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Effect of metal ions on the stable adduct formation of 16α-hydroxyestrone with a primary amine via the Heyns rearrangement

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Abstract

16α-Hydroxyestrone (16α-OHE_1), one of the major estrogen metabolites in humans that may plays a role in cell transformation, has been found to form stable adducts with nuclear proteins. The mechanism for the formation of a stable covalent adduct of 16α- OHE_1 with protein has been postulated via the Heyns rearrangement after Schiff base formation. The Heyns rearrangement on the steroidal D-ring α-hydroxyimine was investigated using 17-(2-methoxyethylimino)estra-1,3,5(10)-triene-3,16α-diol as a model intermediate. Rates of the Heyns rearrangement and hydrolysis of the steroidal α-hydroxyimine were determined by a high-performance liquid chromatography (HPLC) simultaneously. The Heyns rearrangement was demonstrated to be optimum at pH 6.2 and the reaction rate at physiological pH, 7.3–7.5, was more than 90% of that at the optimum pH. On the other hand, modulator(s) to the reactions were also examined. According to our previous finding of the proton-mediated mechanism of the Heyns rearrangement, the effects of cationic metal ions on the reactions were examined with 29 metal chlorides. Five metal ions, Pt^{4+}, Cu^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+}, suppressed the formation of Heyns product significantly while Fe^{2+}, Y^{3+}, Gd^{3+}, and Er^{3+} slightly increased it. The suppression rate was synergistically enhanced by the combination of Pt^{4+} with Co^{2+}, Cu^{2+}, or Ni^{2+}. These results suggest the five metal ions, Pt^{4+}, Cu^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+}, reduce the formation of the Heyns product in vivo and, therefore, would be useful tools to clarify the implication of the stable adduct formation of 16α-OHE_1 with protein.

Keywords: 16α-Hydroxyestrone; Stable adduct; Heyns rearrangement; Steroidal D-ring α-hydroxyimine; Metal ion effect; Reduction of the adduct formation

Epidemiologic studies have implicated estrogen in the etiology of breast cancer [1]. The role of major metabolites such as 2- and 16α-hydroxylated estrogens in this process are still the subject of great discussion. Although 16α-hydroxyestrone (16α-OHE_1), which is a principal 16α-hydroxylated estrogen in humans [2], is suspected to be a strong risk factor epidemiologically [3,4], the genotoxic damage caused by the estrogen metabolite is still controversial [5,6]. Recently, 4-hydroxylated estrogen, a minor catechol estrogen, has been found to form genotoxic nucleic acid adducts, while its geometric isomer, 2-hydroxylated estrogen, formed non-genotoxic adducts [7]. On the other hand, a covalent interaction of 16α-OHE_1 with protein also presents a possible mechanism for the generation of oncogenic event(s). It has been revealed that 16α-OHE_1 is an unique long-acting uterotropic estrogen, that the affinity to the estrogen receptor is relatively low [8], and that it forms a stable adduct with the estrogen receptor [9]. 16α-OHE_1 is supposed to form a stable covalent adduct at the 17-position with the primary amino group in protein via the Schiff base (imine) formation followed by Heyns rearrangement of the D-ring α-hydroxyimine as depicted in Fig. 1 [10]. Previously, we have demonstrated that an α-hydroxyimine of 16α-OHE_1 gave a fairly stable adduct, N-(3-hydroxy-16-oxoestra-1,3,5(10)-trien-17β-yl)amine derivative in aqueous methanol (MeOH) by the Heyns rearrangement. Although the formation rate of the Heyns product may be influenced by biologic and environmental factors, these internal or external factors have not been resolved. Modulators for adduct formation are urgently needed to clarify the implication of 16α-OHE_1 in carcinogenic event(s). According to our proton-mediated mechanism of the Heyns rearrangement proposed previously [11], metal ions are presumed to interact with the reaction intermediate as well as the proton. In this study we

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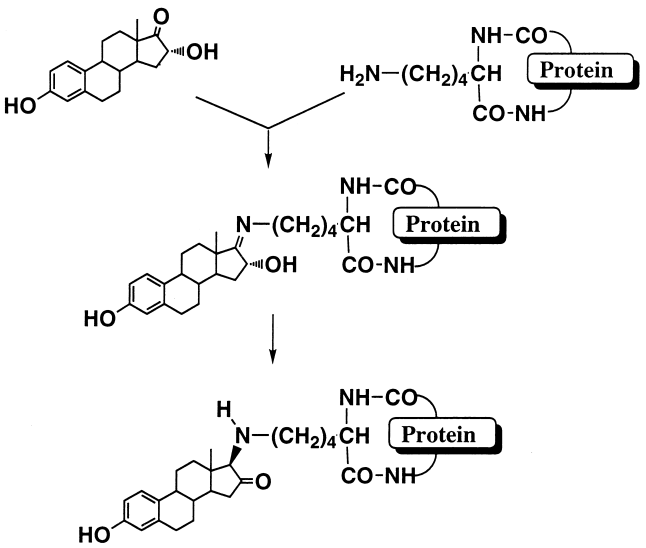

Fig. 1. Mechanism for the stable adduct formation between 16wα-OHE_1 and protein.

examined the effects of metal cations on the Heyns rearrangement and the stability of the steroidal α-hydroxyimine using newly developed HPLC for simultaneous determination of the Schiff base and products.

1. Experimental

1.1. Materials

Estrone (E_1), estradiol (E_2), and estriol (E_3) were purchased from Steraloids Inc. (Wilton, NH, USA). 2-Methoxyethylamine (2-MEA) was obtained from Tokyo Kasei Kogyo Ltd. (Tokyo, Japan). 16α-OHE_1 diacetate was prepared from E_1 by the method of Leeds et al. with minor modifications [12]. 3-Hydroxy-17β-(2-methoxyethylamino)-esestra-1,3,5(10)-trien-16-one (16-oxo,17β-amine), 17β-(2-methoxyethylamino)estra-1,3,5(10)-triene-3,16α-diol (16α-OH,17β-amine) hydrochloride and 17β-(2-methoxyethylamino)estra-1,3,5(10)-triene-3,16β-diol (16β-OH,17β-amine) hydrochloride were prepared by the methods described previously (Fig. 2) [11]. 6α-Hydroxyestradiol (6α-OHE_2) was prepared from E_2 by the method of Wintersteiner and Moore [13]. 16β-Hydroxyestradiol (16-epiE_3) was produced by sodium borohydride ($NaBH_4$) treatment of 16-oxoestradiol (16-oxoE_2) which was prepared from 16α-OHE_1 by keto-enol rearrangement under basic condition.

1.2. Apparatus

The apparatus used for HPLC was a LC-10AD solvent delivery system equipped with a SPD-10A spectrophotometer (at 280 nm) and a CHROMATOPAC C-6A data processor (Shimadzu, Kyoto, Japan).

1.3. Determination of the products of the Heyns rearrangement and hydrolysis of 16α-OH,17-imine

17-(2-Methoxyethylimino)estra-1,3,5(10)-triene-3,16α-diol (16α-OH,17-imine) was prepared from 16α-OHE_1 and 2-MEA in MeOH as reported previously [11]. The 16α-OH,17-imine was determined to be approximately 95% of the total estrogen in the original preparation by the follow-

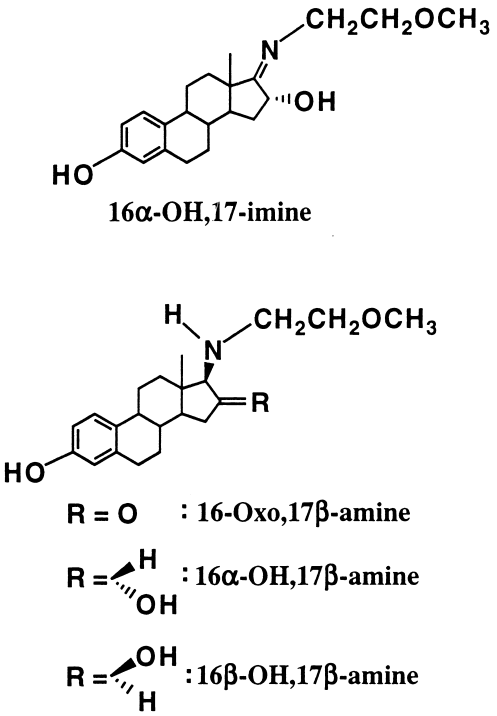

Fig. 2. Structures of the major products after reduction with $NaBH_4$ of the steroidal α-hydroxyimine and its rearranged or hydrolyzed products.

Table 1
Epimeric ratio of the $NaBH_4$ reduction of carbonyl group at 16- or 17-position

Estrogen	α-Configuration	β-Configuration
16-oxo,17β-amine	3.1	96.9
16-oxo,17β-OH	9.8	90.2
16α-OHE_1	93.6	6.7

The ratio was determined by HPLC using the peak areas.

ing HPLC method. 16α-OH,17-imine (27 mM at the final concentration) was incubated in a mixture of 50 mM Tris HCl buffer (various pH condition or pH 6.2) with or without a metal ion and MeOH (1:1 v/v) for 10 min at 37°C. The reaction was terminated by the addition of $NaBH_4$. Then the resulting mixture was loaded onto a Sep-Pak C_{18} cartridge after the addition of 6α-OHE_2 (the internal standard (*IS*) for HPLC) and water, washed thoroughly with water and eluted with MeOH (5 ml). The eluate was subjected to HPLC on a Puresil C_{18} column (5 μm, 150 × 4.6 mm internal diameter; Millipore-Waters, Milford, MA, USA) using 0.5 (w/v)% potassium phosphate buffer (pH 6.8)/MeOH (1:1 v/v) as the eluent.

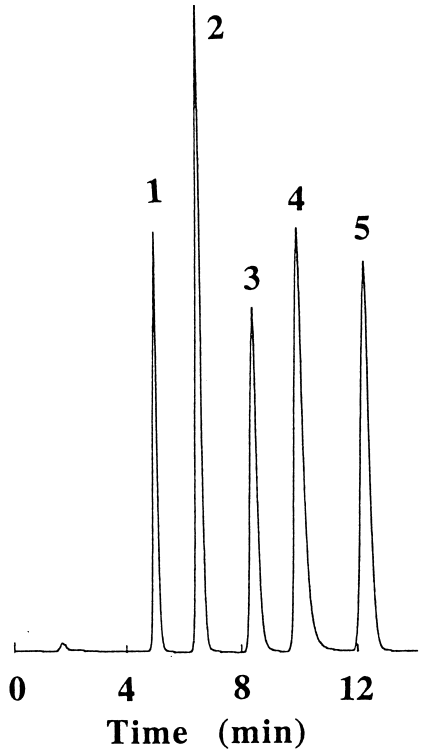

Fig. 3. Chromatogram of the estrogen derivatives. Mobile phase: 0.5% potassium phosphate buffer (pH 6.8)/MeOH (1:1, v/v). Flow rate: 1.0 ml/min. UV absorption of the estrogen derivatives were monitored at 280 nm. The numbers on the peaks indicate: **1**; 6α-OHE_2 (*IS*), **2**; E_3, **3**; 16α-OH,17β-amine, **4**; 16β-OH,17β-amine, **5**; 16-epiE_3.

2. Results and discussion

The formation of stable adduct of 16α-OHE_1 to several proteins has been investigated using a radio-labeled ligand. The radioactivity was retained preferentially by nuclear components such as histones [14] and the estrogen receptor [9] after incubation with ^{3}H-labeled 16α-OHE_1, while E_3 failed to show covalent binding [14]. The two-step mechanism for the formation of a covalent adduct of 16α-OHE_1 with protein has been proposed to proceed via Schiff base (α-hydroxyimine) formation with an amino group of proteins followed by Heyns rearrangement of the α-hydroxyimine. Formation of the Schiff base of 16α-OHE_1 at the 17-position with the ϵ-amino group of lysine in aqueous medium has been revealed in the presence of sodium cyanoborohydride [15]. Furthermore, we have proved that the Heyns rearrangement proceeded on the steroidal α-hydroxyimine with the lysinyl ϵ-amino group in neutral condition and gave Nϵ-(3-hydroxy-16-oxoestra-1,3,5(10)-trien-17β-yl)-L-lysine, which is considered to be a partial structure of the stable 16α-OHE_1-protein adduct [16]. However the reaction condition as well as the effector of the Heyns rearrangement of the steroidal α-hydroxyimine have not been clarified. As it is difficult to analyze the Heyns product formed in proteins, a Schiff base from 16α-OHE_1 with 2-MEA, a model amino compound, was utilized to demonstrate the pH effect and search for the effector related to the rearrangement.

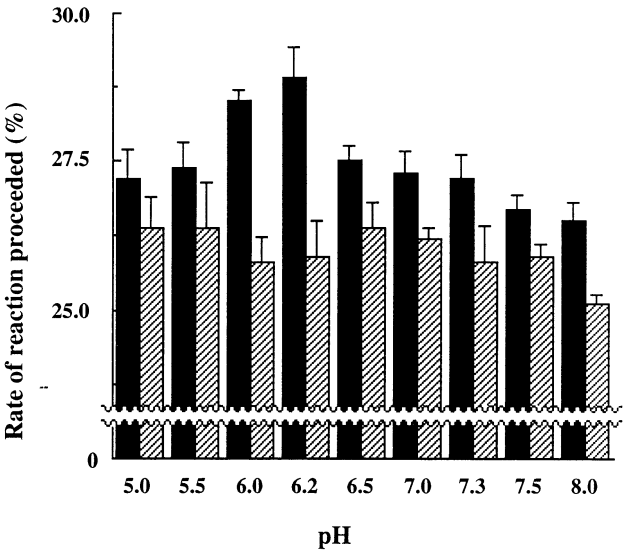

Fig. 4. Effect of pH on the Heyns rearrangement and the hydrolysis of the steroidal D-ring α-hydroxyimine. 16α-OH,17-imine was incubated in a mixture of 50 mM Tris HCl buffer (pH 5.0–8.0) and MeOH (1:1, v/v) for 10 min at 37°C and then treated with $NaBH_4$. The estrogen derivatives in the solution were subjected to HPLC analysis. ■, the stable adduct (16-oxo-17β-amine); ▨, hydrolyzate (16α-OHE_1 + 16-oxoE_2).

Table 2
Metal chloride used for the examination of effects on the Heyns rearrangement and hydrolysis of 16α-hydroxyestrone 17-imine

Valency	Compound
Divalent	$FeCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$, $BaCl_2$, $MgCl_2$, $CaCl_2$, $SrCl_2$
Trivalent	$FeCl_3$, $CrCl_3$, $AlCl_3$, YCl_3, Lanthanides ($LaCl_3$, $CeCl_3$, $PrCl_3$, $NdCl_3$, $SmCl_3$, $EuCl_3$, $GdCl_3$, $TbCl_3$, $DyCl_3$, $HoCl_3$, $ErCl_3$, $YbCl_3$)
Tetravalent	$PtCl_4$, $SeCl_4$

2.1. HPLC system for determination of the products from the steroidal α-hydroxyimine

After incubation of 16α-OH,17-imine in an aqueous MeOH, 16-oxo,17β-amine (the stable adduct), 16α-OHE_1 (the primary hydrolyzed product) and 16-oxoE_2 (the secondary product from 16α-OHE_1 by keto-enol rearrangement) were present in the resulting mixture in addition to the Schiff base theoretically. Therefore, simultaneous HPLC analysis of these products and 16α-OH,17-imine was performed to evaluate the rates of the Heyns rearrangement and hydrolysis of 16α-OH,17-imine. However, the α-hydroxyimine was too labile to be detected directly by the system. Hence, the incubation mixture was treated with $NaBH_4$ to transform it into a stable form, 16α-OH,17β-amine. The other estrogen derivatives that co-existed were also affected by this treatment. The carbonyl containing estrogen derivatives, 16-oxo,17β-amine, 16-oxoE_2 and 16α-OHE_1, yielded corresponding hydroxyl products with α- and β-configurations, while 16α-OH,17-imine gave 16α-OH,17β-amine quantitatively: the epimeric ratios of the reduction products are listed in Table 1. The 4 major reduced products (16α-OH, 17β-amine, 16β-OH,17β-amine, E_3 and 16-epiE_3) and 6α-OHE_2 (used for *IS*) were well separated from each other by HPLC on a Puresil C_{18} column using a mixture of 0.5 (w/v)% potassium phosphate buffer (pH 6.8) and MeOH (1:1 v/v) as the eluent (Fig. 3). The linear relationships of the peak height ratios of the 4 reduced products to the *IS* were observed in the range of 0.1:1 to 6.4:1 (data not shown). The extraction of the estrogen derivatives in the reduced mixture using a Sep-Pak C_{18} cartridge was satisfactory.

Accordingly, the amounts of 16α-OH,17-imine, 16-oxo,17β-amine, 16α-OHE_1 and 16-oxoE_2 in the reaction mixture were determined from the major reduced products with a correction based on the epimeric ratios in Table 1. All the rates (mean ± standard deviation, $n = 5$) of hydrolysis and Heyns rearrangement in this paper were expressed as the percent of a cumulative amount of 16α-OHE_1 and 16-oxoE_2 (observed in a few experiment in the presence of metal ion(s), but was less than 3% of 16α-OHE_1 formed) and an amount of 16-oxo,17β-amine relative to the total estrogens determined, respectively.

2.2. Effect of pH on the Heyns rearrangement and hydrolysis of the steroidal α-hydroxyimine

The rates of the Heyns rearrangement and hydrolysis of the steroidal α-hydroxyimine were examined in a mixture of 50 mM Tris HCl buffer with various pH (5.0−8.0) and MeOH (1:1 v/v). As illustrated in Figure 4, the rate of hydrolysis seemed to be less affected by pH than that of Heyns rearrangement in the pH range examined. The hydrolysis rate appeared to be decreased but was insignificant, at pH 6.0 and 6.2. Contrarily, the rate of the Heyns rearrangement of 16α-OH,17-imine was significantly increased in these pH conditions. These results led us to conclude that the reaction condition with a pH of approximately 6.2 was optimal for the Heyns rearrangement of the steroidal D-ring α-hydroxyimine.

Although the Heyns rearrangement was seemed to be decreased at the physiological pH according to the amount of Heyns products at pH 7.3 and 7.5, it was still more than 90% (94% and 92%, respectively) of that at the optimum pH

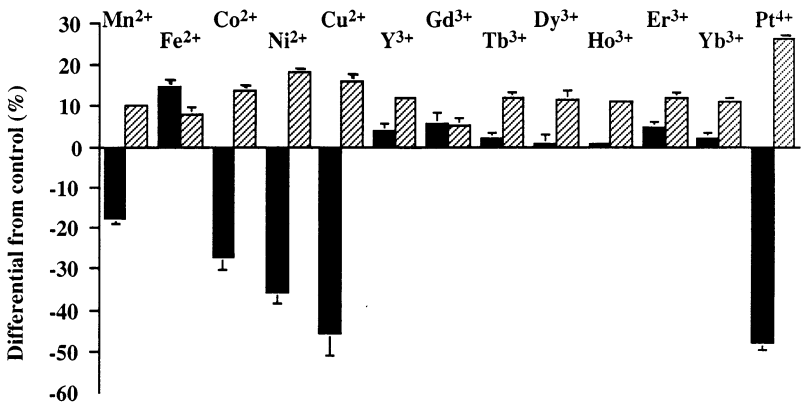

Fig. 5. Effect of metal ion on the Heyns rearrangement and the hydrolysis of the steroidal D-ring α-hydroxyimine. 16α-OH,17-imine was incubated in a mixture of 50 mM Tris HCl buffer (pH 6.2) containing metal chloride (5 mM) and MeOH (1:1, v/v) for 10 min at 37°C and then treated with $NaBH_4$. The estrogen derivatives in the solution were subjected to HPLC analysis. ■, the stable adduct (16-oxo-17β-amine); ▨, hydrolyzate (16α-OHE_1 + 16-oxoE_2).

Table 3
Reduction of metal ion promoted Heyns rearrangement and hydrolysis of the steroidal α-hydroxyimine by EDTA

Metal ion	Heyns rearrangement	Hydrolysis
Pt^{4+}	54%	31%
Cu^{2+}	92%	92%
Co^{2+}	42%	36%
Fe^{2+}	73%	65%

Concentrations of metal ion and EDTA were 5 mM.

as shown in Fig. 4. Hence, it is conceivable that the Heyns rearrangement may proceed on the Schiff base formed from 16α-OHE_1 with protein in vivo, not only under pathologic conditions but also under physiological conditions. Since the tissue with inflammation is generally acidic, the formation rate of stable adduct may be increased in inflamed (breast) tissue [17].

2.3. Effect of metal ion on the Heyns rearrangement and hydrolysis of the steroidal α-hydroxyimine

Twenty-nine metal chlorides, including 13 lanthanides, were examined for effects on the Heyns rearrangement and hydrolysis of 16α-OH,17-imine (Table 2). Tris HCl buffer was adjusted at pH 6.2 after the addition of metal chloride. Incubation of the 16α-OH,17-imine (27 mM at the final concentration) was performed with a mixture of the buffer and MeOH (1:1 v/v) for 10 min at 37°C. None of the precipitate was observed during the incubation. The first screening was carried out using 5 mM metal ions at the final concentration. Fe^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Pt^{4+}, Y^{3+}, and heavier lanthanide (atomic number: 62–68 and 70) ions affected the Heyns rearrangement and/or hydrolysis of the 16α-OH,17-imine while 13 metal ions (Ba^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+}, Zn^{2+}, Al^{3+}, Cr^{3+}, Fe^{3+}, Se^{4+}, and lighter lanthanide (atomic number: 57–60) ions) did not show any significant effect on both reactions (Fig. 5). The rate of the Heyns rearrangement was modestly enhanced by Fe^{2+} (15%) and slightly by Y^{3+} (4.1%), Gd^{3+} (5.7%) and Er^{3+} (4.6%). All other effective metals, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Pt^{4+}, suppressed the rearrangement rate significantly (18, 27, 36, 45, and 50%, respectively). On the other hand, the hydro-

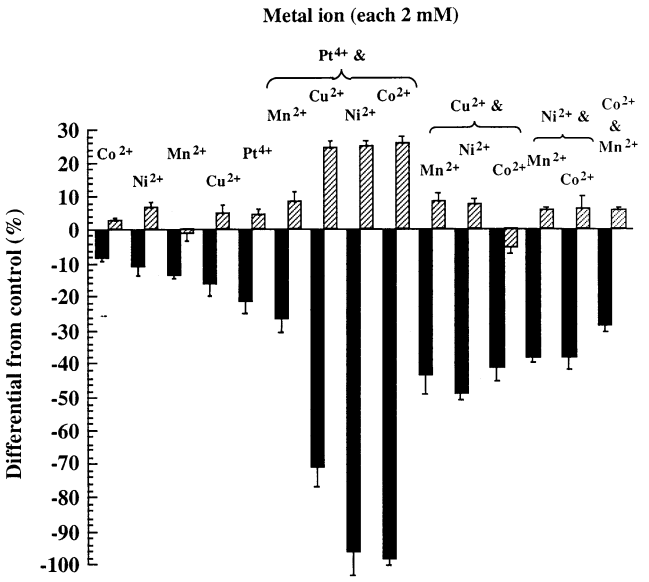

Fig. 7. Combination effect of metal ions on the Heyns rearrangement and the hydrolysis of the steroidal D-ring α-hydroxyimine. 16α-OH,17-imine was incubated in a mixture of 50 mM Tris HCl buffer (pH 6.2) containing metal chloride (each 2 mM) and MeOH (1:1, v/v) for 10 min at 37°C and then treated with $NaBH_4$. The estrogen derivatives in the solution were subjected to HPLC analysis. ■, the stable adduct (16-oxo-17β-amine); ▨, hydrolyzate (16α-OHE_1 + 16-oxoE_2).

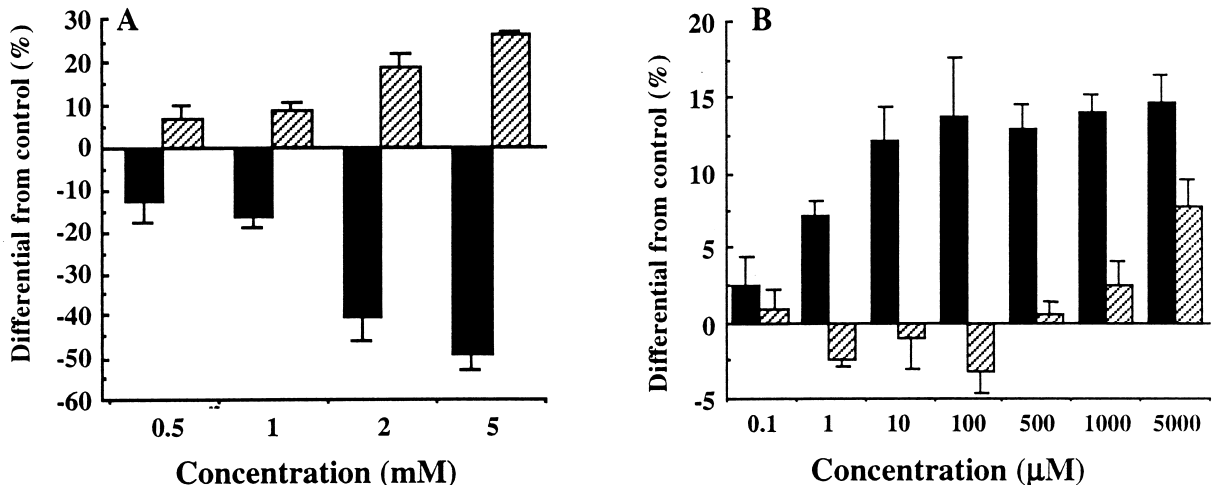

Fig. 6. Effect of metal ion concentration on the Heyns rearrangement and the hydrolysis of the steroidal D-ring α-hydroxyimine. 16α-OH,17-imine was incubated in a mixture of 50 mM Tris HCl buffer (pH 6.2) containing (A) $PtCl_4$ or (B) $FeCl_2$ and MeOH (1:1, v/v) for 10 min at 37°C and then treated with $NaBH_4$. The estrogen derivatives in the solution were subjected to HPLC analysis. ■, the stable adduct (16-oxo-17β-amine); ▨, hydrolyzate (16α-OHE_1 + 16-oxoE_2).

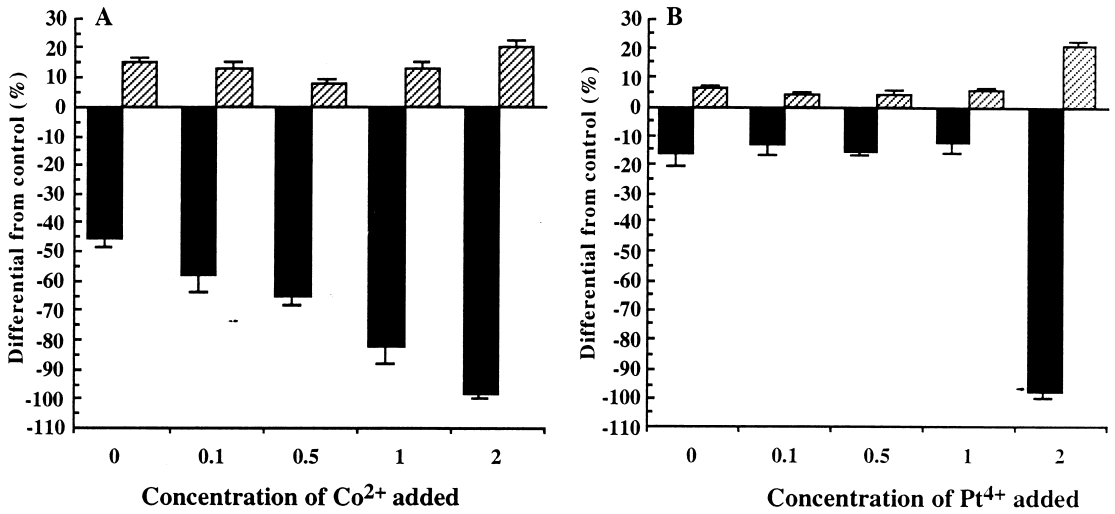

Fig. 8. Effect of supplementary metal ion concentration on the Heyns rearrangement of the steroidal D-ring α-hydroxyimine promoted by Pt^{4+} or Co^{2+}. 16α-OH,17-imine was incubated in a mixture of 50 mM Tris HCl buffer (pH 6.2) containing (**A**) $PtCl_4$ (2 mM) with $CoCl_2$ (0–2 mM) or (**B**) $CoCl_2$ (2 mM) with $PtCl_4$ (0–2 mM) and MeOH (1:1, v/v) for 10 min at 37°C and then treated with $NaBH_4$. The estrogen derivatives in the solution were subjected to HPLC analysis. ■, the stable adduct (16-oxo-17β-amine); ▨, hydrolyzate (16α-OHE_1 + 16-oxoE_2).

lysis rate of the Schiff base was increased by all of the effective metal ions, for example Pt^{4+} increased it by 26% (Fig. 5). Both effects promoted by metal ions were reduced similarly by the addition of a chelating agent, ethylenediaminetetraacetic acid (EDTA) (Table 3). The effects of the rearrangement and hydrolysis of the steroidal D-ring α-hydroxyimine by Pt^{4+}, Cu^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} were in a dose-dependent manner in a range of 0.5–5 mM. The effect by Pt^{4+} concentration is depicted in Fig. 6A. On the other hand, the rate of the Heyns rearrangement increased by the addition of Fe^{2+} up to 100 μM and then became constant (Fig. 6B).

The combination effect among the five effective metal ions that suppressed the Heyns rearrangement were examined at the concentration of 2 mM. As depicted in Fig. 7, significant synergistic effects on the suppression of the rearrangement were observed in the combination of Pt^{4+} with Co^{2+}, Ni^{2+} and Cu^{2+}; 98, 96 and 71%, respectively, while individual metal ions suppressed it by 21, 8, 11 and 16% at this concentration. Modest synergistic effects were also observed in the combinations of Cu^{2+} with Ni^{2+}, Mn^{2+} (alone, 13%) or Co^{2+}; 49, 43, and 41% and Ni^{2+} with Mn^{2+} or Co^{2+}; both 38%. On the other hand, only the additive effect on the Heyns rearrangement was observed in the combination of the metal ions (Fe^{3+}, Y^{3+}, Gd^{3+}, and Er^{3+}), which individually enhanced the rearrangement by a few percent (data not shown). The effect of supplementary metal ion concentration on the Heyns rearrangement and hydrolysis of the steroidal α-hydroxyimine promoted by Pt^{4+} or Co^{2+} is shown in Fig. 8. When various concentrations of Co^{2+} were added to 2 mM of Pt^{4+}, the percent suppression of the Heyns rearrangement was increased by Co^{2+} in a dose-dependent manner. However, in the opposite combination, the synergistic effect was observed only at the concentration of 2 mM of Pt^{4+}. These findings suggest that Pt^{4+} played a predominant role and Co^{2+} worked supplementary in the synergistic effect.

It has been demonstrated that a stable adduct of 16α-OHE_1 with serum albumin and erythrocyte membrane protein accumulated in vivo and in vitro [10,18]. The risk of breast cancer was also revealed to be associated with estrogen 16α-hydroxylation in humans [3] and mice [19]. In these cases, elevated 16α-OHE_1 levels in certain site(s) may allow for the accumulation of the stable adduct formed from 16α-OHE_1 and protein(s). The modified protein may play a role in the genesis of oncogenic event(s) [19,20]. Accordingly, Pt^{4+}, Co^{2+}, Ni^{2+}, and Cu^{2+} would be useful to clarify an implication of the stable adduct formation of 16α-OHE_1 with protein in the oncogenic event as a suppresser of the Heyns rearrangement.

Acknowledgments

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