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Activation of matrix metalloproteinase-2 and -9 by 2- and 4-hydroxyestradiol

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Abstract

Breast cancer patients frequently develop metastases. This process requires the degradation of extracellular matrix proteins which act as a barrier to tumour cell passage. These proteins can be degraded by proteases, mainly the matrix metalloproteinases (MMPs). MMP-2 and -9 which are frequently detected in breast cancer tissues. ProMMPs are released from cancer cells, and their activation is considered to be a crucial step in metastases development. In breast cancer, estrogen metabolism is altered favouring the accumulation of 2- and 4-hydroxyestradiol (2- and 4-OHE_2). These estradiol metabolites can generate free radicals. Since reactive species are known activators of proMMPs, this study was designed to determine if the free radicals generated by 2- and 4-OHE_2 can activate proMMP-2 and -9. Activation of MMPs by hydroxyestradiol was determined by monitoring the cleavage of a fluorogenic peptide and by zymography analysis. Both estradiol metabolites activated the MMP-2 and -9. 4-OHE_2 was a more potent activator than 2-OHE_2, which reflects its higher capacity to generate free radicals. ProMMPs activation was mainly mediated through $O_2^{\bullet -}$, although the free radical $HO^{\bullet}$ also activated the proMMPs but to a lesser extent. ProMMPs activation was not observed with estrogens that cannot generate free radicals, i.e. estradiol, estrone, 2- and 4-methoxyestradiol, and 16α-hydroxyestrone. These results demonstrate that 2- and 4-OHE_2 at a concentration as low as 10^{-8} M can activate the proMMP-2 and -9 and might play an important role in the invasion of breast cancer cells.

Keywords: Free radicals; Estrogens; Matrix metalloproteinase; Metastasis

1. Introduction

Women with breast cancer frequently develop metastases. This process requires the degradation of the extracellular matrix proteins which act as a barrier to tumour cell migration. These proteins can be degraded by proteases, mainly the matrix metalloproteinases (MMPs) [1,2]. In breast cancer, MMP-2 and -9 are frequently detected [3,4]. These MMPs are released under an inactive form (proMMPs) by cancer cells, leukocytes and endothelial cells [5,6]. Under its inactive proMMP form, the propeptide domain covers the active site blocking the access to substrates. This configuration is stabilized through an interaction between a cysteine located at a key position in the propeptide and a zinc atom at the MMP active site [7,8]. Activation of proMMPs is considered to be a crucial step in metastases development [1].

ProMMPs can be activated by low concentrations of such reactive oxygen species as hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) and superoxide anion ($O_2^{\bullet -}$) [9,10]. This activation of proMMPs is inhibited by the addition of catalase or superoxide dismutase (SOD), which supports the involvement of H_2O_2, $HO^{\bullet}$ and $O_2^{\bullet -}$ in the activation process [9]. It is suggested that free radicals can dissociate the cysteine–zinc interaction between the propeptide and the active site allowing the autocleavage of the propeptide and the release of active MMP [11].

Abbreviations: APMA, *p*-aminophenylmercuric acetate; $CuSO_4$, copper sulfate; Cu(II), copper(II); E_2, estradiol; E_1, estrone; ER, estrogen receptor; HPLC–MS, high pressure liquid chromatography–mass spectroscopy; H_2O_2, hydrogen peroxide; 2-OHE_2, 2-hydroxyestradiol; 4-OHE_2, 4-hydroxyestradiol; 2-OHE_1, 2-hydroxyestrone; 4-OHE_1, 4-hydroxyestrone; 16α-OHE_1, 16α-hydroxyestrone; $HO^{\bullet}$, hydroxyl radical; 2-$MeOHE_2$, 2-methoxyestradiol; 4-$MeOHE_2$, 4-methoxyestradiol; 2-$MeOHE_1$, 2-methoxyestrone; 4-$MeOHE_1$, 4-methoxyestrone; MMPs, matrix metalloproteinases; PBS, phosphate-buffered saline; RFU, relative fluorescent unit; $O_2^{\bullet -}$, superoxide anion; SOD, superoxide dismutase

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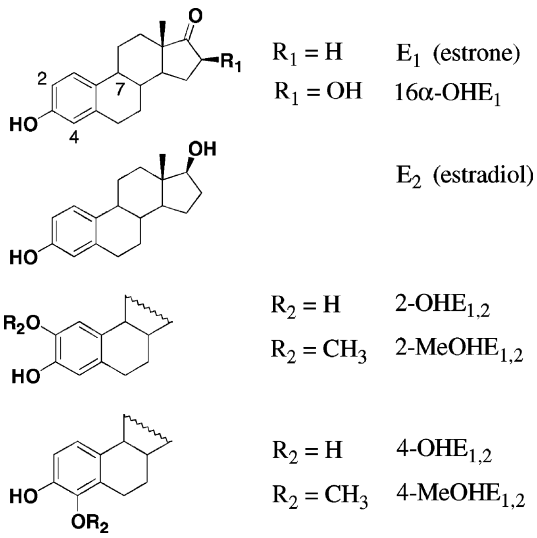

Fig. 1. Structure of estradiol and its principal metabolites.

Estrogens are involved in breast cancer development. In these tumours, estradiol metabolism is altered leading to the accumulation of the hydroxyestrogens 2- and 4-hydroxyestradiol (2- and 4-OHE_2) as well as 2- and 4-hydroxyestrone (2- and 4-OHE_1) [12–14]. These hydroxyestrogens have two hydroxyl groups on their aromatic ring A, leading to a structure called catecholestrogen (Fig. 1). These hydroxyestrogens can be methylated on position 2 or 4 by the catechol-*O*-methyltransferase to produce the 2- and 4-methoxyestradiol (2- and 4-$MeOHE_2$) and also 2- and 4-methoxyestrone (2- and 4-$MeOHE_1$). Alteration of the estradiol metabolism can also lead to the accumulation of 16α-hydroxyestrone (16α-OHE_1), which is suggested to be related to the development of breast cancer [15,16]. However, the mechanisms involved regarding 16α-OHE_1 are still largely unknown.

In the presence of cytochrome P-450 oxidase, 2- and 4-hydroxyestrogens under their catechol form are converted into semiquinone estrogen. We have demonstrated, as have others, that the conversion of catecholestrogen to semiquinone estrogen can also be performed through a non-enzymatic pathway using the copper (Cu^{2+}) ion [17,18]. This radical structure of the hormone then reacts with molecular oxygen to generate the $O_2^{\bullet -}$, while the hormone is converted into quinone estrogen. Both hydroxyl groups on the aromatic ring of estrogen are required for the generation of free radicals, since the methoxy metabolites (2- and 4-$MeOHE_2$ and 2- and 4-$MeOHE_1$) are unable to generate free radicals [17].

We have also demonstrated that the 4-hydroxy derivatives generate about 2.5 times more free radicals than their equivalent 2-hydroxy, leading to DNA strand breaks. No significant difference was measured when comparing to the hydroxyestrogens derived from either estradiol or estrone [17]. Considering the accumulation of the 4-hydroxyestrogens in breast tumours, it is likely that the local production of free radicals by 4-OHE_1 and 4-OHE_2 is high [12,19].

The role of free radicals generated by estrogens in metastases development has never been determined. The present study was designed to determine whether the hydroxyestrogens can activate proMMP-2 and -9.

2. Materials and methods

2.1. Chemicals and reagents

Cupric sulfate ($CuSO_4$) was obtained from the Fisher Scientific Co. (Fair Lawn, NJ). *p*-Aminophenylmercuric acetate (APMA), 2-$MeOHE_2$, 2-OHE_2, 4-OHE_2, 16α-OHE_1, estradiol, estrone, ascorbic acid, gelatin, mannitol and superoxide dismutase (Cu, Zn–SOD) were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON). 4-$MeOHE_2$ was obtained from Steraloids Inc. (Newport, RI). Chelex-100 resin was obtained from Bio-Rad (Richmond, CA). Fluorogenic peptide MMP Substrate III, proMMP-2 and -9 were obtained from Calbiochem (San Diego, CA).

2.2. Removal of endogenous metal ions from buffers and water

Water and buffers used were treated with Chelex-100 resin to remove endogenous metal ions as described previously [17]. This treatment was performed prior to the addition of $ZnCl_2$ or $CaCl_2$. To verify that our solutions were metal free, the ascorbate assay was performed [20]. Briefly, an ascorbate solution (0.1 M) was prepared using reagent grade ascorbic acid and ultra pure water. To perform the assay, 4 μl of 0.1 M ascorbate was added to 3 ml of the solution to be tested. This resulted in an initial absorbance of approximately 1.8 at 265 nm, which was then followed for 15–30 min. In a successfully demetaled buffer, the loss of ascorbate should be <0.5% after 15 min. A larger decrease of absorbance would indicate that a significant concentration of transition metals remained.

2.3. Oxygen consumption

Oxygen (O_2) consumption was monitored for 15 min with a Clark oxygen electrode (YSI-53, Yellow Spring, OH) following mixing of estrogens (40 μM) with Cu^{2+} (10 μM) in air-saturated PBS, pH 7.4, at 37 °C. The PBS had been treated overnight with Chelex-100 resin to remove endogenous metal ions.

2.4. HPLC–MS analyses of quinone estrogens

Formation of quinone estrogens was detected by HPLC–MS analysis. 4-OHE_2 and 4-$MeOHE_2$ (100 μM)

were incubated with the Cu^{2+} (100 μM) in PBS for 30 min at 37 °C. The resulting mixture was injected in a HPLC Alliance equipped with a X-Terra C_{18} column (Waters, Milford, MA) and eluted at a flow rate of 1 ml/min using Chelex-treated water (49.7%), acetonitrile (49.7%) and acetic acid (0.6%) as mobile phase. The separated products were then ionized by APCI and analyzed with a mass spectrometer platform from Micromass (Manchester, UK) equipped with the MassLynx software (Micromass, Manchester, UK). The HPLC–MS analyses were performed by Néokimia (Sherbrooke, Que., Canada).

2.5. Activation of proMMP-2 and -9 by estrogens

ProMMP-2 or -9 (0.17 μg) was incubated for 2 h at 37 °C with one of the estrogens tested (10^{-5} or 10^{-8} M) or 0.01% ethanol (solvent used to dissolved the hormones) in 20 μl of reaction buffer (proMMP-2: 50 mM borate pH 7.4, 5 mM $CaCl_2$, 20% glycerol, 0.005% Brij-35, 0.01 mM $ZnCl_2$; proMMP-9: 100 mM Tris pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.01% Brij-35). Cu^{2+} (10 μM) was added to the estrogens to initiate the generation of free radicals. In parallel, free radical scavengers were added, i.e. Cu, Zn–SOD at 200 U/ml to neutralize the $O_2^{\bullet -}$ or mannitol at 10 mM to scavenge the $HO^{\bullet}$. After activation of MMP-2 or -9, the samples were then incubated with 50 μM (MMP-2) or 22 μM (MMP-9) of the fluorogenic peptide MMP Substrate III (Calbiochem, San Diego, CA). This peptide contains the MMP cleavable Gly–Leu bond and the EDANS–Dabcyl as fluorophore–quencher combination. After enzymatic cleavage by active MMPs, quenching is eliminated and fluorescence can be measured. The enzymatic activity was recorded according to the variation of relative fluorescent unit RFU/s. Care was taken to always work at the saturated concentration of the fluorogenic substrate. The kinetics of peptide cleavage were then followed for 30 min using the 96-well plate reader Synergy HT, BIO-TEK Instruments (Winooski, VT) set at a $\lambda_{ex} = 340$ nm and a $\lambda_{em} = 485$ nm. As a positive control, APMA (1 mM) was incubated with proMMP-2 and -9 for 2 h at 37 °C, and the level of MMP activation was measured using the fluorogenic peptide as described.

2.6. Zymogram analysis

Aliquots of 4 ng proMMP-2 were exposed to 4-OHE_2 (10^{-5} M) and Cu^{2+} (10 μM) at 37 °C for 2 h. Activation of proMMP-2 with 1 mM APMA was also performed under the same conditions and used as a positive control. Samples were applied on a 12% polyacrylamide–SDS gel containing 0.2% gelatin and electrophoresed at 150 V during 3 h at 4 °C. After removal of SDS from the gel by incubating in 2.5% Triton X-100 (30 min, room temperature), the gel was incubated at 37 °C for 18 h in 50 mM Tris–HCl, pH 7.5, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton X-100, and stained with Coomassie blue R-250 [21].

2.7. Statistical analysis

Each assay was repeated three–six times. Data are expressed as the mean ± standard deviation.

3. Results

3.1. Generation of free radicals by estradiol metabolites

According to current models, a catechol compound such as the 2- and 4-hydroxyestrogens can be converted to a semiquinone estrogen in the presence of transition metal ions which act as electron acceptors. A second electron can then be transferred to O_2 producing the quinone estrogen and the radical $O_2^{\bullet -}$. This free radical can dismutate into H_2O_2. The Fenton reaction could then occur in the presence of Cu^+ or Fe^{2+} to produce the radical $HO^{\bullet}$.

To demonstrate that 2- and 4-OHE_2 can generate $O_2^{\bullet -}$, the consumption of molecular oxygen was measured using a Clark electrode. Endogenous metal ions in water and buffer solutions were previously removed following treatment with the resin Chelex-100. The ascorbate assay was performed to certify that our solutions were metal ion free [20].

Although 2- and 4-OHE_2 underwent a slow oxidation in aqueous solution (data not shown), the presence of Cu^{2+} was required to significantly enhance oxygen consumption. The addition of Cu^{2+} alone did not produce any $O_2^{\bullet -}$, as evidence by the absence of oxygen consumption. The best estrogen was 4-OHE_2 metabolite which consumed O_2 at a level 2.7-fold higher than its equivalent 2-hydroxy metabolite (Fig. 2).

The same assay was repeated with the methylated forms of 2- and 4-OHE_2, i.e. 2- and 4-$MeOHE_2$, as well as with estradiol, estrone and 16α-OHE_1. As shown in Fig. 2, the methylated metabolites 2- and 4-$MeOHE_2$ did not consume any oxygen in the presence of Cu^{2+}, indicating that it is the catechol form of the estrogen metabolites that is required for O_2 consumption. Regarding estradiol, estrone and

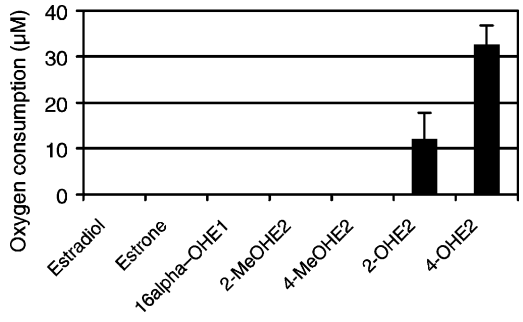

Fig. 2. Oxygen consumption by estrogens. Estrogen (40 μM) was mixed in a solution of PBS at 37 °C. Cu^{2+} (10 μM) was then added to initiate the consumption of oxygen which was monitored for 15 min with a Clark oxygen electrode (2-OHE_2: $P < 0.01$ vs. estradiol, $n = 3$; 4-OHE_2: $P < 0.01$ vs. estradiol, $n = 3$).

16α-OHE_1, these estrogens have only one hydroxyl group in position 3 of the aromatic ring A. As a consequence, they do not possess a catechol structure as seen in the 2- and 4-OHE_2 and so no O_2 consumption was measured.

3.2. Formation of quinone estrogen in the presence of Cu^{2+}

The transfer of an electron to O_2 should be accompanied by the conversion of the semiquinone estrogen to its quinone form. The presence of quinone estrogen was recently confirmed in our laboratory by HPLC chromatography combined with a mass spectra analysis [22]. After a 30 min incubation of 4-OHE_2 in the presence of Cu^{2+}, the reaction mixture was separated by HPLC and the elution peak was ionised by APCI to add a hydrogen ion. The resulting compound gave a signal corresponding to the molecular mass of the quinone estrogen plus 1 (MH+). Two hallmarks of the quinone estrogen were also present, a signal corresponding to the addition of sodium (MNa+) and a second one due to the elimination of one water molecule (MH+(−H_2O)) [22]. The blue shift absorbance from a maximum at 280 nm for 4-OHE_2 to 246 nm for the quinone estrogen also corresponded to the shift previously reported for catechol compound conversion to quinone [23]. The mass spectra analysis was repeated with 4-$MeOHE_2$. As anticipated, no quinone was detected with 4-$MeOHE_2$, supporting the thesis that the methylation of a hydroxyl group on the aromatic ring A forbids transfer of an electron to O_2 and the formation of quinone estrogen.

3.3. Activation of proMMP-2 and -9 by estrogens

Metastases development is closely related to the activation of some proMMPs, such as proMMP-2 and -9 for breast cancer. ProMMPs activation can be achieved by low concentrations of reactive oxygen species such as H_2O_2,[1] $HO^{\bullet}$ and $O_2^{\bullet -}$ [9,10]. Since some estradiol metabolites generate these reactive species, we investigated whether they can activate the proMMP-2 and -9.

The first series of assays was performed using the latent MMP-9 (proMMP-9), incubated with the different estrogens added at a concentration of 10^{-5} M and incubated for 2 h at 37 °C. To initiate the generation of free radicals, Cu^{2+} (10 μM) was added. The level of MMP-9 activation was determined using a fluorogenic peptide, the MMP Substrate III (Calbiochem).

Significant activation of proMMP-9 (Fig. 3) was observed after the addition of either 2- or 4-OHE_2, and 4-OHE_2 induced a 3.6-fold greater activation than 2-OHE_2. This observation correlated with the respective capacity of 4- and 2-OHE_2 to generate free radicals, as measured by O_2 consumption (Fig. 2). On the other hand, the addition of Cu^{2+} alone at 10 μM did not activate the proMMP-9, indicating that the presence of 2- or 4-OHE_2 was necessary. For the sake of comparison, the level of activation obtained by adding 4-OHE_2 was 0.40 ± 0.04 RFU/s ($P < 0.01$ versus background, $n = 6$) compared to a level of 1.58±0.16 RFU/s with the chemical activator APMA added at 1 mM and used as a positive control.

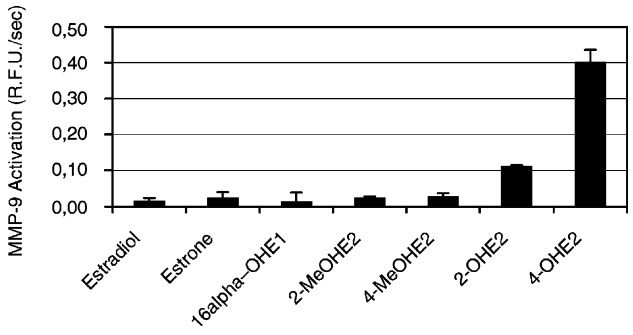

Fig. 3. Activation of proMMP-9 by estrogens. ProMMP-9 (0.17 μg) was incubated for 2 h at 37 °C with one of the estrogens tested (10^{-5} M) or 0.01% ethanol. Cu^{2+} (10 μM) was added at the beginning of the kinetic to initiate the generation of free radicals. The level of MMP-9 activation was measured using a fluorogenic peptide (MMP Substrate III). The kinetic of peptide cleavage was followed for 30 min using the 96-well plate reader set at a $\lambda_{ex} = 340$ nm and a $\lambda_{em} = 485$ nm (2-OHE_2: $P < 0.01$ vs. estradiol, $n = 3$; 4-OHE_2: $P < 0.01$ vs. estradiol, $n = 6$).

The proMMP-9 activation assay was also performed with the other estrogens. As expected, estradiol, estrone, 16α-OHE_1, 2-$MeOHE_2$ and 4-$MeOHE_2$ did not increase the level of MMP-9 activity much higher than the background level (Fig. 3). The metabolites derived from estrone, i.e. 2- and 4-OHE_1 as well as 2- and 4-$MeOHE_1$, were not included in the present study since we have previously shown that the estrone and estradiol metabolites have identical capacities to generate free radicals [22].

To determine whether the hydroxyestrogens can activate other proMMPs, 4-OHE_2 was incubated in the presence of proMMP-2. As seen in Fig. 4, the activation level obtained

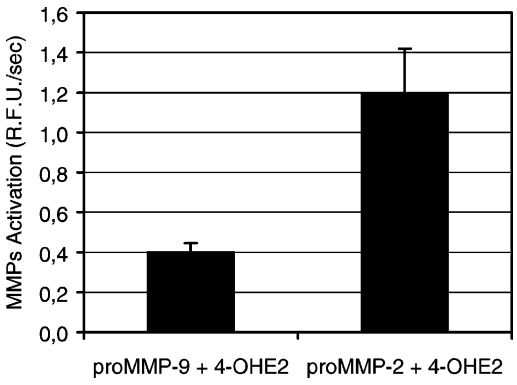

Fig. 4. Activation of proMMP-2 and -9 by 4-OHE_2. ProMMP-2 and -9 (0.17 μg) were incubated for 2 h at 37 °C with 4-OHE_2 (10^{-5} M) and Cu^{2+} (10 μM). The level of MMPs activation was measured using a fluorogenic peptide (MMP Substrate III) as previously described (proMMP-9 + 4-OHE_2 vs. proMMP-9 alone, $P < 0.01$, $n = 6$; proMMP-2 + 4-OHE_2 vs. proMMP-2 alone, $P < 0.01$, $n = 3$).

[1] Idem.

Fig. 5. Cleavage of proMMP-2 propeptide by 4-OHE_2. ProMMP-2 (4 ng) was incubated with 4-OHE_2 (10^{-5} M) and Cu^{2+} (10 μM) for 2 h at 37 °C. Activation of proMMP-2 with 1 mM APMA was also performed under the same conditions and used as a positive control. Samples were then analysed using a gelatin zymography assay.

with MMP-2 was three-fold higher than that measured with MMP-9 (alone proMMP-9 = 0.038 ± 0.004 RFU/s, n = 6, proMMP-9 + 4-OHE_2 = 0.40 ± 0.04 RFU/s, n = 6, $P < 0.01$; alone proMMP-2 = 0.01 ± 0.03 RFU/s, n = 3, proMMP-2 + 4-OHE_2 = 1.19 ± 0.23 RFU/s, n = 3, $P <$ 0.01).

3.4. Cleavage of the proMMP-2 propeptide by 4-OHE_2

Activation of proMMPs requires the removal of the propeptide that covers the active site of the protease. This configuration is stabilized through an interaction between a cysteine located at a key position in the propeptide and a zinc atom at the MMP active site [7,8].

For more insight into the mechanisms of proMMP activation by the hydroxyestrogens, cleavage of the propeptide of proMMP-2 was determined by gel zymography. This assay distinguished between proMMP and MMP which migrated according to their molecular weights. Gel zymography is a non-reducing acrylamide gel polymerized with the MMP-2 substrate, gelatin. During electrophoresis, proMMP-2 is artificially activated without losing its propeptide. After migration, the gel is incubated under appropriate conditions that allow all pro- and cleaved metalloproteinase species to locally digest the gelatin. Areas digested by metalloproteinases appear as clear bands after staining with Coomassie blue. ProMMP-2 activated by APMA was included as a control.

Human proMMP-2 has a molecular weight of 72 kDa while its activation by APMA yielded an active species of 65 kDa [24]. The zymography assay demonstrated that the proMMP-2 is converted to its active MMP-2 form when incubate in the presence of 4-OHE_2 (Fig. 5). These data suggest that free radicals generated by 4-OHE_2 can disrupt the interaction between the SH group of Cys^{73} located in the propeptide domain and the zinc atom at the MMP-2 active site.

3.5. MMP activation at a physiological concentration of hydroxyestrogens

A relatively high concentration of estradiol metabolites (10^{-5} M) was used in the initial assays of proMMPs activation. However, circulating estrogen concentrations in premenopausal women are around 10^{-9} to 10^{-10} M [25]. The estrogen concentration in breast tumours is higher reaching 10–50-fold the concentration found in blood [26]. In addition, higher enzymatic activities leading to the synthesis of hydroxyestrogens were also detected in breast tumours compared to normal tissue [12–14]. Therefore, it is reasonable to expect a level of 4-OHE_2 around 10^{-8} M in breast tumours. Thus, the enzymatic assay was repeated to determine if 4-OHE_2 can still activate proMMPs at a concentration as low as 10^{-8} M.

Table 1
MMPs activation at physiological concentration of 4-OHE_2

	Enzymatic activity (RFU/s)	
	10^{-5} M 4-OHE_2	10^{-8} M 4-OHE_2
MMP-2	1.19 ± 0.23	0.48 ± 0.11
MMP-9	0.40 ± 0.04	0.26 ± 0.06

Under these conditions, the level of MMP-2 and -9 activated by 4-OHE_2 in the presence of 10 μM Cu^{2+} was still high, reaching, respectively, 0.48 and 0.26 RFU/s, compared to 1.19 and 0.4 RFU/s when the estradiol metabolite was incubated at 10^{-5} M (Table 1). In the absence of Cu^{2+}, the level of MMPs activation with 10^{-8} M 4-OHE_2 was negligible, i.e. 0.01 RFU/s, suggesting that the activation at physiological concentrations of 4-OHE_2 was related to the generation of free radicals. For the sake of comparison, no significant activation of proMMP-2 and -9 (RFU/s <0.002) was obtained when APMA was added at the same concentration as 4-OHE_2, i.e. 10^{-8} M.

3.6. Role of Cu^{2+} in proMMP activation

At a concentration of 10^{-8} M of 4-OHE_2, the addition of Cu^{2+} was required to activate proMMP-2 and -9. However, when the concentration of 4-OHE_2 was increased to 10^{-5} M, proMMP-2 and -9 were activated even in the absence of Cu^{2+}, although at a lower level (Table 2). The production of free radicals by 4-OHE_2 requires the participation of transition metals such as Cu^{2+}. The quantity of such ions was estimated in the preparation of proMMP-2 and -9 supplied by Calbiochem (San Diego, CA) using the ascorbate assay

Table 2
Role of Cu^{2+} in proMMPs activation

	Enzymatic activity (RFU/s)	
	10^{-5} M 4-OHE_2	10^{-8} M 4-OHE_2
MMP-2		
$+Cu^{2+}$	1.19 ± 0.23	0.48 ± 0.11
$-Cu^{2+}$	0.17 ± 0.01	0.01 ± 0.01
MMP-9		
$+Cu^{2+}$	0.40 ± 0.04	0.26 ± 0.06
$-Cu^{2+}$	0.31 ± 0.03	0.01 ± 0.01

Table 3
Role of free radicals in proMMPs activation

	Enzymatic activity (RFU/s)	
	10^{-5} M 4-OHE_2	10^{-8} M 4-OHE_2
MMP-2		
−SOD/mannitol	1.19 ± 0.23	0.48 ± 0.11
+SOD	0.51 ± 0.20[a]	0.13 ± 0.08[b]
+Mannitol	0.91 ± 0.49[c]	0.23 ± 0.15[d]
MMP-9		
−SOD/mannitol	0.40 ± 0.04	0.26 ± 0.06
+SOD	0.29 ± 0.06[e]	0.03 ± 0.04[f]
+Mannitol	0.27 ± 0.06[g]	0.15 ± 0.13[h]

[a] vs. MMP-2 − SOD/mannitol + 4-OHE_2 10^{-5} M, $P = 0.002$, $n = 4$.
[b] vs. MMP-2 − SOD/mannitol + 4-OHE_2 10^{-8} M, $P = 0.001$, $n = 4$.
[c] vs. MMP-2 − SOD/mannitol + 4-OHE_2 10^{-5} M, $P = 0.106$, $n = 5$.
[d] vs. MMP-2 − SOD/mannitol + 4-OHE_2 10^{-8} M, $P = 0.02$, $n = 5$.
[e] vs. MMP-9 − SOD/mannitol + 4-OHE_2 10^{-5} M, $P = 0.03$, $n = 4$.
[f] vs. MMP-9 − SOD/mannitol + 4-OHE_2 10^{-8} M, $P < 0.001$, $n = 4$.
[g] vs. MMP-9 − SOD/mannitol + 4-OHE_2 10^{-5} M, $P < 0.01$, $n = 4$.
[h] vs. MMP-9 − SOD/mannitol + 4-OHE_2 10^{-8} M, $P = 0.19$, $n = 4$.

and Cu^{2+} as standard. In the preparations of proMMP-2 and -9, the concentrations of Cu^{2+}-like transition metals were, respectively, 1.85 and 1.35 μM. These data suggested that impurities in the proMMP-2 and -9 preparations could initiate the production of free radicals when the quantity of 4-OHE_2 is elevated.

3.7. Identification of the free radicals involved

To provide further evidence that MMPs activation induced by hydroxyestradiol was mediated by free radicals, the scavengers Cu, Zn–SOD and mannitol were added. The resulting MMPs activity was measured using the fluorogenic peptide MMP Substrate III, as previously described.

Addition of Cu, Zn–SOD, which neutralizes $O_2^{\bullet -}$, led to an important reduction of MMP-2 and -9 activation initiated at either 10^{-5} or 10^{-8} M of 4-OHE_2 (Table 3). Inhibition by mannitol, the scavenger of $HO^{\bullet}$, was still important but less pronounced than that obtained with Cu, Zn–SOD. These results suggest that $O_2^{\bullet -}$ generated by this estradiol metabolite could play an important role in the activation of MMP-2 and -9.

Catalase could not be used in our study since it interfered with the enzymatic assay used to quantify the level of MMP activation.

4. Discussion

Estrogens are known to stimulate the proliferation of hormone-dependent breast cancer cells through an interaction with estrogen receptors. However, the effect of estrogens on invasion and metastases development has been much less studied. Using estrogen receptor (ER)-positive MCF-7 and T47D cells, it has been shown that the addition of estradiol stimulated cell growth but inhibited invasion and motility [27]. Supporting these data, the release of MMP-2 and -9 from MCF-7 cells was not altered by estradiol [28,29]. As expected, no effect of estradiol was observed on ER-negative MDA-MB-231 breast cancer cells [30].

In this study, we report that the invasiveness of breast cancer cells could be enhanced by the estradiol metabolites 2- and 4-OHE_2. Indeed, these hormones generate free radicals that convert the inactive proMMP-2 and -9 to their active forms. This activation of proMMPs was measured at a concentration as low as 10^{-8} M of both hormones, a concentration of hydroxyestrogens that can be found in breast tumours. Estradiol concentrations in plasma typically vary from 10^{-9} to 10^{-10} M [25], but higher levels can be reached in breast tissues. Aromatase, the enzyme catalyzing the rate-limiting step in estrogen biosynthesis, is widely present throughout the body. Breast tissue itself contains aromatase, both in its fatty components and in its epithelial cells, and can synthesize estrogen in situ. Immunohistochemical staining studies to detect aromatase activity in breast tumours demonstrated high levels of aromatase staining in individual cells, supporting the concept that aromatase might act in an autocrine or paracrine fashion in breast tissue [31–33]. Consequently, Reed et al. demonstrated that up to 83% of tumour estrogen levels could result from in situ aromatase [34].

Estradiol and estrone in breast tissues can then be efficiently converted to their hydroxyestrogens equivalents. Indeed, a study reporting the activity of the 2- and 4-hydroxylase from isolated malignant breast tumours demonstrated that 2.58×10^{-4} M 2-hydroxyestrogens and 2.69×10^{-4} M 4-hydroxyestrogens can be synthesized in only 30 min [12]. The high level of 2- and 4-hydroxylase in breast tumours results in the accumulation of 2- and 4-hydroxylated estrogens at concentrations 50-fold higher than detected levels of estradiol and estrone [35]. The average level of 2-hydroxyestrogens in normal and malignant tissues reached 8.87×10^{-7} and 1.56×10^{-6} M, while concentrations of 1.3×10^{-6} and 2.1×10^{-6} M were measured for the 4-hydroxestrogens (data reported by the authors in femtomoles per milligram tissue were converted to mol/l using a density of 1.0 g/ml for breast tissues). Hydroxyestrogen accumulation in breast tissues is also not related to the presence of estrogen receptors, since similar levels of 2- and 4-hydroxyestrogens were detected in ER+ and ER− breast tumours [35]. Considering the accumulation of 2- and 4-OHE_2 in breast tumours, the local production of free radicals by the hydroxyestrogens is likely to exceed the concentrations needed to activate proMMPs in the current study [12,19].

Generation of free radicals by hydroxyestrogens requires the presence of a transition metal like Cu^{2+}. Although its concentration is more elevated in breast tumours compared to normal tissues, micromolar levels of free Cu^{2+} as used in our study would not be reached in vivo [36]. However, the 2- and 4-hydroxyestrogens are easily oxidised in cell culture media showing that other transition metals are already

present either bound to proteins or free in the cellular environment.

Activation of proMMPs by the 2- and 4-hydroxyestrogens is mediated by $O_2^{\bullet -}$ and $HO^{\bullet}$. The presence of free radical scavengers in breast tissues could, therefore, regulate the level of proMMPs activation. Breast cancer tissues are known to be under an oxidative stress which results in an up-regulation of antioxidant mechanisms [37,38]. On the other hand, we have previously demonstrated that the process of free radical generation by 2- and 4-OHE_2 can be enhanced by some antioxidants. Indeed, hydroxyestrogens under their semiquinone forms give an electron to oxygen generating $O_2^{\bullet -}$, while the hormone is converted to its quinone estrogen structure. This quinone estrogen in the presence of an electron donor like NADH is reconverted to semiquinone estrogen creating a redox cycle that amplifies the generation of free radicals by 2- and 4-OHE_2. This redox cycle between semiquinone and quinone estrogens can also be maintained by some antioxidants. Indeed, we have reported that some antioxidants such as *N*-acetylcysteine at low concentrations can give an electron to the quinone estrogen generating the semiquinone estrogen and consequently maintaining the semiquinone–quinone estrogen redox cycle. Therefore, the addition of this antioxidant results in an amplification of free radical generation [39]. On the other hand, other antioxidants such as glutathione at certain concentrations, neutralized free radicals and reduced oxidative damage mediated by 2- and 4-OHE_2.

Deleterious effects of some antioxidants has been reported in women with breast cancer [40]. Some cancer patients consume large doses of vitamins and minerals, especially antioxidants to neutralize free radicals [40,41]. However, mega-doses of antioxidants such as β-carotene and Vitamin C have led to an increase of recurrence and to a decrease of survival for women diagnosed with unilateral breast cancer and who have been treated with the usual chemotherapy and radiotherapy treatments [40]. If 2- and 4-OHE_2 estradiol metabolites have an important impact on breast cancer progression, a deleterious outcome of antioxidant consumption might be related to their capacity to maintain the semiquinone–quinone estrogen redox cycle, and lead to an enhancement of free radical generation by 2- and 4-OHE_2.

Since antioxidants can either neutralise free radicals or maintain the semiquinone–quinone estrogen redox cycle, the capacity of hydroxyestrogens to activate proMMPs in breast tumours remains to be confirmed.

However, supporting the potential impact of 2- and 4-OHE_2 in breast cancer progression, our data indicate that the reaction between proMMPs and 2- and 4-OHE_2 is very efficient. Indeed, APMA at 1 mM was added as a chemical activator of proMMPs. According to the zymogram analysis, more than 95% of the proMMP-2 were activated by the APMA (Fig. 5), resulting in an enzymatic activity of 5.61 ± 0.63 RFU/s as measured by the cleavage of the MMP Substrate III. A lower level of enzymatic activity was obtained with 4-OHE_2, i.e. 0.48 ± 0.11 RFU/s. However, this estradiol metabolite was added at a concentration of only 10^{-8} M, while no significant activation was obtained when APMA was added at 10^{-8} M. In addition to the very low concentration of 4-OHE_2 used, the concentration of proMMP-2 in the enzymatic assay was also very low, i.e. 1.18×10^{-7} M, suggesting that the reaction is very efficient and likely to occur in vivo.

Using 4-OHE_2 and proMMP-2 as examples, the likely steps in the activation of proMMPs by hydroxyestrogens are as follows:

$$\text{4-OHE}_2 + \text{Cu}^{2+} \rightarrow \text{4-OHE}_2\text{–semiquinone} + \text{Cu}^{+}$$

$$\text{4-OHE}_2\text{–semiquinone} + \text{O}_2 \rightarrow \text{4-OHE}_2\text{–quinone} + \text{O}_2^{\bullet -}$$

$$\text{O}_2^{\bullet -} + \text{proMMP-2} \rightarrow \text{MMP-2}$$

Our study demonstrates that activation of proMMP-2 by 4-OHE_2 proceeds through the cleavage of the propeptide, which covers the active site blocking the entry to substrate. This configuration is stabilized through an interaction between a cysteine located at a key position in the propeptide and the zinc atom at the MMP active site [7,8]. This configuration is common to most proMMPs. It is suggested that free radicals can dissociate the cysteine–zinc interaction leading to autocleavage of the propeptide and the release of an active MMP [11,42]. Our data indicate that $O_2^{\bullet -}$ generated by 4-OHE_2 at 10^{-8} M was the principal intermediary to activate the proMMPs, while at 10^{-5} M of the hormone, both $O_2^{\bullet -}$ and $HO^{\bullet}$ could trigger cleavage of the propeptide. Supporting the involvement of free radicals, a higher level of MMPs activation was obtained with 4-OHE_2 compared to 2-OHE_2, which corresponds to their relative capacity to generate free radicals. Furthermore, we note that of the estrogens tested, those that cannot generate free radicals are unable to activate the proMMPs.

The 2- and 4-OHE_2 could also be seen as universal activators of proMMPs. The mechanisms by which 2- and 4-OHE_2 activate proMMP-2 and -9 involve the generation of free radicals and without the need of a specific amino acid sequence in the propeptide, as is normally required for the cleavage mediated by a protease. Therefore, it is possible that 2- and 4-OHE_2 could induce the cleavage of other proMMPs.

In summary, we have demonstrated that 2- and 4-OHE_2 at levels potentially present in breast cancer tissues can activate proMMP-2 and -9. Since activation of MMPs is a key step in tumour invasion of surrounding tissues, these observations offer a novel and potentially important mechanism by which estrogen metabolites may promote metastases in women with breast cancer.

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