IN VITRO INACTIVATION OF OESTROGEN RECEPTOR BY NUCLEI

Prevention by phosphatase inhibitors

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1. Introduction

Nuclear loss of oestrogen receptor following its nuclear translation is required for some hormonal responses [1–3]. In a mouse uterus cell-free system nuclei inactivate oestrogen-free and oestrogen-bound receptor of cytosol as well as the nuclear receptor [4]. This nuclear inactivating activity could have a role in the in vivo nuclear turnover of oestrogen receptor and in mechanism of oestrogen action [4]. These experiments show that the receptor inactivating activity is present in nuclei and nuclear extracts of oestrogen target tissues but not in nuclei and nuclear extracts of non-target tissues. Furthermore they suggest that a dephosphorylation process is involved in the inactivation of receptor by nuclei.

2. Materials and methods

Female Swiss mice (~30 g) were used. The following buffers were used: 10 mM Tris–HCl (pH 7.4) containing 1 mM dithiothreitol without (TD buffer) or with 1 mM EDTA (TED buffer). Tissues were homogenized as in [5] in buffer with 0.25 M added sucrose (TD– or TED–sucrose buffers). Purified nuclei were prepared layering a suspension of 750 × *g* pellet in TED–sucrose buffer on top of TED buffer containing 2.2 M sucrose and centrifuging the sample at 150 000 × *g* for 75 min at 2°C. Nuclear extracts were prepared from nuclei suspended in TED buffer, shaken at 0°C for 30 min, then sonicated as in [4]. The sonicated sample was centrifuged at 150 000 × *g* for 10 min at 2°C and the supernatant (called nuclear extract) utilized. [^{3}H]Oestradiol–cytosol receptor complex was obtained by incubating uterus cytosol with [^{3}H]oestradiol-17β (12×10^{-9} M, spec. act. 101 Ci/mmol) alone (high specific activity [^{3}H]-oestradiol) and in presence of a 1000-fold excess of cold oestradiol (low specific activity [^{3}H]oestradiol) at 0°C for 2 h. Specific oestradiol binding activity was determined as the difference between cytosol binding at 0°C for 2 h of high and low specific activity [^{3}H]oestradiol-17β. Bound oestradiol was separated from free-hormone by treatment with dextran-coated charcoal. The receptor inactivating experiments were performed incubating at 25°C for the indicated times [^{3}H]oestradiol–cytosol receptor complex (1×10^{-12} mol oestrogen binding sites/ml incubation mixture) in absence (control) and in presence of either 750 × *g* pellet or nuclei or nuclear extracts. Samples were then immediately cooled at 0°C, centrifuged at low speed to sediment 750 × *g* pellet or nuclei and the supernatants treated with dextran-coated charcoal to measure the oestrogen binding activity. Centrifugation at low speed was omitted in the case of control and samples incubated with nuclear extracts. Proteins and DNA were assayed as in [6].

3. Results and discussion

Table 1 shows the loss of [^{3}H]oestradiol–receptor complex after incubation at 25°C in presence of nuclei and nuclear extracts from different tissues. As expected from [4] the [^{3}H]oestradiol–receptor complex is partially inactivated by addition of uterus nuclei. Also nuclei from mammary gland, as well

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Table 1
Inactivation of [^{3}H]oestradiol-17β–cytosol receptor complex by nuclei and nuclear extracts from different tissues

	DNA / Protein	DNA in incubation mixture (μg/ml)	Loss of complex in cytosol (%)
Uterus nuclei	0.39	6.7	30
Mammary gland nuclei	0.26	6.1	24
Liver nuclei	0.23	5.0	2
Quadriceps muscle nuclei	0.23	7.9	0
Uterus nuclear extract	–	–	30
Liver nuclear extract	–	–	0

Uterine cytosols were first labelled with high or low specific activity [^{3}H]oestradiol-17β for 2 h at 0°C and then incubated in TED–sucrose buffer (pH 7.4) at 25°C for 20 min in absence (control) and in presence of either nuclei or nuclear extracts from different sources. The extracts were prepared from amounts of nuclei equal to those added as intact nuclei to cytosol. After incubation, cytosols incubated with nuclei were centrifuged at 3200 × g to remove nuclei. Thereafter, cytosols were treated with charcoal and the specific oestrogen-binding activity assessed. A 6% inactivation of control was found and subtracted from the inactivation of cytosol added with nuclei or nuclear extracts. Values in table are averages from 3 different experiments

as nuclear extract from uterus, inactivate receptor; on the contrary, nuclei from liver and quadriceps muscle and nuclear extract from liver are totally ineffective. These results show that the receptor inactivating activity is specific to oestrogen target tissues and support the hypothesis that this activity has a physiological role. Another promising result is that the receptor-inactivating activity can be quantitatively extracted from nuclei, making possible purification and characterization of molecule(s) responsible for this activity.

The possibility that the inactivation of oestrogen receptor involves a proteolytic process was then investigated. The effect of several protease inhibitors on [^{3}H]oestradiol-17β bound cytosol receptor incubated

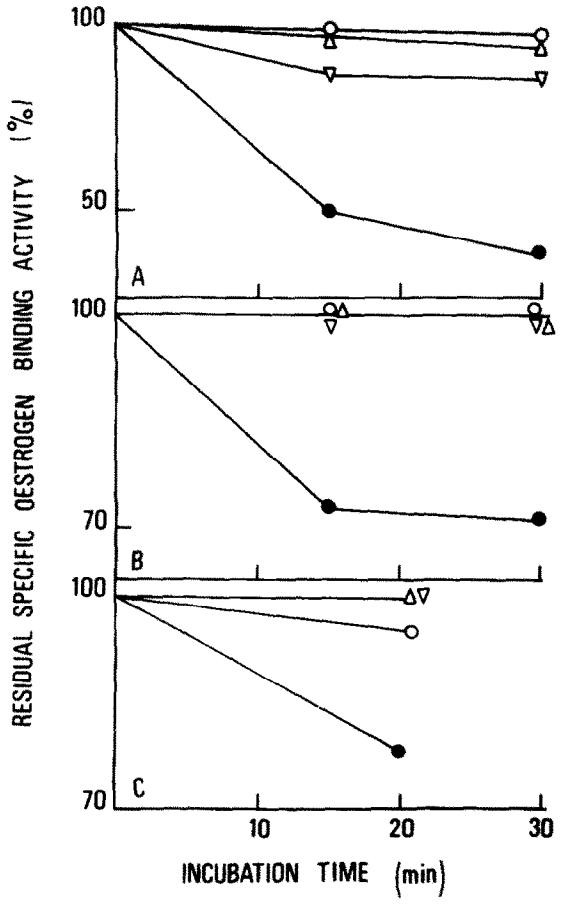

Fig.1. Effect of phosphatase inhibitors on time-dependent inactivation of [^{3}H]oestradiol–receptor complex by nuclei. Cytosol in buffer with sucrose added, was pre-labelled at 0°C for 2 h at 0°C with high and low specific activity [^{3}H]-oestradiol-17β, then incubated at 25°C for the indicated times in presence of: (A) an equivalent amount of 750 × g pellet; (B) nuclei purified from the same amount of 750 × g pellet used in A; or (C) nuclear extract prepared from the same amount of nuclei used in B. The samples were incubated either (●) without inhibitors or (○) with 20 mM NaF, or (△) 5 mM Na_2MoO_4 or (▽) 0.5 mM $ZnCl_2$. In the experiment in presence of $ZnCl_2$, EDTA was omitted from buffers.

at 25°C for 20 min in presence of an equivalent amount of 750 × *g* pellet from mice uterus was studied. 0.88 mM phenylmethyl sulphonyl fluoride, 0.1 mM L-1-tosyl-amide-2-phenyl-ethylchloromethyl chetone, 1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl chetone HCl and 5 μg/ml egg-white trypsin inhibitor do not inhibit the inactivation of receptor by 750 × *g* pellet (not shown). From these experiments proteolytic activities do not seem to be responsible for the inactivation process of receptor by nuclei.

We have then studied the effect of known phosphatase inhibitors on receptor inactivation by 750 × *g* pellet, nuclei and nuclear extract from uterus. Fluoride, molybdate and zinc inhibit several phosphatases including phosphoprotein phosphatase [7–9]. They almost completely inhibit the inactivation of [^{3}H]-oestradiol–receptor complex by 750 × *g* pellet, or purified nuclei or nuclear extract (fig.1). These results strongly suggest that the inactivation of oestrogen binding activity by nuclei in the in vitro system is due to a dephosphorylation process. More work, however, is necessary to verify if the dephosphorylated molecule(s) is the receptor itself or molecules regulating the receptor activity.

The possibility that steroid receptor can be inactivated by a dephosphorylation process has already been formulated for the steroid-free form of glucocorticoid receptor [10,11]. Our results however for the first time suggest that nuclei are able to inactivate a steroid receptor by such a process. Since nuclei have a central role in the mechanism of action of steroids on the target tissues, the inactivation process of the receptor by nuclei observed here could be tightly related to the mechanism of action of oestrogens.

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