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TAMOXIFEN AND OESTROGEN BOTH PROTECT THE RAT MUSCLE AGAINST PHYSIOLOGICAL DAMAGE

R. W. KOOT,[1] G. J. AMELINK,[1,2] M. A. BLANKENSTEIN[3] and P. R. BÄR[1,2]*

[1]Department of Neurology, Research Laboratory, [2]Janus Jongbloed Research Centre and [3]Department of Endocrinology, University of Utrecht, Utrecht, The Netherlands

Summary—Tamoxifen (TX), an oestrogen antagonist, was used to characterize the protective effect of oestradiol (E2) on exercise-related creatine kinase (CK) release from skeletal muscle of the rat. Subcutaneous administration of TX for 3 weeks in female rats had a profound antioestrogen effect as evidenced by a reduced weight of the uterus. The CK release after electrical stimulation of the isolated soleus muscle, previously shown to be E2-dependent, was markedly reduced (30–50%) after treatment with TX; this observation points to an E2-like protective action of TX instead of E2-antagonism. This effect was dose-dependent (0.25–1.00 mg/kg) and was not seen when TX was given shortly (24 h) before the experiments. In ovariectomized females, that show more CK leakage due to the lack of circulating E2, both E2- and TX-treatment resulted in a 60% reduction of the CK leakage. Muscles from male rats, treated with TX, showed a similar response: after contractions the CK release was significantly lower. We conclude that TX, like E2, reduces contraction-induced muscle damage in the rat and, thus, has E2-agonistic properties on skeletal rat muscle.

INTRODUCTION

Long-term, intensive exercise usually leads to a certain degree of muscle damage [1, 2]. This muscle damage is characterized by the release of several intracellular proteins from fibres into the circulation, e.g. creatine kinase (CK) [3], myoglobin [4, 5], and a few other proteins (for a review see [6]). In healthy subjects the release of these proteins can be seen as part of a physiological degeneration–regeneration cycle of muscle fibres following strenuous exercise, but there are some diseases in which there is a pathologically increased release of these proteins. For example, boys with Duchenne's muscular dystrophy have extremely high levels of CK activity in their circulation in rest and after exercise [7] whereas carriers of this disease, although usually without clinical symptoms, may have slightly raised values in rest, and an exaggerated increase in serum CK activity after exercise [8]. Myoglobin is a more sensitive index of such increased muscle fragility than CK [5, 9]. An increase of serum CK is also seen in nearly all cases of McArdle's disease (myophosphorylase deficiency) [10].

An experimental design in which rats run for 2 h on a treadmill enabled a more detailed study of CK elevations during and immediately after exercise [11]. In this model several factors were investigated which could prevent or diminish CK release: physiological effectors such as oestradiol (E2) [12] and drugs such as dantrolene sodium [13]. Previous studies had shown a sex-linked difference: after the same amount of exercise male rats had a considerably higher CK leakage from muscle than female rats [11], consistent with observations in humans [14]. Hormonal manipulation by treating male and ovariectomized (OVX) female rats with E2 clearly showed that E2 lowers the CK release [12]. The protective influence of E2 on muscle damage was confirmed in a carefully controlled *in vitro* system [15], in which short treatment (24 h) with E2 did not alter CK release. These observations, combined with the fact that there are oestrogen-receptors in rat skeletal muscle [16] may indicate that a multi-step process is involved in the protection of the muscle membrane, with some receptor-mediated E2 action as its first step. We addressed this question by using an oestrogen antagonist [tamoxifen (TX)] to block the E2-receptor. TX has a low incidence of side effects and is at present the antioestrogen of choice for the treatment of breast cancer; therefore we considered TX to be the most suitable antioestrogen drug.

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*To whom correspondence should be addressed at: Department of Neurology AZU, Room G03.228, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands.

In view of the fact that the rat is closest to man with respect to its response to TX [17], we chose to use the rat in our experiments.

EXPERIMENTAL

Animals

Six-week-old male and female Wistar (CPB WU stain, HSD Zeist, Holland) rats were used in all experiments. Treatment was started at 3 weeks of age (about 50 g). In experiment 1, female rats were given either TX or vehicle (olive oil) for 3 weeks (details follow). In experiment 2, 16 female rats were OVX at 21 days of age, divided into 4 groups and treated with E2, TX or both. Experiment 3 consisted of 2 groups of male rats that were administered either TX or vehicle for 3 weeks. All rats had chow and water *ad libitum* and were housed in light (6.00–18.00 h) and temperature controlled rooms at 22°C.

Surgery

Ovariectomy was performed through two small dorsal paraspinal incisions between the iliac crest and the lower ribs. After the ovaries had been carefully exposed, they were clamped between 2 mosquito clamps and removed after ligation of the surrounding tissue in order to establish correct haemostasis. Muscle and skin incisions were closed in 2 layers with Vicril. To check the endocrinological effects of TX and E2 treatment, the uteri of controls and treated animals were removed after muscle dissection. The abdomen was opened and the uterus was severed at the connection between vagina and cervix uteri. Next, the uteri were weighed and photographed.

Muscle preparation and stimulation

Soleus muscle preparation and stimulation *in vitro* have been extensively described by Amelink *et al.* [15]. In short: soleus muscles were dissected out from rats that had been anaesthetized first, and then killed by cervical dislocation. After careful weighing the muscles were attached to small glass holders and placed in glass tubes containing 6 ml Krebs–Ringer buffer (pH 7.40) at 37°C. Before electrical stimulation the muscle length was adjusted to give maximal twitch tension. Muscle were stimulated with biphasic pulses of 0.1 ms duration, amplified to 25 V/900 mA. Trains of pulses (duration 500 ms, rate 0.2/s) were given for 30 min at a frequency of 100 Hz. After the muscles had been mounted and the optimum muscle length had been determined, the bathing medium was refreshed; 30 min later (T = −30), the first sample was taken and stimulation was started. At the end of stimulation (T = 0), the medium was replaced with fresh buffer; this was repeated every 30 min up to 240 min. In our experimental set up 4 soleus muscles of 2 animals could be studied simultaneously, so that each of the experiments discussed below consists of several sets of paired observations. Results are the mean of at least 6 muscles in every group.

Enzyme analysis

At 30 min intervals the medium in each of the 4 tubes was removed with the aid of a 4-channel peristaltic pump and immediately replaced with fresh carbogen-saturated medium warmed to 37°C. The medium removed was kept on ice and CK activity was determined within 2 h of sampling by means of an non-activated enzyme-linked assay; the production of NADPH was followed at 334 nm and 30°C. All activities are expressed as mU/30 min.

Drug treatment

E2 and TX were suspended in sterile filtered olive oil and injected subcutaneously (s.c., 0.1 ml) once a day. Controls received an injection of 0.1 ml olive oil s.c. without any additions (vehicle) at the same time and during the same period. As a decreased growth rate is reported after TX treatment [18], both controls and treated rats were weighed before and after treatment.

Experiments

1. TX treatment and CK release from soleus muscles of intact female rats. Three-week-old female rats received 0.25, 0.5 or 1.0 mg/kg TX (ICI46.474, a gift from ICI Farma, Holland) or vehicle for 3 weeks. Then, the soleus muscles were taken out and the CK release *in vitro* was determined after stimulation. To study whether TX has an acute effect, 6-week-old female rats were given a large dose of TX (5.0 mg/kg) 24 h before the *in vitro* assay.

2. TX treatment and CK release from soleus muscles of OVX females. Sixteen 21-day-old female rats were OVX and assigned to 4 groups of 4 animals. These groups received daily injections for 3 weeks of (1) vehicle, (2) 40 μg/kg E2, (3) 5.46 mg/kg TX (molar ratio 100:1 with respect to E2) and (4) both E2 (40 μg/kg) and TX (5.46 mg/kg). After this treatment period

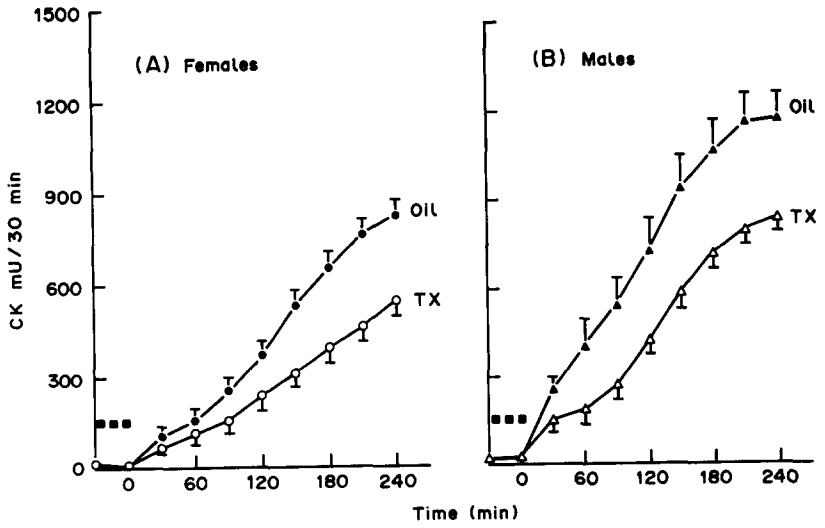

Fig. 1. The effect of TX on the CK release from soleus muscles of non-OVX female rats (A) and normal male rats (B) after electrical stimulation *in vitro*. One group received only the vehicle, olive oil (Oil), the other group 0.5 mg/kg TX. (A) The mean enzyme release is significantly lower in the TX treated group ($F = 7.431$, $df = 22$, $P = 0.012$). In both groups, there is a significant increase of CK efflux with time ($F = 113.306$, $df = 9$, $P < 0.001$). The rate of increase of CK efflux with time is different for TX treated and oil treated female rats ($F = 4.012$, $df = 9$, $P = 0.01$). (B) The release profiles of TX treated and oil treated male rats are significantly different ($F = 7.843$, $df = 14$, $P = 0.014$). Both groups show a significant increase of CK efflux with time ($F = 81.515$, $df = 9$, $P = 0.001$). Between the groups there is a different rate of increase of CK efflux with time ($F = 4.889$, $df = 9$, $P = 0.033$). The bars indicate the SEM. The ■ indicate the 30 min stimulation period. CK release is expressed in mU/30 min.

the soleus muscles were taken out and CK efflux after stimulation measured as described. The uteri of these animals were removed and weighed to check the effect of treatment.

3. TX treatment and CK release from soleus muscles of male rats. Three-week-old male rats were given 0.5 mg/kg TX or vehicle for 3 weeks. After this period the soleus muscles of both groups were taken out and CK efflux was determined after electrical stimulation as described above.

Statistical analysis

Data were analysed with an analysis of variance, followed by Student's *t*-test. Results are presented as mean ± SEM. In experiment 1 and 3 the efflux curves were analysed by using multivariate analysis. *F* values, degrees of freedom (*df*) and resulting *P* values are given where appropriate.

RESULTS

1. TX treatment and CK release from soleus muscles of intact female rats. Figure 1(A) shows the CK efflux from soleus muscles of control treated female rats (21-day-old, 0.5 mg/kg TX). A significant reduction of CK release was found in the TX-treated group: the cumulative CK efflux, i.e. the total amount of CK released during the experiment (the area under the efflux curve), was markedly lower for TX treated rats than for controls (2.33 ± 0.34 vs 3.69 ± 0.36 U) ($P = 0.012$). In Table 1 the effect of different doses of TX on the cumulative CK efflux of female rat soleus muscles is shown: there is a dose-related protective effect. TX, given in a high dose 24 h before the experiment, did not have an effect on the CK release *in vitro* (not shown).

2. TX treatment and CK release from soleus muscles of OVX females. The cumulative CK efflux from rat soleus muscles of 4 groups is shown in Fig. 2. All rats were OVX before treatment. As reported previously [15], ovariectomy results in an increased cumulative CK release (4.15 ± 0.51 U). E2 treatment restored the cumulative CK efflux to values even below those of untreated, intact females (2.05 ± 0.29 U). Administering TX to OVX animals

Table 1. The dose-dependent effect of TX treatment on cumulative CK release

TX (mg/kg)	% of control
0.00	100
0.25	71
0.50	63
1.00	52

The total amount of CK released after treatment with 3 doses of TX is given as a percentage of the leakage after treatment with the vehicle (olive oil).

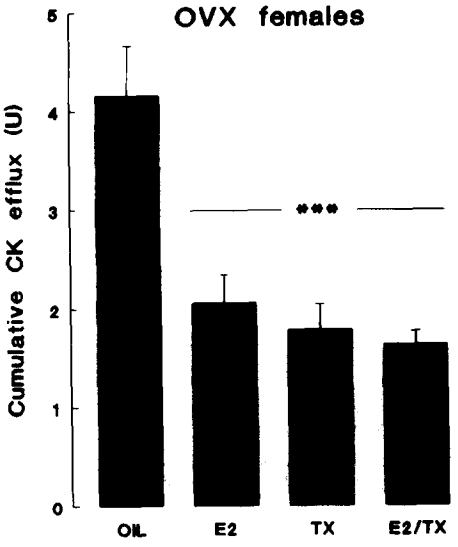

Fig. 2. The effect of TX and E2 on contraction-induced cumulative CK release from soleus muscles in OVX female rats. Olive oil (OIL), TX (5.46 mg/kg), E2 (40 μg/kg) or both E2 and TX (E2/TX, 40 μg/kg and 5.46 mg/kg) were administered for 3 weeks. Bars indicate the SEM; *** indicate that the 3 treatments resulted in leakage that was significantly different from non-treated animals ($P < 0.002$).

reduced the CK efflux to a level just below that of E2 treated rats (1.78 ± 0.26 U). The lowest CK release was achieved by injecting both TX and E2 (1.63 ± 0.14 U); this value is not significantly lower than that of the individual treatments. All 3 treatments were highly significant when compared to OVX control animals ($P < 0.002$), but did not differ significantly between each other.

3. TX treatment and CK release from soleus muscles of male rats. The cumulative CK efflux from soleus muscles of males treated for 3 weeks with TX is markedly reduced compared to the control group (4.02 ± 0.39 U vs 6.27 ± 0.71 U) [$P = 0.014$, Fig. 1(B)]. Note also the difference in the absolute amount of CK released: as shown previously, males release more CK than females [15].

The effect of TX treatment on the weight of the uterus

To verify whether the ovariectomy was performed correctly and to quantify the biological response to treatment with E2 and TX, the uteri were weighed and photographed to visualize the differences. The left hand part of Fig. 3 shows clearly that, as expected, in non-OVX female rats TX antagonizes the uterotrophic effect of circulating E2: the normal weight of the uteri was 472 ± 62 mg, that of TX treated animals was 150 ± 16 mg ($P < 0.001$). The right hand part of Fig. 3 shows data of the uteri of OVX females. Non-treated OVX animals have a very small, undeveloped uterus (40 ± 6 mg); E2 treatment restores the weight to a certain extent (230 ± 18 mg) ($P < 0.001$, compared to untreated OVX animals). TX treatment also results in a small increase in uterus weight (78 ± 3 mg) ($P = 0.001$). Combined treatment results in a weight that is about the same as after TX treatment alone (85 ± 3 mg). These data confirm that the molar ratio E2/TX (1:100) is such that the effect on the uterus of TX overrules the effect of E2, as also seen by Furr *et al.* [18]. The effect of treatment is visualized by the size of the uterus (Fig. 4).

DISCUSSION

We found a protective effect of TX on rat skeletal muscle in both sexes, indicated by a decreased CK release after electrically-induced contractions. Thus, TX mimics the oestradiol effect we have described earlier in intact animals [11, 12] and in isolated muscle in the same *in vitro* system [15]. The underlying mechanism of the E2-protection on rat skeletal muscle has not been clarified so far. To test our hypothesis that this protective effect of E2 is receptor-mediated we used TX, a well-known antioestrogen available for clinical application with low side effects. From our results it is clear that TX had its normal strong antioestrogenic

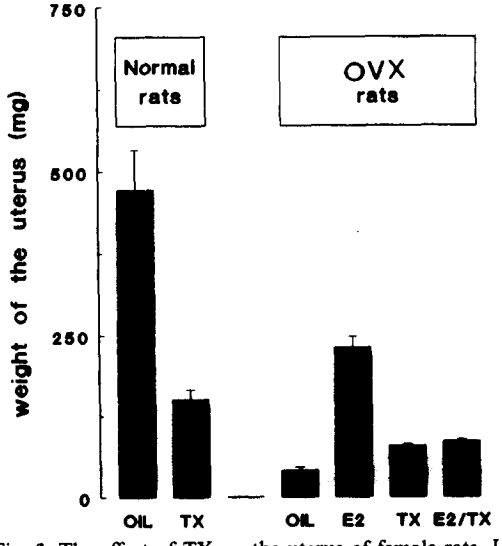

Fig. 3. The effect of TX on the uterus of female rats. Left hand part: normal female rats treated with olive oil (OIL) or TX (0.5 mg/kg). Right hand part: OVX female rats treated with OIL, E2 (40 μg/kg), TX (5.46 mg/kg) or both E2/TX (40 μg/kg and 5.46 mg/kg). Values are the mean weight in mg of 3–6 uteri. Bars indicate the SEM.

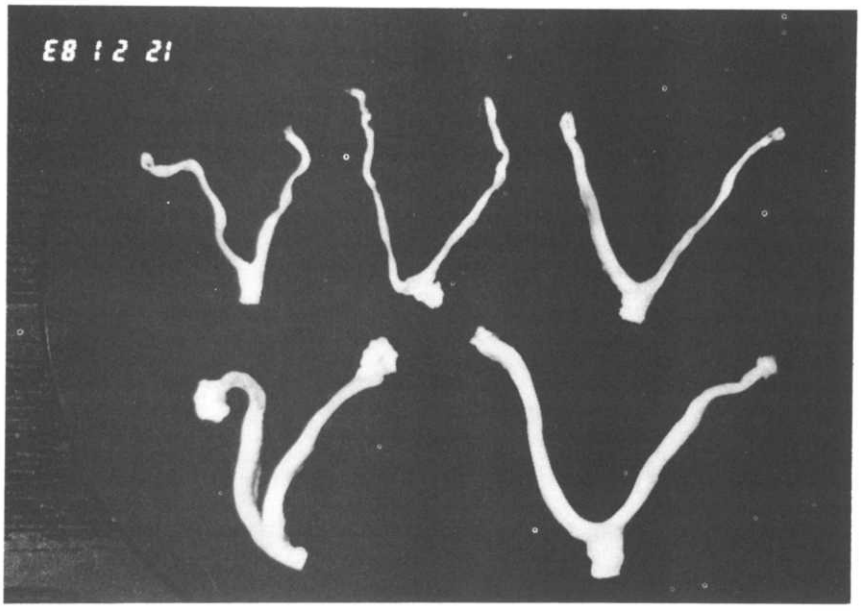

Fig. 4. The uteri of female rats: (clockwise, starting bottom left) normal untreated; OVX, TX treated; OVX, untreated; OVX, E2/TX treated; and OVX, E2 treated.

effect on E2 target tissues such as the uterus; the weight of uteri of TX-treated female rats was about one third that of control animals. However, no antioestrogenic effect (E2 antagonism would cause more muscle damage) on the protection of skeletal muscle could be found; instead, TX rather mimicked the E2 effect of lowered CK release. It therefore seems that TX has an agonistic effect on the skeletal muscle, while in the same experiments it had an antagonistic effect on the uterus.

The oestrogen receptor has been generally accepted as the place of interaction of antioestrogens. However, a small proportion of patients with E2 receptor negative tumours do still benefit from TX therapy. Receptor research supports the notion that the direct interaction of antioestrogens with some enzymes (phospholipid/Ca^{2+}-dependent protein kinase [19], Ca^{2+}/calmodulin-dependent cAMP phosphodiesterase [20]) represents an alternative mechanism to explain the antioestrogenic activity of TX, be it at high concentrations of TX. The recent publication of a positive effect of TX in POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, M protein and skin changes) in which the authors reported that TX seemed to stimulate the activity of natural killer lymphocytes [21], also points in the direction of an alternative mechanism to explain some of the biological effects of TX.

There are at least two major binding sites for triphenylethylene derivatives like TX; the nuclear oestrogen receptor [22], and a cytosolic, mostly microsomal, membrane associated protein, the triphenylethylene antioestrogen binding site (AEBS or TABS [23, 24]). Nuclei might have AEBS as well [25], but $<1\%$ of the total and maybe due to contamination of the preparations [26]. AEBS are not sex-steroid hormone receptors and are thought not to be target sites for steroid hormones like E2 [23]. AEBS are present in nearly all tissues in the rat: uterus, liver, kidney, spleen, ovary, brain and colon but only in negligible amounts in muscle, heart and serum [23, 24]. The functional role of AEBS remains undefined (for a review see Ref. [27]).

One of the main metabolites after oral administration of TX, both in humans [28] and rats [29], is 4-hydroxy-TX. This metabolite has a relative low affinity for AEBS and a much higher affinity for the oestrogen receptor than TX [30], at least as strong as that of E2 [31]. Treating rats with s.c. injections instead of oral administration may therefore cause a shift in metabolite concentrations. Different authors suggest however that conversion of TX to 4-hydroxy-TX and *N*-desmethyl-TX may be an advantage but is not a requirement to inhibit oestrogen action [32].

The fact that expression of oestrogen antagonizing effects of TX depends on the species may be a result of differences in interpretation of the drug–oestrogen receptor complex by the cell [17]. For each species, and particularly in the rat, the antagonizing effects could correlate

with the presence or absence of AEBS since the observed antioestrogen effect of TX so far concerns tissues with AEBS in different concentrations, but never in low amounts. As mentioned above the amount of AEBS in rat skeletal muscle is negligible. Our results show agonist, oestrogen-like activity of TX in the absence of AEBS. A speculation about the mechanism may be that the absence of AEBS affects the properties of the oestrogen receptor, although Dahlberg [16] described that the oestrogen receptor in rat muscle was similar to those in "classical target organs". So, if only the combined interaction of AEBS and oestrogen receptor results in antioestrogenic activity of TX then, in rat skeletal muscle TX may act as an agonist.

Further investigations are in progress to give support to this hypothesis and to find out if our observations may lead to another application of TX in the future, namely to protect the skeletal muscle against wasting, for example in patients with muscle disease. In these studies new antioestrogens, especially pure antagonists (e.g. ICI 164.384 [33]) that do not bind to the AEBS, will undoubtedly be important tools to elucidate the mechanism by which E2 and TX exert their protective effect on the rat skeletal muscle.

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