) (Fig. 1A and 1C). Although the expression levels of hepatic VTG1 and ER-α were also suppressed by treatment with 0.1 μM tamoxifen plus E2, no statistically significant differences were observed (Fig. 1A and 1C). In the presence of 3.7 nM E2, expression levels of hepatic VTG2 mRNA were significantly reduced in the 0.1 and 1 μM tamoxifen treatment groups, as compared with E2 treatment alone ($p < 0.05$) (Fig. 1B).

Effects of Menadione on Expression Levels of Estrogen-responsive Genes

Expression levels of ER-α mRNA in the livers of male medaka exposed to 0.1 and 1 μM menadione for 8 hr were significantly lower when compared to controls ($p < 0.05$) (Fig. 1C). With regard to induction of hepatic VTG1 and VTG2 mRNA expression after exposure to 0.1 and 1 μM menadione, no differences compared to the control group were seen (Fig. 1A and 1B). On the other hand, treatment with 1 μM menadione in the presence of 3.7 nM E2 distinctly suppressed expression levels of hepatic VTG2 and ER-α mRNA in males, similarly to tamoxifen treatment ($p < 0.05$) (Fig. 1B and 1C). In the presence of E2, induction of hepatic VTG1 mRNA was not suppressed by treatment with 0.1 or 1 μM menadione (Fig. 1A). In both the 10 and 100 μM menadione treatment groups in the presence or absence of E2, all test fish died within 30 min and four hr, respectively (data not shown).

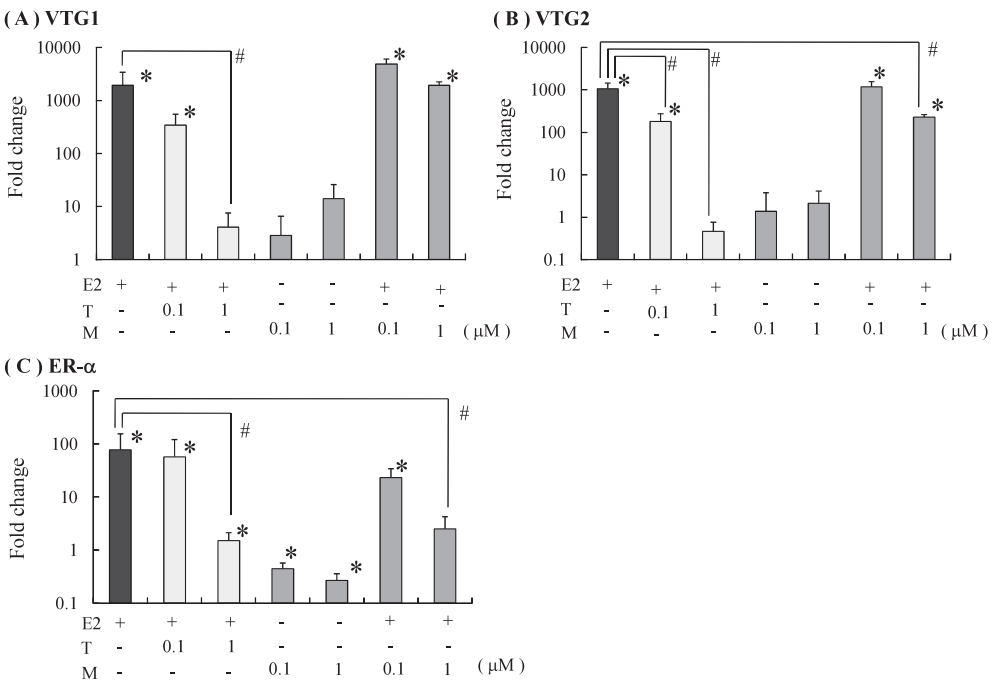

Fig. 1. Anti-estrogenic Effects of Tamoxifen and Menadione on VTG1 (A), VTG2 (B) and ER-α (C) mRNA Expression under Dark Condition in Male Medaka Liver

Three male medaka in each treatment groups were treated with 0.1, 1 μM menadione or tamoxifen in the presence or absence of E2 for 8 hr. Bars represent mean mRNA expression values ± S.D. (relative to β-actin mRNA expression). M; menadione. T; tamoxifene. +; in treatment. Asterisk (*) denotes significant differences *vs*. control group. # and bar that joins E2 and test chemical-treated groups indicate significant differences *vs*. E2 treatment group ($p < 0.05$).

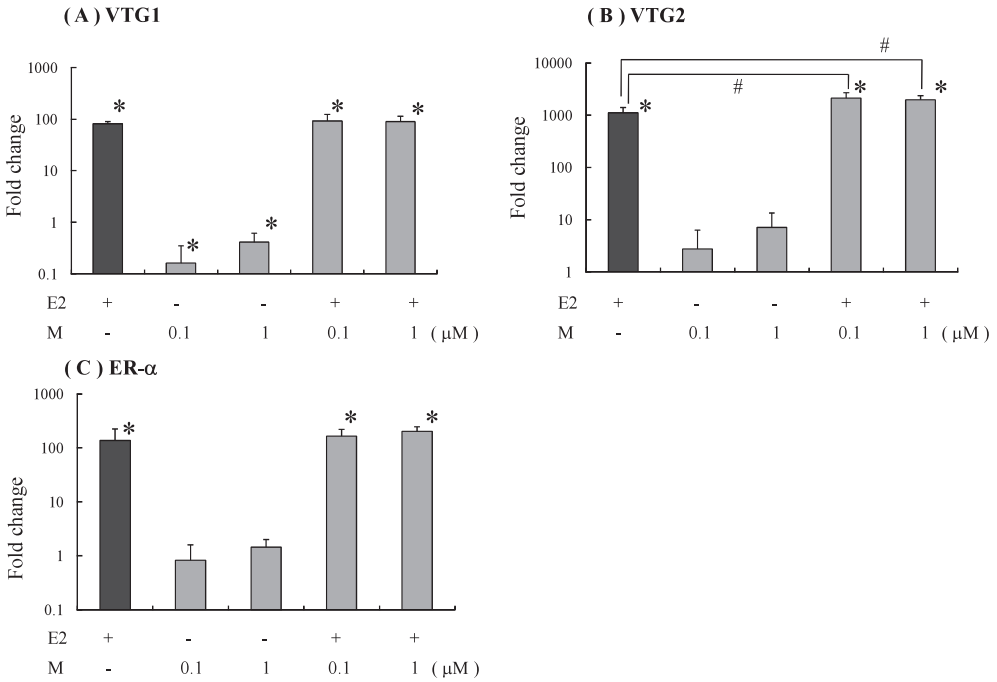

Fig. 2. Anti-estrogenic Effects of Menadione on VTG1 (A), VTG2 (B) and ER-α (C) mRNA Expression under Fluorescent Light Condition in Male Medaka Liver

Three male medaka in each treatment group were treated with 0.1, 1 μM menadione in the presence or absence of E2 for 8 hr. Data bars represent mean mRNA expression values ± S.D. (relative to β-actin mRNA expression). M; menadione. +; in treatment. Asterisk (*) denotes significant differences *vs.* control group. # and bar that joins E2 and test chemical-treated groups in the Figure indicate significant differences *vs*. E2 treatment group ($p < 0.05$).

Effects of Fluorescent Light on Anti-estrogenic Potential of Menadione

After exposing menadione to fluorescent light for 8 hr, the changes in the anti-estrogenic effects of menadione were investigated. In contrast to treatment under dark conditions, no significant changes in gene induction were observed in presence of E2 (Fig. 2A and C), despite the fact that VTG1 and ER-α mRNA suppression was observed under dark conditions (Fig. 1A and C). In addition, VTG2 mRNA induction was slightly increased by menadione in the presence of E2 (Fig. 2B). Thus, under fluorescent light, menadione did not suppress E2 activity, and showed reduced anti-estrogenic activity.

Changes in Menadione Spectrum after Fluorescent Light Exposure

The absorbance spectrum between 200 nm and 400 nm was measured. The spectrum was altered by fluorescent light immediately after the start of exposure, and was altered substantially after 2 days of treatment. Major peaks positioned between 250 and 270 nm were diminished, and new peaks appeared at 230 nm (Fig. 3). These changes were related to fluorescent light exposure time. Under this condition, the decomposition rate of menadione was estimated at 1.2 μmol/h. Expose test solutions were 1 μmol/l and 0.1 μmol/l, which contain 0.2 μmol/200 ml and 0.02 μmol/200 ml menadione respectively. Therefore 0.1 and 1 μM menadione which used for *in vivo* treatment in this study are complete decomposed by fluorescent light within 8 hr.

DISCUSSION

Menadione is a synthetic vitamin K3, and is reportedly toxic to mammalian hepatic cells and invertebrates.[7, 10] Jung *et al*. studied the anti-estrogenic activity of various chemical substances by yeast two-hybrid assay, reporter gene assay and competitive binding assay. They reported that menadione and other two chemicals (pentachlorophene and hexachlophene) bind to estrogen receptor competitively with E2, thus confirming anti-estrogenic activity *in vitro*.[15] However, the *in vivo* anti-estrogenic potency of menadione remains uncertain.

In this paper, we investigated the *in vivo* anti-estrogenic action of menadione based on estrogen-

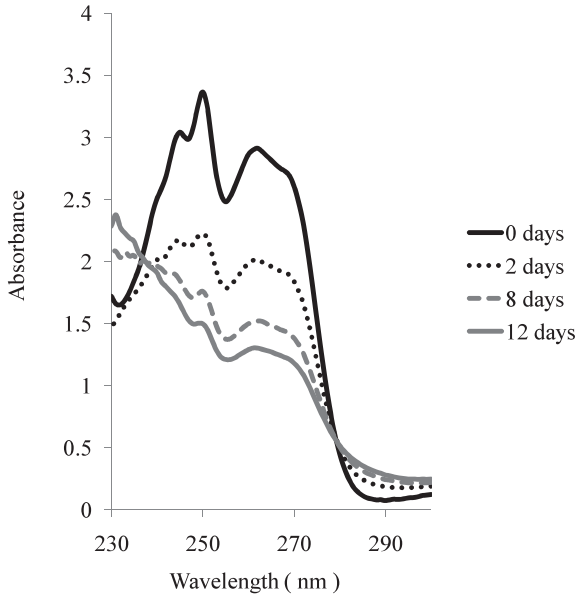

Fig. 3. Changes in Menadione Spectrum after Fluorescent Light Exposure

responsive gene (VTG 1, 2 and ER-α) expression in the liver of male medaka. Tamoxifen has been reported to be a partial agonist of E2 in male medaka, but it usually works as an antagonist in female and E2-treated male medaka.[17] Tamoxifen suppressed estrogen-responsive genes in the presence of E2. At 1 μM, tamoxifen showed particularly marked suppression when compared to 0.1 μM, and thus, tamoxifen exerts dose-dependent suppression. Sun *et al*. investigated plasma VTG concentrations in male and female medaka treated with various concentrations of tamoxifen for 21 days, and found that plasma VTG levels dose-dependently increased in male medaka. However, plasma VTG levels decreased in females treated with high levels of tamoxifen.[17] This suggests that tamoxifen shows partial agonistic activity due to competitive inhibition of E2, depending on its concentration *in vivo*. Taken together with our results, the data confirm that tamoxifen acts as a partial agonist in fish liver.

On the other hand, in male medaka treated with menadione, the induction pattern of estrogen-responsive genes was different from that with tamoxifen treatment. In the absence of E2, VTG1 and VTG2 mRNA was not induced, while ER-α mRNA levels in the liver were suppressed. In the presence of E2, however, menadione suppressed hepatic VTG2 mRNA and ER-α mRNA expression in livers in a dose-dependent manner. These results suggest that menadione shows anti-estrogenic activity *in vivo*. Although Jung *et al*. reported menadione binding activity to ER *in vitro*,[15] menadione did not induce estrogen-responsive genes *in vivo*. Thus, it is possible that menadione is a full E2 antagonist *in vivo*. Previously we showed VTG2 mRNA expressions lower than VTG 1 mRNA on non-treated male medaka liver and induction of VTG2 mRNA by E2 and estrogenic chemical treatment was more significant than that of VTG1 mRNA.[4] In this paper, we demonstrated that VTG2 mRNA was also more sensitive for anti-estrogenic effect than VTG1 mRNA.

Menadione was also found to lose anti-estrogenic potency under fluorescent light. These results suggest that the anti-estrogenic potency of menadione is diminished by light irradiation. Vitamin K compounds, including menadione, are known to be affected by sunlight, irradiation and radioactivity. As menadione is toxic to invertebrates and is light sensitive, menadione is candidate biocide for ship ballast water, which contains numerous microorganisms.[13] Raikow *et al*. investigated the changes in menadione toxicity under sunlight conditions, and found that toxicity to invertebrates is diminished by exposure to sunlight, thus suggesting that light exposure results in a structural change. Here, we also demonstrated that the anti-estrogenic activity is diminished, even after brief exposure to fluorescent light.

Mee *et al*. investigated the light decomposition of menadione by gas chromatograph mass spectrometry. They suggested that menadione is readily converted to an oxide or epoxide under sunlight.[14] It is likely that the same reaction occurred in our study. However, the molecular details of the structural changes are unclear. Further investigations into the molecular details and correlations with anti-estrogenic activity *in vivo* are necessary.

In conclusion, we demonstrated that menadione shows anti-estrogenic activity *in vivo*, resulting suppression of hepatic estrogen-responsive genes, such as VTG2 and ER-α, in male medaka, and that this activity is based on its molecular structure. This is the first report describing the *in vivo* anti-estrogenic activity of menadione. The relationship between anti-estrogenic potency and molecular structure of menadione will be investigated in the future, and this will help in understanding the structural relationship of anti-estrogenicity.

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