# Transcriptional effects of metal ions on the bovine oxytocin and the thymidine kinase-ERE promoter through the estrogen receptor α in MDA-MB 231 breast cancer cell line

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**Key words:**
metal Ion, oxytocin, estrogen receptor, breast cancer cell line.

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***Abstract:***

**BACKGROUND:** Some of metal ions as environmental pollutants show estrogenic activity. This xenostrogenic compounds can be caused carcinogenicity in organs. The mechanism of carcinogenicity of metal ions is not clarified. **OBJECTIVES:** In this study, we investigated the Transcriptional effects of variety of metal ions on the bovine oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in MDA-MB 231 breast cancer cell line. **METHODS:** Cells were plated into flask (75cm$^2$) at 1.3 density or into 12- well plates (Nunc) at a density of 100000 cells per well and were transfected with a total of 3 µg of plasmid DNA using calcium phosphate coprecipitation. Oestrogen and some metal ions were used for stimulation of transfected cells. **RESULTS:** Our results showed that copper and cadmium ions activating specifically the oxytocin promoter, and cobalt and possibly, mercury ions activating specifically the ERE-controlled promoter and the majority of the ions did not affect transcriptional activation significantly. **CONCLUSIONS:** The study revealed that some metal ions show estrogenic activity by classical or non-classical mechanisms as well as some metal ions exhibit estrogenic activity by undetermined mechanisms in transfected MDA-MB 231 cell line.

## Introduction

Estrogen hormones have effects at all levels of biological organisation and influence growth, differentiation, and function of the tissues of the female reproductive system, such as uterus, ovary, and breast, as well as of non-reproductive tissues such as bone (Beato, 2000; Klaassen, 2001; Jensen and Jacobson, 1962) and the cardiovascular system (Barrett-Connor and Stuenkel, 1999; Klaassen, 2001) in both sexes.

Paradoxically, estrogen can be both a beneficial and a harmful molecule. Unfortunately, however, estrogens are clearly carcinogenic in humans and rodents but the molecular pathways by which these hormones induce cancer are only partially understood (Key and Beral, 1992; Key and Pike, 1988). Two distinct mechanisms of estrogen carcinogenicity have been outlined. Stimulation of cell proliferation and gene expression by binding to the estrogen receptor is the first and important mechanism in hormonal carcinogenesis, named classic method (Stoica et al., 2000). However, estrogenicity is not sufficient to explain the carcinogenic activity of all estrogens, because some estrogens are not carcinogenic (Key and Beral, 1992; Key and Pike, 1988). Increasing evidence of a second mechanism of

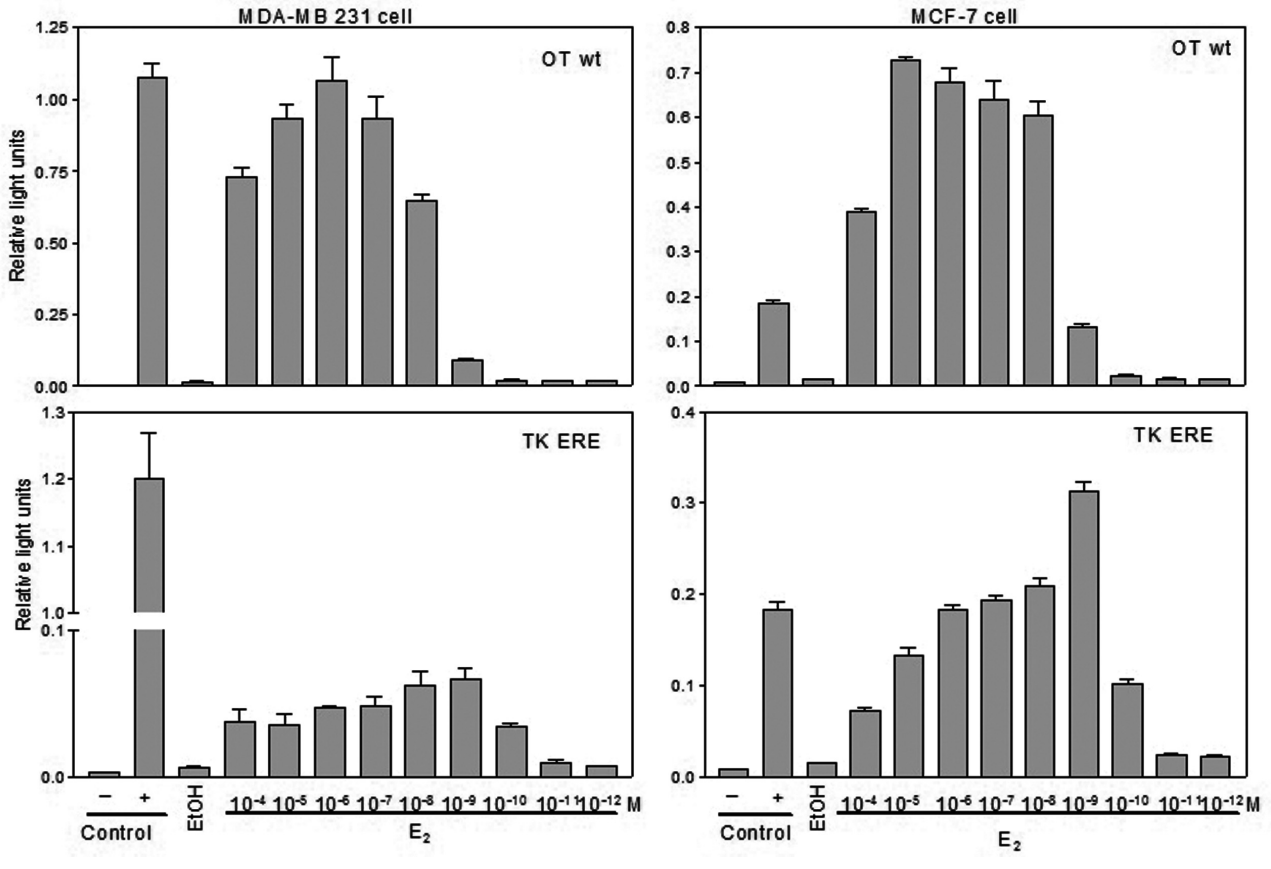


Figure 1. Activation of the oxytocin promoter (OTwt) and thymidine kinase-ERE promoter (TK ERE) by estrogen receptor α (ERα)/ estradiol complex. MDA-MB 231 and MCF-7 cells were transfected with the OTwt construct or TK ERE construct and ERα and treated with different amounts of the estradiol ($E_2$).

carcinogenicity has focused attention on catechol estrogen metabolites, which are less potent estrogens than estradiol, but can directly or indirectly damage DNA, proteins and lipids. Estrogen carcinogenesis has been investigated in experimental animal models (Key and Beral, 1992).

The general agreement is that estrogens are involved in the etiology of breast cancer (Ray et al., 2001). Breast cancer is the most common cancer in women worldwide (Parkin et al., 2001), with more than one million new cases being estimated every year and is the leading cause of death in women between the age of 35 and 47 (Stoica et al.,2000).

Xenoestrogens which are defined as chemical substances entering the body from the external environment may mimic or interfere with the action of endogenous estrogenic hormones (Ignar-Trowbridge et al., 1993). Metals represent a new class of endocrine disruptors. (Garsia-Morales et al., 1994; Martin et al., 2003; Stoica et al., 2000).

Estrogen-like molecules are thought to function predominantly through estrogen mediated activation of transcription via estrogen responsive elements (ERE). Both estrogen receptor α and estrogen receptor β can interact with various cell cycle transcriptional factors (Klinge, 2001; Kuiper et al., 1998), but the molecular mechanisms of metal ions effects are not well understood. The study presented here now aims at the elucidation of classical (bind to its nuclear receptor) and non-classical mechanisms (act via other mechanism than attachment to its nuclear receptor) by a detailed analysis of the components of the regulatory system controlling the oxytocin promoter. Transient transfection experiments using the breast cancer cell line MDA-MB 231 were performed and are presented here.

## Materials and Methods

**Cell Culture:** The MDA-MB 231 breast cancer

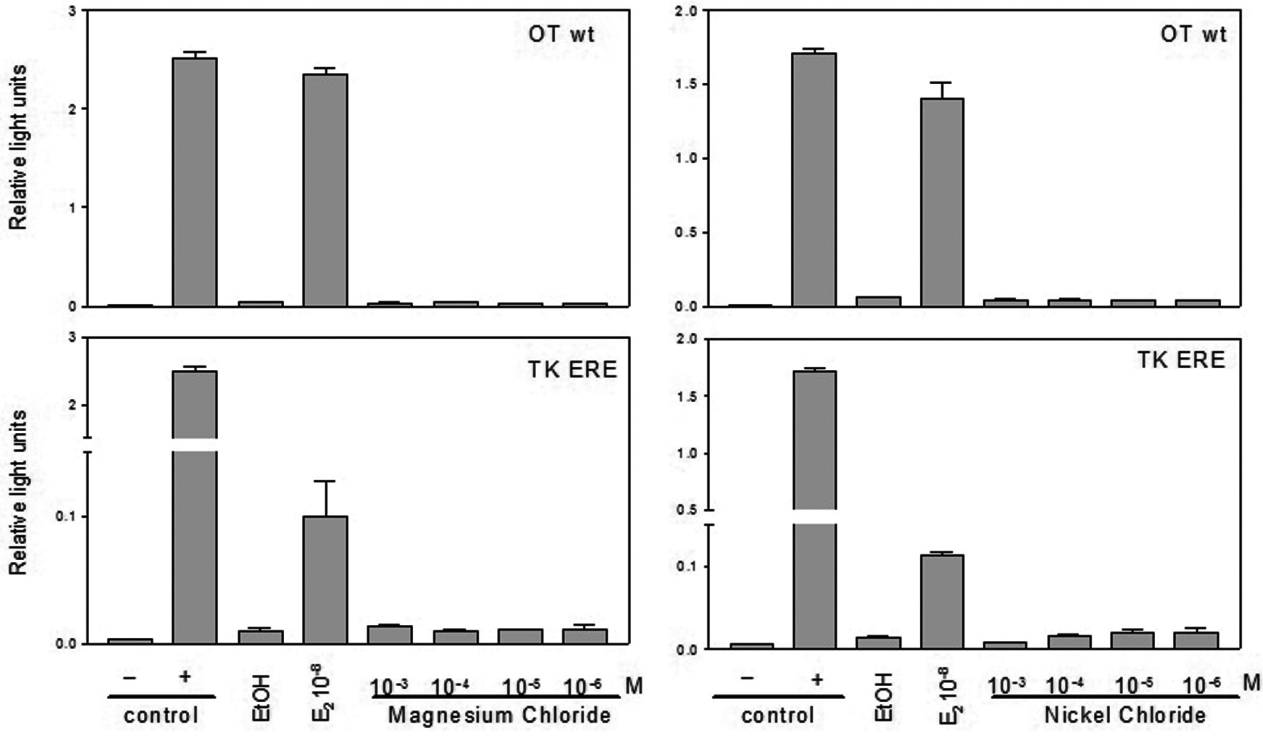


Figure 2. Transcriptional effects of magnesium chloride and nickel chloride on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter (OTwt) or the thymidine kinase-ERE promoter (TK ERE) and estrogen receptor α (ERα) and treated with different concentrations of magnesium chloride or nickel chloride.

cell line was used because it has shown to be devoid of functional estrogen receptor α and estrogen receptor β (Stedronsky et al., 2002). MDA-MB 231 cells were plated out in small 25 $cm^2$ culture flasks (Nunc, Wiesbaden, Germany) in phenol red free Dulbecco,s modified Eagle's medium (DMEM) (Gibco, Karlsruhe, Germany) supplemented with 10% of steroid-free (charcoal-stripped) foetal calf serum (CCS) plus 1% of L-glutamine (Sigma, Taufkirchen, Germany) and 0.5% of penicillin/ streptomycin (Sigma) and incubated at 37°C / 5% $CO_2$. Before reaching confluence, cells were detached from the bottom of the flask by the addition of 1.5ml 1x Viralex TM-EDTA solution (0.05% trypsin, 0.5 mM EDTA) followed by incubation at 37°C for 2 min. Cells were harvested by centrifugation for 2 minutes at 6500 g and resuspended in culture medium. The resuspended cells were re-plated either into another flask (75$cm^2$) at 1.3 density or into 12-well plates (Nunc) at a density of 100000 cells per well for transfection. In addition, MCF-7 cells were applied as control.

**Metals:** Cupric chloride (Merck), zinc chloride (Fluka), lithium chloride (Merck), magnesium chloride (Merck), cupric sulphate (Sigma-Aldrich), lead chloride (Sigma-Aldrich), mercury chloride (Sigma-Aldrich), zinc sulphate (Sigma-Aldrich), and cobalt chloride (Fluka) were used for stimulation of transfected cells.

**Transfection Assays:** The majority of methods and materials are characterized in detail in the previous publication (Koohi et al., 2005). In each well of 12-well plates (Nunc)$10^5$ MDA-MB 231 were seeded and on the following day transfected with a total of 3 µg of plasmid DNA using calcium phosphate coprecipitation (Profection Mammalian Transfection System, Promega, Mannheim, Germany). The transfected plasmid DNA included 1µg of

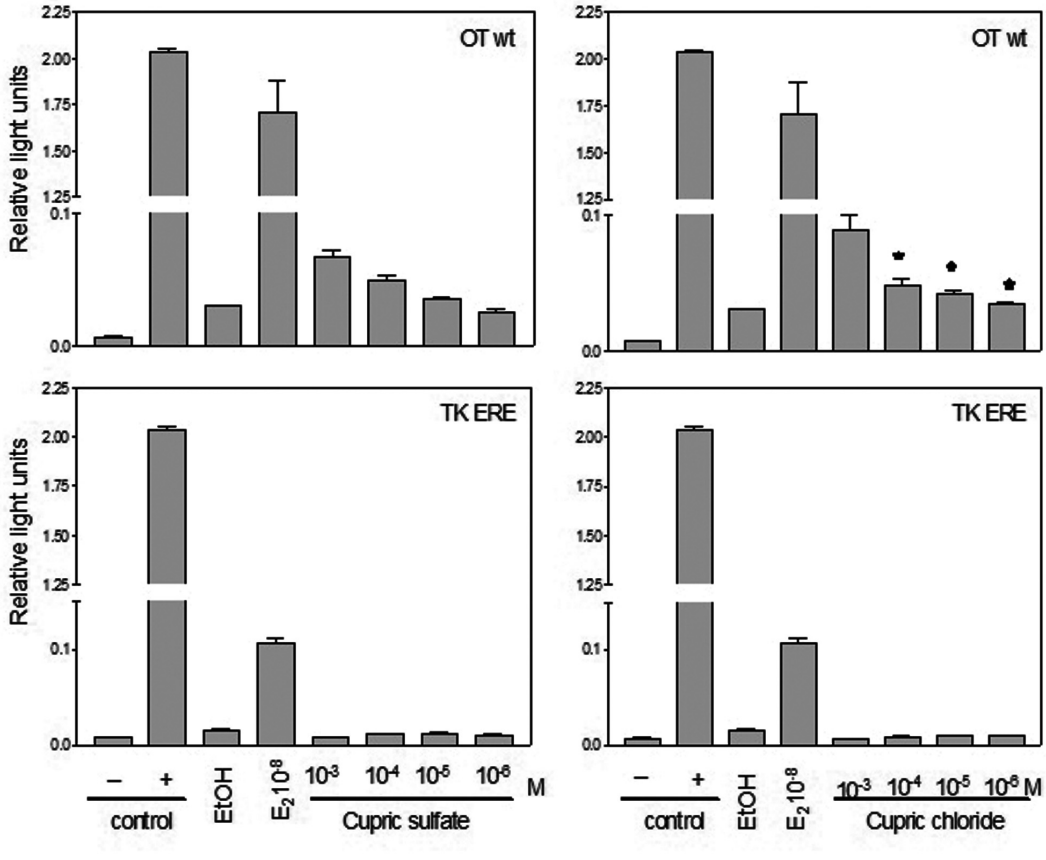


Figure 3. Transcriptional effects of cupric sulphate and cupric chloride on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter (OTwt) or the thymidine kinase-ERE promoter (TK ERE) and estrogen receptor α (ERα) and treated with different concentrations of cupric sulphate or cupric chloride.

promoter-luciferase plasmid, 1.5µg of the relevant ER expression vector (see below), and 0.5µg of a LacZ control vector driven from the CMV early promoter. Following transfection, cells were incubated for 16 h, then medium was changed, and cells were stimulated with oesterogen and metal ions in 2.5 ml fresh culture medium per well. In the control group oestrogens and metal ions did not add. After a further 24 h incubation, cells were washed twice in PBS and then lysed in 1×Passive Lysis Buffer (Promega) before determination ofluciferase and β-galactosidase activities (Koohi et al., 2005). After correction for transfection efficiency, results were expressed as relative light units (means + S.D. for triplicate wells). All experiments were repeated at least twice with identical results. Statistical significance for the differences in promoter activities was assessed by one-way ANOVA followed by the Newman-Keuls test, or unpaired t-tests with Welch's correction using the GraphPad Prism 3.0 software package (GraphPad Software Inc., San Diego, CA).

**DNA constructs:** All promoter-reporter constructs were as previously described (Koohi et al., 2005). Either the bovine oxytocin promoter (-183 to +17) (Ruppert et al.,1984) was used (OXT), inserted into the pGL3-Basic vector (Promega), or the thymidine kinase promoter controlled by a single vitellogenin ERE (TKERE) was used to drive a similar luciferase reporter construct (Stedronsky et al., 2002). As negative and positive controls, respectively, we used the pGL3-Basic plasmid, containing neither promoter nor transcriptional enhancer sequences, and the pGL3-Control vector, expressing luciferase under the control of the SV40 promoter and enhancer (both from Promega). Erα was generated from an expression construct comprising the human ERα cDNA controlled by a CMV viral promoter (Koohi et al., 2005). A version of Erα where in the

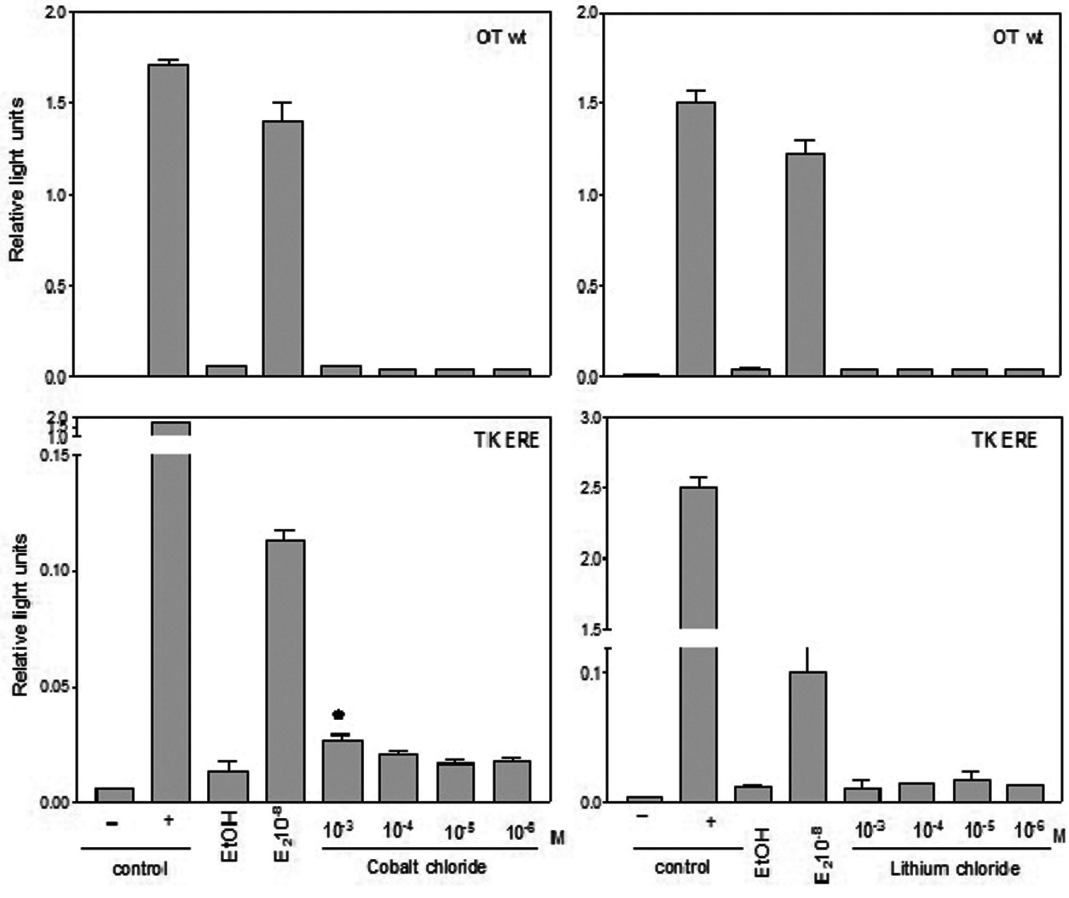


Figure 4. Transcriptional effects of cobalt chloride and lithium chloride on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α (ERα) and treated with different concentrations of cobalt chloride or lithium chloride.

DNA-binding domain (DBD) was mutated so that the receptor was no longer able to interact directly with an ERE as in Koohi et al. result (Koohi et al., 2005). A human ERβ (hERβ) expres-sion vector was obtained as a generous gift from Dr. Katrin Stedronsky (Institute for Hormone and Fertil-ity Research, Hamburg, Germany) and the bovine ERβ (bERβ) expression construct was prepared as in Walther et al (Walther et al., 1999) and driven from a CMV promoter.

**Cell Toxicity Assay:** All compounds used were additionally tested for any cytotoxic effects at the concentrations used, which could influence the results obtained. Firstly, cells were checked for integrity following a 24 h incubation with the test substance by staining washed cells for 3 h with 2% neutral red, and after further washing in PBS, photometrically measuring the absorbed dye at 540 nm. Secondly, following exposure to the test substances, cells were replated into T75 flasks, and subjected to 7 days of culture, after which all colonies in the flasks were stained with 10% Giemsa (Merck) and cells counted.

## Results

**Promoter-Specific Stimulation of Transcription by Metal Ions:** In early experiments, the MDA-MB 231 and MCF-7 cells were transfected and treated with increasing amounts of 17β-estradiol ($E_2$) (Figure 1). The results indicated that 17β-estradiol had dose-dependent and agonistic effects on the oxytocin promoter in MDA-MB 231 and MCF-7 cells in the presence of transfected estrogen receptor. Half-maximal stimulation was achieved at a concentration around $10^{-10}$ M at the thymidine kinase-ERE promoter, whereas $10^{-9}$ to $10^{-8}$ M estradiol were

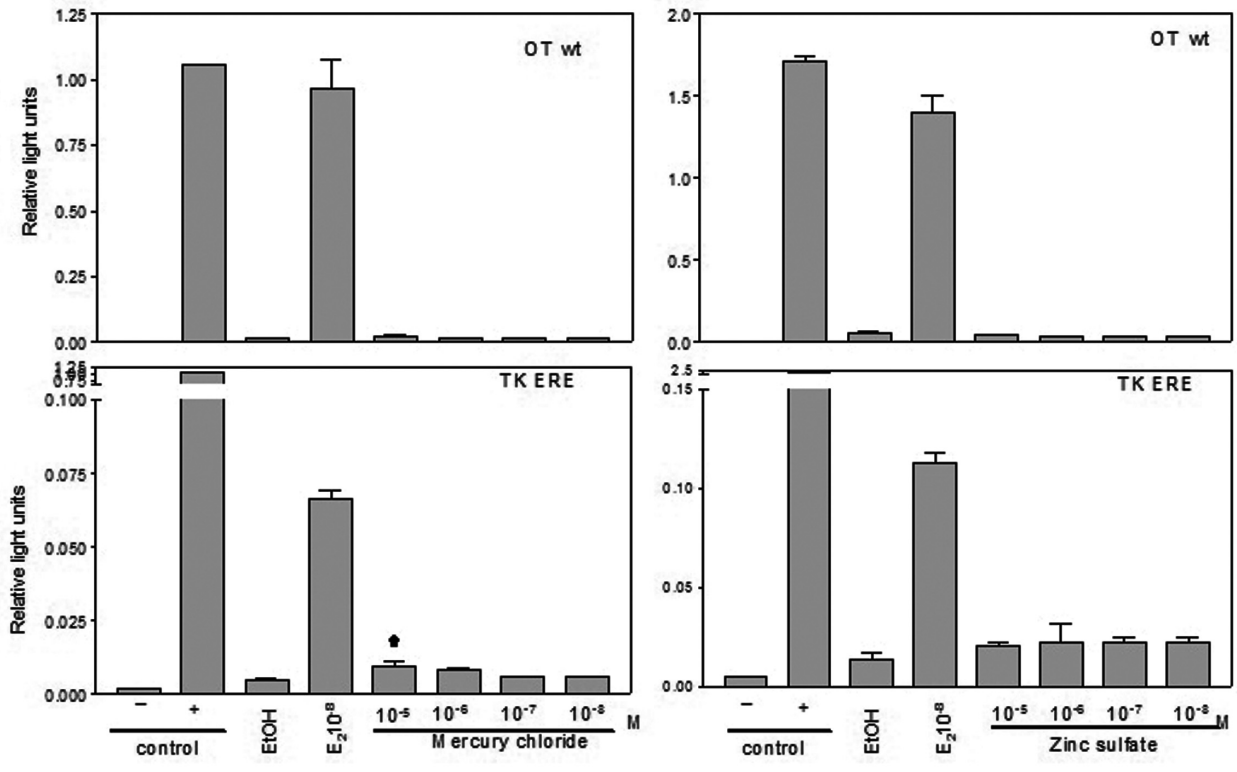


Figure 5. Transcriptional effects of mercury chloride and zinc sulphate on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α (ERα) and treated with different concentrations of mercury chloride or zinc sulphate.

Table 1. Transcriptional activity of metal ions on the oxytocin or the thymidine kinase ERE (TK ERE) promoter in MDA-MB 231 cells.

| Compound \ promotors | oxytocin | TK ERE |
|---|---|---|
| **Magnesium chloride** | - | - |
| **Nickel chloride** | - | - |
| **Cupric chloride** | + | - |
| **Cobalt chloride** | - | + |
| **Lithium chloride** | - | - |
| **Mercury chloride** | - | + |
| **Zinc sulfate** | - | - |
| **Cadmium chloride** | + | - |

necessary to achieve half-maximal stimulation of the wild type oxytocin promoter. Without co-transfection of estrogen receptor α, no estrogen-dependent up-regulation could be detected in MCF-7 cells indicat-ing that the cells may had insufficient expression of the endogenous estrogen receptor for this promoter (data not shown).

After preliminary experiment MDA-MB 231 cells were transfected with oxytocin or the thymidine kinase-ERE promoter and an expression vector containing the human estrogen receptor α. The transfected cells were treated with increasing concentration of metal ions, such as magnesium and nickel ions (Figure 2), cupric chloride or cupric sulphate (Figure 3), cobalt chloride or lithium chloride (Figure 4), mercury chloride or zinc sulphate (Figure 5) and cadmium chloride (Figure 6). The results show no agonistic effects of magnesium and nickel ions on the two promoters.

These results demonstrate an agonistic activity of copper ions with a half-maximal stimulation at a concentration around $10^{-4}$M specifically on the oxytocin promoter. In addition, only a weak agonistic

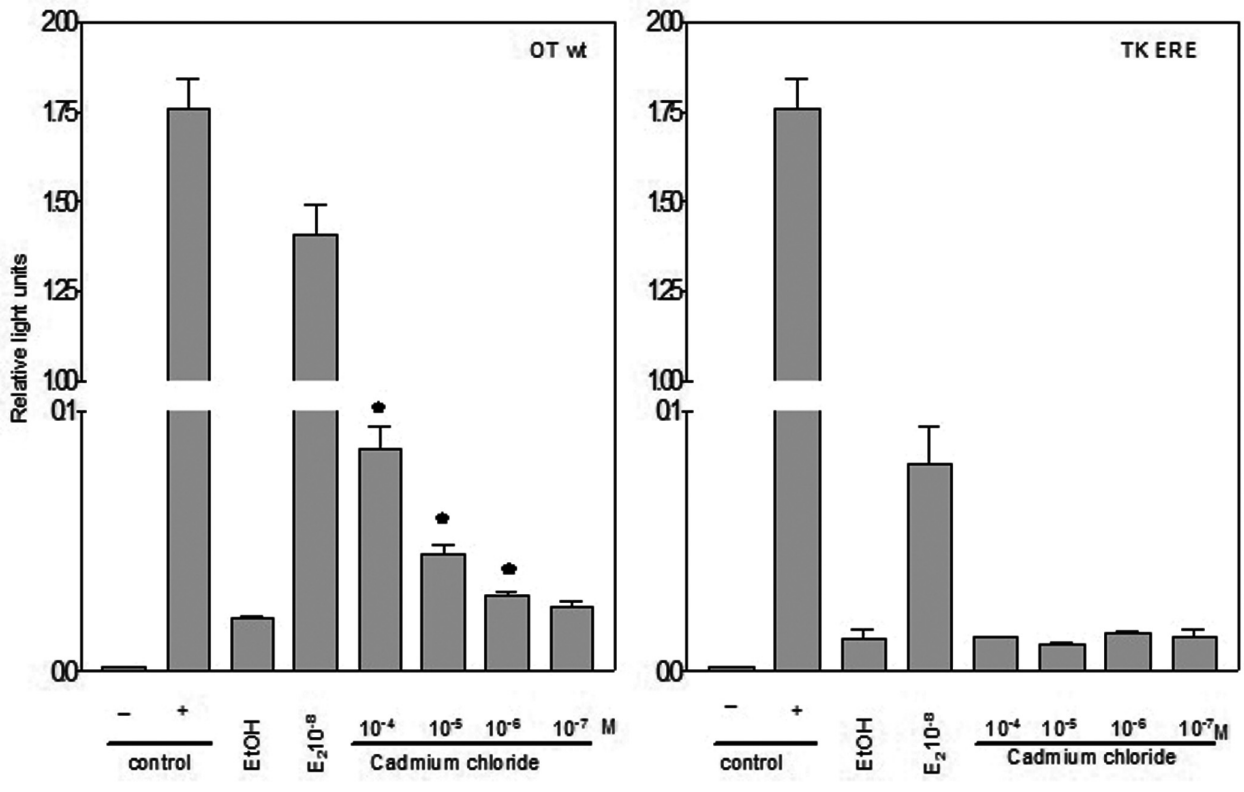


Figure 6. Transcriptional effects of cadmium chloride on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor ? in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α (ERα) and treated with different concentrations of the cadmium chloride.

effect of cobalt ions could be detected that appears to be selective for the thymidine kinase-ERE promoter. Also, only with mercury ion could a weak dose-dependent agonistic activity be detected on the thymidine kinase-ERE promoter. Unfortunately, it was not possible to employ higher concentration of mercury or zinc salts, like the ones used in the experiment with other metals described above, due to the high toxicity of mercury and zinc ions. The results demonstrate that cadmium chloride has a significant agonistic effect on the oxytocin promoter with half-maximal stimulation reached at a concentration of approximately $10^{-5}$ M to $10^{-4}$ M. This effect, as the effect of copper ions, is totally selective for the oxytocin promoter.

In summary, from the metal ions selected from the literature as having estrogen-like effects, several showed a total selectivity for promoters regulated by either the classical ERE-dependent mechanism (cobalt and, possibly, mercury), or the non-classical mechanism ( copper and cadmium). Other metal ions, for which no transcriptional activity could be detected in this assay system, apparently exert their effects by still other mechanisms.

## Discussion

Environmental contamination by toxic heavy metal ions such as cadmium, copper, zinc, and mercury from various sources (e.g., volcanic activity, weathering of rocks, and industrial, mineral mining, and agriculture activities) has been a problem for decades because these metals are not easily eliminated from the ecosystem. The molecular mechanisms that form the basis for the cellular toxicity of metal ions are manifold. Many metal ions are able to react with functional groups in proteins, namely sulfhydryl-, hydroxyl-, amino-, and carboxyl-groups. The major

mechanisms of metal toxicity comprise the general induction of oxidative stress (Valko et al., 2005), as well as the specific exchange of the zinc stabilizing the conformation of "zinc finger" proteins (Predki and Sarkar, 1992). In addition, it has been shown that metal ions inhibit DNA repair processes, thus increasing the rate of mutations that lead to carcinogenesis (Hartwig and Schwerdtle, 2002). Besides these general and specific toxic effects, metal ions can also exert estrogen-like activities. Divalent metal ions have been shown to activate estrogen receptor (Martin et al., 2003; Stoica et al., 2000), thus directly exerting estrogenic effects. Some of these environmental chemicals exert their estrogen-like effects by non-classical mechanisms (Steaimetz and Young, 1996). Environmental factors probably play a prominent role in breast cancer etiology. Breast cancer incidence has been rising steadily in many countries and it has been suggested that part of the increase may be due to such unexplained environmental factors (Ray et al., 2001).

In the present study, Metal ions were tested in the MDA-MB 231 transfection system. Whereas the majority of the ions did not affect transcriptional activation significantly, promoter-specific activation by some ions clearly could be demonstrated, with copper and cadmium ions activating specifically the oxytocin promoter, also cobalt and possibly, mercury ions specifically the ERE-controlled promoter. Whereas the estrogenic potential of these ions was expected from the results of other studies, the total selectivity for the oxytocin promoter, controlled by the described non-classical mechanism of estrogen action, or the classical ERE-controlled promoter was surprising. Even though the metal ions have been tested before (Garsia-Morales et al., 1994; Martin et al., 2003) for estrogenic effects on the pS2 promoter shown to contain an ERE (Barkhem et al., 2002) and the progesterone receptor promoter apparently controlled by AP-1 and Sp1 sites (Schultz et al., 2005), comparable differences in the estrogenic potentials could not be detected. The mechanism of metal ion action on estrogen receptor (Martin et al., 2003; Stoica et al, 2000) appears to comprise non-competitive binding to the ligand binding domain and to require a functional AF-2 domain. However, even for cadmium, whose estrogenic potential was detected first (Garsia-Morales et al., 1994), the mechanism of action could not yet be clarified in detail. Nevertheless, the importance of cadmium, as a common environmental toxic substance mimicking the effects of estrogen in vivo has been established (Henson and Chedrese, 2004; Johnson et al., 2003). Therefore the elucidation of the mechanisms control-ling the hormone-like activities of metal ions will be helpful for the development of screening procedures assessing the biological effects of these environ-mental pollutants.

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# مطالعه‌ی اثرات یون فلزات بر روی رونویسی ژن اکسی توسین گاوی و پروموتور تیمیدین کیناز- ERE بوسیله گیرنده‌ی نوع آلفا استروژن بر روی سلول‌های لاین سرطانی سینه MDA-MB 231

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## چکیده

**زمینه مطالعه:** برخی از یون‌های فلزی به عنوان آلوده کننده‌ی محیط دارای خصوصیات استروژنیک نیز می‌باشند. این ترکیبات زنواستروژنیک می‌توانند در برخی ارگان‌ها موجب سرطانزایی شوند. مکانیسم سرطانزایی این یون‌های فلزی هنوز مشخص نمی‌باشد. **هدف:** در این مطالعه اثرات ترجمه‌ای برخی از یون‌های فلزی بر روی ژن اکسی توسین گاوی و پروموتور تیمیدین کیناز ERE _ توسط گیرنده‌ی نوع آلفا استروژن در سلول‌های لاین سرطانی سینه MDA-MB 231 مورد بررسی قرار گرفته است. **روش کار:** سلول‌ها در فلاسک‌های cm2 ۷۵ با تراکم یک‌سوم یا در پلیت‌های ۲۴ چاهکی (Nunc) با تعداد ده هزار سلول در هر چاهک قرار داده شده وسپس با استفاده از DNA ۳mg پلاسمید با استفاده از روش coprecipitation کلسیم فسفات ترانسفکشن صورت گرفت. استروژن و برخی از یون‌های فلزی جهت تحریک سلول‌های ترانسفکت شده مورد استفاده قرار گرفت. **نتایج:** نتایج این مطالعه نشان داد که یون‌های مس و کادمیوم باعث افزایش فعالیت پروموتور اکسی توسین شده و یون‌های کبالت و احتمالاً جیوه به طور معنی داری باعث فعال شدن پروموتور کنترل شده‌ی ERE شده و دیگر یون‌های فلزی باعث افزایش معنی دار در فعالیت رونویسی نشدند. **نتیجه‌گیری نهایی:** نتایج این مطالعه نشان داد که، برخی یون‌های فلزی فعایت استروژنیک از نوع کلاسیک یا غیر کلاسیک از خود نشان داده، همچنین برخی دیگر از یون‌های فلزی فعالیت استروژنیک با مکانیسم ناشناخته در سلول‌های لاین MDA-MB 231 از خود نشان دادند.

**واژه‌های کلیدی:** یون فلزی، اکسی توسین، گیرنده‌ی استروژن، سلول‌های لاین سرطانی سینه.

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