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Neuroprotection by Tamoxifen in Focal Cerebral Ischemia is Not Mediated by an Agonist Action at Estrogen Receptors but is Associated with Antioxidant Activity

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Abstract

We have previously shown that tamoxifen can induce marked neuroprotection after middle cerebral artery occlusion (MCAo) in rats and have described two possible mechanisms of action: namely inhibition of EAA release and inhibition of nNOS activity. In this study we tested other potential mechanisms. Namely, agonist action at estrogen receptors and an antioxidative action. Tamoxifen-treated rats had significantly improved neurobehavioral deficit scores after 24 hours and showed ~75% reduced infarct volumes. These were unaffected by ICI 182,780 (a high affinity and pure receptor antagonist) administered intravenously, or intracisternally to avoid possible lack of brain penetration, 15 minutes before intravenous administration of tamoxifen. In rats subjected to 2 hours MCAo followed by 22 hours reperfusion, a 1.8-fold and 2.9-fold increase of F₂-IsoPs and F₄ neuroprostanes, respectively, as relatively stable markers of oxidative damage, were measured in the ischemic hemisphere compared with the corresponding contralateral hemisphere or sham controls. Tamoxifen given at 3 hours after the start of ischemia reduced the IsoPs and NeuroPs to sham control levels, and also inhibited their production by chemically induced lipid peroxidation in brain homogenates. These data are consistent with at least part of tamoxifen's marked neuroprotection in focal cerebral ischemic injury being due to its antioxidant activity but not by an acute action on estrogen receptors (212 words).

Keywords

middle cerebral artery occlusion; isoprostanes; neuroprostanes; ICI 182,780; behavior, infarct volume; male Sprague-Dawley rats

Introduction

Cerebral ischemic damage is considered to develop as a result of a rapidly diminished energy supply causing progressive increases in the extracellular levels of excitatory amino acids (EAAs), cellular calcium influx and increases in reactive oxygen and nitrogen species (Atwell

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and Laughlin 2001;Dirnagl et al. 1999;Ginsberg 2003;Siesjo and Wieloch 1996). We have previously shown that tamoxifen, a drug widely used in the treatment of breast cancer (MacGregor and Jordan, 1998), and also used at higher doses in brain cancers (Pollack et al, 1997), reduces infarct size by more than 80% in both the reversible (Kimelberg et al, 2000) and permanent (Kimelberg et al, 2003) middle cerebral artery occlusion models in the rat.

Tamoxifen has several potential neuroprotective actions, including: 1) inhibition of EAA release via swelling activated anion channels (Kirk and Kirk, 1994), 2) Inhibition of neuronal nitric oxide synthase (nNOS), both *in vitro* and *in vivo* (Osuka et al. 2001;Renodon et al. 1997), 3) scavenging of reactive oxygen species (ROS) and reactive nitrogen species (RNS) species *in vivo* (Obata and Kubota 2001), and 4) action at estrogen receptors (Dhandapani and Brann, 2002). Effects in the central nervous system (CNS) are likely to be further facilitated by its known ability to cross the blood-brain barrier (BBB) and accumulate in the brain (Biegon et al. 1996). Only mechanisms 1) and 2) have been evaluated in *in vivo* stroke models in the sense of whether tamoxifen neuroprotection correlates with inhibition of these processes (Osuka et al, 2001;Feustel et al, 2004). In this study, we tested whether the neuroprotective effect of tamoxifen depends on its action at estrogen receptors by seeing if neuroprotection by tamoxifen was affected by estrogen blockade by ICI 182,780, a high affinity estrogen receptor antagonist ($IC_{50} = 0.29$ nM) devoid of any partial agonism both *in vitro* and *in vivo* (Wakeling et al, 1991;Howell et al, 2000). We also tested whether tamoxifen shows antioxidant activity in a reversible middle cerebral artery occlusion (MCAo) model under the same conditions where it shows protection by testing whether an increase in F_2 – isoprostanes (IsoPs) and F_4 – neuroprostanes (NeuroPs), as biomarkers of oxidative damage, were inhibited (Marin et al, 2000;Milatovic et al, 2005;Montine et al, 2002). IsoPs are considered accurate markers of general free radical damage to brain tissue, while NeuroPs uniquely reflect free radical damage to neuronal membranes *in vivo* (Montine et al, 2004). We also checked whether tamoxifen could directly inhibit production of IsoPs in brain homogenates.

Methods

Middle Cerebral Artery Occlusion

All animal procedures were in accordance with the “Guidelines for Care and Use of Laboratory Animals” and were approved by the institutional animal care and use committee. Male Sprague-Dawley rats (300 to 350 g, Taconic, Germantown, NY) were anesthetized with isoflurane in a sealed chamber after 50 mg/kg atropine sulfate (Sigma, St. Louis, MO) had been given intramuscularly. They were then tracheally intubated and mechanically ventilated with 2.0% isoflurane in 30% O_2 /balance N_2 . Blood gas analysis verified that $PaCO_2$, PaO_2 , pH and mean arterial blood pressure were maintained within the normal physiological ranges. Body temperature was monitored with a rectal probe and maintained between 37.0 °C and 37.5 °C with a heating pad. *Temporalis* muscle temperature was used to reflect brain temperature and was maintained between 36.0 °C and 37.0 °C with a heating lamp. All these procedures have been previously described (Kimelberg et al, 2000;Zhang et al, 2005).

Reversible middle cerebral artery occlusion was performed as described by Longa et al. (Longa et al. 1989), as used previously in our laboratory (Kimelberg et al, 2000;Zhang et al, 2005). Briefly, a 4-0 nylon intraluminal suture was introduced into the right internal carotid artery (ICA) via the external carotid artery (ECA). The common carotid artery and ICA were temporarily clipped and the suture placed into the ECA stump and threaded into the ICA with the ICA clip removed, and gently advanced ~20 mm until resistance was felt. The suture was left in place for 2 hours and then slowly withdrawn. All animals showed a right side weakness (circling and/or falling to right) when they recovered from anesthesia, verifying ischemia induced neurological deficits. Body and brain temperature were maintained throughout the

experiment until each animal was completely recovered from anesthesia and had been returned to its cage.

Intravenous or Intracisternal Injection of ICI 182,780

For intracisternal (i.c.) injection, rats were placed in the ear bars of a stereotaxic frame and the occipital membrane was punctured with the needle of a Hamilton syringe as described by Martinez et al (2000). Correct positioning of the needle into the cisterna magna was determined by the reflux of clear cerebrospinal fluid (CSF) into the syringe. Fifteen minutes before the onset of ischemia, 10 µg of ICI 182,780 (Tocris, Ellisville, MO) in 10 µl dimethyl sulfoxide (DMSO) was manually injected in the same manner over a period of 20 seconds. For intravenous (i.v.) administrations, the right jugular vein was exposed so that a catheter could be inserted. Tamoxifen (5 mg/kg, Sigma, St. Louis, MO) or the vehicle, DMSO, was slowly administered at the initiation of ischemia over a period of 15 minutes at a rate of 0.01 ml/minute controlled by a syringe pump (PHD 2000 Infusion, Harvard Apparatus). ICI 182,780 (1 mg/kg) was given i.v. 15 minutes before the initiation of ischemia. After 24 hours neurological status was scored, the animals were then sacrificed and the brains examined for infarct volume.

Measurements of IsoPs and NeuroPs

For experiments on antioxidant activity, tamoxifen (5 mg/kg) or the vehicle was administered at 3 hours after the initiation of ischemia, i.e., 1 hour after reperfusion. After 24 hours animals were anesthetized with CO₂ which took around ~1 minute and killed by decapitation, using a guillotine. The whole brain was placed in a metal matrix at 4 °C and sectioned at 2 mm thickness from the anterior tip of the frontal lobe. Four cross-sections (from 2- to 10- mm anterior coordinate) were subsequently selected and redissected on ice into four compartments: ipsilateral striatum, ipsilateral cortex, contralateral striatum, and contralateral cortex. These samples were immediately frozen in liquid N₂ and stored at – 80 °C, prior to IsoP and NeuroP purification. The total period from decapitation to placing the samples in liquid N₂ was 8~10 minutes.

The technique used to measure IsoPs and NeuroPs was the same as previously described (Montine et al, 2002;2004). Briefly, samples of dissected brain (40-200 mg) were removed from the –80 °C freezer, placed on dry ice, and the tissue rapidly weighed on a microbalance. Tissue was homogenized in 5 ml ice-cold chloroform: methanol (2:1, v/v) and BHT (0.005% v/v) to prevent autooxidation. Two ml of 0.9% NaCl was then added, and the sample kept at room temperature for 1 hour with occasional shaking. The sample was then centrifuged, and the aqueous and protein layers removed and discarded. The remaining chloroform layer was dried under a stream of nitrogen, and re-suspended in 0.5 ml methanol. Compounds esterified in phospholipids were hydrolyzed using chemical saponification by adding 0.5 ml 15% aqueous potassium hydroxide. The tissue sample was acidified to pH 3 with 1 M HCl and diluted to 12 ml with pH 3 H₂O. Approximately one ng of internal standard [²H₄]15-F_{2t}-IsoP for IsoP was then added to the mixture. The mixture was then vortexed and applied to a C18 Sep-Pak column preconditioned with 5 ml methanol and 5 ml of water (pH 3). The column was washed sequentially with 10 ml of water (pH 3) and 10 ml heptane and eluted with 10 ml ethyl acetate/heptane (50:50, v/v). The ethyl acetate/heptane eluate from the C18 Sep-Pak was then dried over anhydrous Na₂SO₄ and applied to a silica Sep-Pak. The column was then washed with 5 ml of ethyl acetate/heptane (75:25, v/v) and the compounds eluted with 5 ml ethyl acetate/methanol (50:50, v/v). The ethyl acetate/methanol eluate was evaporated under a stream of nitrogen. Compounds were converted to pentafluorobenzyl esters by treatment with a mixture of 40 ml of 10% pentafluorobenzyl bromide in acetonitrile and 20 ml of 10% *N,N*-diisopropylethylamine in acetonitrile at 40°C for 20 minutes. The reagents were dried under nitrogen and the residue subjected to TLC using a solvent system of chloroform/ethanol (97:3, v/v). Approximately 2–5 mg of the methyl ester internal standard was chromatographed on a

separate lane and visualized by spraying with a 10% solution of phosphomolybdic acid in ethanol followed by heating. Compounds migrating in the region 0.5 cm to 2 cm above the methyl ester standard were scraped and extracted from the silica gel with 1 ml ethyl acetate. The ethyl acetate was dried under nitrogen and compounds were converted to trimethylsilyl ether derivatives by adding 20 ml BSTFA and 10 ml dimethylformamide and incubated at 40°C for 5 minutes. The reagents were dried under nitrogen and the compounds re-dissolved in 15 ml of undecane for analysis. Gas Chromatography (GC) was performed using a 15 mm, 0.25 mm diameter, 0.25 mm film thickness, DB1701 fused silica capillary column (Fisons, Folsom, CA). Column temperature was 190 to 300°C at 15°C/min. with methane carrier gas flows of 1 ml/min. Negative ion chemical ionization MS was performed using a Hewlett–Packard HP5989A instrument interfaced with monitoring ions for F₂-IsoPs (m/z 569), the [²H₄]15-F_{2t}-IsoP internal standard (m/z 573) and F₄-NeuroPs (m/z 593). Ion source temperature was 250°C, and electron energy 70 eV. These samples were measured by the Vanderbilt group in a blinded fashion.

Lipid peroxidation in rat brain homogenate was induced with 5 mM 2,2'-Azobis(2-methylpropionamide) dihydrochloride (AAPH) following 2 h incubation at 37°C. For the experiments with tamoxifen, rat brain homogenates were incubated with vehicle or tamoxifen (1 µM or 50 µM) for 15 min at 37°C followed by the addition of AAPH to a final concentration of 5 mM, and subsequent incubation for an additional 2 hr. Brain homogenates were prepared from 3 rat cerebral hemispheres as 0.1 g of brain/ ml of 1 M phosphate buffer pH 7.

Neurobehavioral Deficit Scoring

Neurobehavioral deficit scoring was based on the 18 point scale described by (Garcia et al. 1995), as used previously in our laboratory (Zhang et al, 2005). Neurological status was scored at 24 hours after the ischemia. The individual evaluating neurobehavioral deficits was blinded as to whether vehicle or drugs were administered. The neurobehavioral scale consisted of the following six tests: 1) spontaneous activity (0 to 3 points); 2) symmetry in the movement of four limbs (0 to 3 points); 3) forepaw outstretching (0 to 3 points); 4) climbing (1 to 3 points); 5) body proprioception (1 to 3 points); and 6) response to vibrissae touch (1 to 3 points). The score given to each rat at the completion of the evaluation is the summation of all six individual test scores. Three is the minimum neurological score and 18 is the score exhibited by normal rats.

Measurement of Infarct Volume

Infarct volume was assessed using 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO) staining, as used previously in our laboratory (Kimelberg et al, 2000). Twenty four hours after ischemia, rats were injected with 120 mg of pentobarbital. The brain was then removed, and cut into 2 mm sections. The slices were placed in a Petri dish containing 2% TTC for 30 minutes, and periodically agitated so that no slices continuously rested on the bottom. Lesion volumes were calculated from summed, measured areas (ImageJ 1.33u, NIH) of unstained tissue in mm² multiplied by 2 mm slice thickness. The individual measuring the infarct size was blinded as to whether vehicle or drugs were administered.

Statistical Analysis

Analysis of infarct volume and neurobehavioral scores was by two way analysis of variance (ANOVA). The General Linear Model module in Statistica (StatSoft) was used. We tested for a 1) a between-group effect of tamoxifen administration or vehicle, 2) a between-group effect of ICI administration or vehicle (regardless of route), and 3) their interaction. Comparisons were made between the groups by the Newman-Keuls test. For analysis of IsoPs and NeuroPs levels, statistics were utilized by repeated measures ANOVA. For each group of animals we tested for a between-group effect of treatment. A within-animal effect of ipsilateral or

contralateral to the ischemia was also included. An interaction effect was also tested. Post-hoc tests were by Newman-Keuls multiple range test with $\alpha = 0.05$. Striatal and cortical areas were tested separately. Differences were considered statistically significant at $p < 0.05$.

Results

Effects of ICI 182,780 on Tamoxifen-induced behavioral improvement and infarct size

ICI 182,780 was injected into both the cisterna magna (i.c.) and intravenously (i.v.) 15 minutes before initiation of ischemia while tamoxifen was given i.v. at the time of ischemia (see Materials and Methods). Both effects on behavioral score and infarct volume were studied. For the neurobehavioral score there was a statistically significant effect of tamoxifen ($p < 0.001$), but no statistically significant effect of ICI ($p = 0.41$) and no statistically significant interaction ($p = 0.09$) indicating that ICI had no detectable effect on the tamoxifen neuroprotection. Behavioral scores were better in the tamoxifen or tamoxifen/ICI 182,780 (i.v.) and tamoxifen/ICI 182,780 (i.c.)-treated groups compared to the corresponding vehicle or ICI 182,780 (i.v. or i.c.)-treated groups (Figure 1). Also, no significant differences were found among vehicle, ICI 182,780 (i.v.) and ICI 182,780 (i.c.) treated groups, nor among tamoxifen, tamoxifen plus ICI 182,780 (i.v. or i.c.) treated groups (Figure 1).

Infarct volume was measured 24h after initiation of ischemia with TTC with the same regimen of injections as described above. For the infarct volume there was a statistically significant effect of tamoxifen ($p < 0.001$) but no statistically significant effect of ICI ($p = 0.84$) and no statistically significant interaction ($p = 0.88$) indicating that ICI had no detectable effect on the tamoxifen neuroprotection (see Figure 2). Mean infarct volumes were 286 mm^3 (SD: 133; $n = 5$) in vehicle - treated animals and 303 mm^3 (SD: 156; $n = 4$) in ICI 182,780 (i.v., 1 mg/kg) treated animals, which were significantly reduced to 92 mm^3 (SD: 44; $n = 5$) in the animals given tamoxifen (5 mg/kg) at the initiation of ischemia and to 95 mm^3 (SD: 54; $n = 5$) in the animals given tamoxifen (5 mg/kg) plus ICI 182,780 (i.v., 1 mg/kg), respectively. Infarct volume was 251 mm^3 (SD: 52; $n = 5$) in ICI 182,780 (i.c., 10 μg) treated animals and was also significantly less at 86 mm^3 (SD: 43; $n = 5$) in the animals given 5 mg/kg tamoxifen plus ICI 182,780 (i.c., 10 μg). No significant differences were found among vehicle, ICI 182,780 (i.v.) and ICI 182,780 (i.c.) treated groups, nor among tamoxifen, tamoxifen plus ICI 182,780 (i.v.) or ICI 182,780 (i.c.) treated groups. Representative examples of the staining are shown in Figure 3.

Thus tamoxifen or tamoxifen plus ICI 182,780 (i.v. or i.c.) treated rats had significantly improved neurobehavioral deficit scores after 24 hours and showed significantly reduced infarct volumes, giving a reduction of $\sim 75\%$ in all cases. The fact that neuroprotection by tamoxifen was maintained when co-administered with the pure estrogen receptor antagonist ICI 182,780, given both i.c. and i.v., indicates that actions at estrogen receptors are not involved either, peripherally or centrally.

Antioxidant Activity of Tamoxifen

Tamoxifen at both 5 and 10 mg/kg given 3 hours post initiation of ischemia was as effective a neuroprotectant as when it was administered simultaneously with ischemia (Kimelberg et al, 2000,2003). To see whether this protection correlated with antioxidant activity under the same conditions we tested the antioxidant effects of 5 mg/kg tamoxifen given 3 hours after initiation of ischemia as measured by effects on isoprostane appearance. We chose to study effects on isoprostane levels as these compounds are emerging as stable markers of lipid peroxidation and hence free radical damage in cerebral ischemia (Marin et al, 2000),

For IsoPs in the striatum, there was a significant effect of treatment ($p = 0.015$) and a significant interaction effect ($p = 0.025$), reflecting a difference in the response of ischemic versus non-ischemic tissue to the effect of tamoxifen. The IsoPs levels in the ischemic vehicle-treated striatum were significantly higher than all other striatal tissue (all p values < 0.038). No other striatal tissue, including the ischemic striatal tissue in tamoxifen-treated animals, showed significantly different IsoPs levels (Newman-Keuls multiple range tests). For IsoPs in the cortex there was a significant effect of treatment ($p < 0.001$), and a significant interaction effect ($p = 0.003$), reflecting a difference in the response of ischemic versus non-ischemic tissue to the effect of tamoxifen. Again, the IsoPs levels in the ischemic vehicle-treated cortex were significantly higher than all other cortical tissue (all p values < 0.001). No other cortical tissue, including the ischemic cortical tissue in tamoxifen-treated animals, showed significantly different isoprostane levels (Newman-Keuls multiple range tests).

For NeuroPs in the striatum, there was a significant effect of treatment ($p < 0.001$), and significant repeated measures and interaction effects ($p = 0.02$ and 0.01), reflecting a difference in the response of ischemic versus non-ischemic tissue to the effect of tamoxifen. The NeuroP levels in the ischemic vehicle-treated striatum were significantly higher than all other striatal tissue (all p values < 0.05). No other striatal tissue, including the ischemic striatal tissue in tamoxifen-treated animals, showed significantly different neuroprostane levels (Newman-Keuls multiple range tests). For NeuroPs in the cortex, there was a significant effect of treatment ($p = 0.007$) and significant repeated measures and interaction effects ($p = 0.048$ and 0.02), reflecting a difference in the response of ischemic versus non-ischemic tissue to the effect of tamoxifen. The NeuroP levels in the ischemic vehicle-treated cortex were significantly higher than all other cortical tissue (all p values < 0.02). No other cortical tissue, including the ischemic cortical tissue in tamoxifen-treated animals, showed significantly different NeuroP levels (Newman-Keuls multiple range tests).

Thus, as shown in Figure 4a,b significant increases of IsoPs (1.8-fold) and NeuroPs (2.9-fold) were observed in the ipsilateral hemisphere of rats submitted to MCAo for 2 hours followed by 22 hours reperfusion. Treatment with tamoxifen (5 mg/kg, i.v.) at 1 h after reperfusion reduced ischemia-induced IsoP production to the levels in normal (intact, non-operated) or sham-operated animals or the levels on the contralateral sides as measured 24 hours after onset of ischemia, which were all the same. The NeuroP levels were reduced 86% for the ipsilateral cortex and 80% for the striatum and these were significant at the $p < 0.05$ level. In addition, tamoxifen also significantly ($p < 0.05$) reduced the increase of NeuroP in the contralateral cortex to normal and sham levels. In sham-operated rats, neither vehicle nor tamoxifen altered IsoP and NeuroP concentration significantly.

Because there is no evidence that tamoxifen directly inhibits the peroxidation of arachidonic acid we tested its ability to inhibit isoprostane and neuroprostane production after treating brain homogenates with AAPH (see methods). The results are shown in figure 5. As can be seen the formation of F₂-IsoPs and F₄-NeuroPs, were totally suppressed at 50 μ M and by $\sim 50\%$ at 1 μ M tamoxifen but the latter effect did not reach statistical significance ($p = 0.058$). Reaction times and volumes were same for all groups, vehicle+vehicle, vehicle+AAPH, tamoxifen+AAPH ($n = 3$ per group).

Discussion

We have previously shown that tamoxifen markedly reduced infarct volume and neurobehavioral deficits after MCAo in the rat (Kimelberg et al, 2000;2003) and these persist at very high protective levels of $\geq 90\%$ for up to 14 days after initiation of ischemia (Zhang et al, 2005). In the present study we further explored mechanisms of action of tamoxifen that could lead to this marked neuroprotection. These studies are necessary because tamoxifen has

a number of actions consistent with the neuroprotection observed (see introduction). The elucidation of the number of sites of action of tamoxifen, by giving a more complete understanding of how tamoxifen works, will allow more rational design of therapy.

Actions of tamoxifen on estrogen receptors

Although tamoxifen is primarily an estrogen receptor antagonist it has some action as an agonist depending on tissue and tamoxifen concentration (MacGregor and Jordan, 1998). Therefore, the question arises if any of its effects are similar to the protective effects in experimental stroke exerted by estrogens. Estrogen effects are diverse and include not only actions at estrogen receptors (ER) resulting in changes in bcl-2 gene levels, activation of MAP kinases and/or effects on the cAMP-PKA-CREB pathway (Dhandapani and Brann, 2002; Hurn and Brass, 2003), but also direct effects on $[Ca^{2+}]_i$, inhibition of anion channels and antioxidant activity (Green and Simpkins, 2000). Although the protective actions of estrogens have been observed primarily with chronic treatment as replacement therapy in ovariectomized female rats (Green and Simpkins, 2000; Hurn and Brass, 2003), it has also been shown that higher concentrations of 17 β -Estradiol (E2) are neuroprotective in male rats (Toung et al, 1998). Estradiol at high doses was also effective when given up to 3 h, together with a slow-release depot, after permanent MCAo in ovariectomized female rats, and these acute effects were attributed to the antioxidant action of high dose estrogen (Yang et al, 2000).

Although estrogen is very effective with pretreatments of ~ 1 week in ovariectomized rats this efficacy has not translated to effectiveness in clinical trials (Hurn and Brass, 2003). Note, that even if tamoxifen was acting as a partial ER agonist, it is effective when given acutely up to 3 h after initiation of MCAo, unlike the pretreatment with estrogens which is the only one thought to be due ER-induced synthetic or other long term pathways (Dhandapani and Brann, 2002; Hurn and Brass, 2003). In the present study we tested whether the neuroprotective effect of tamoxifen is maintained by tamoxifen when ICI 182,780, a pure ER antagonist, was co-administered. Since ICI 182,780 has been shown not to penetrate the BBB (Wade et al, 1993), we administered it intravenously and intracisternally, thus testing whether tamoxifen induced neuroprotection involves an action on either peripheral or central estrogen receptors (Hurn and Brass, 2003). In this case we gave tamoxifen at the time of the occlusion and ICI 182,780 just prior to tamoxifen, since we assume that there is no time-dependent change in mechanism as the amount of protection is the same within this time window (Kimmelberg et al, 2000, 2003). A novel finding in this study therefore, is that tamoxifen's protection persists when administered with either intravenous injection or intracisternal injection of pure ER blocker, ICI 182,780, in the rMCAo model in rats. Thus the protective effects of tamoxifen are unlikely to involve interactions with ERs in either the periphery or the CNS since these should have been inhibited by the intravenous or intracisternal pretreatment with ICI 182,780, respectively.

ICI 182,780 dose, timing and neuroprotection

We used a single dose of ICI 182,780 to conserve animals and the dose used was based on the data in the paper by Wade et al. (1993), which also showed that the brain is inaccessible to ICI 182,780 which was the reason we used intracisternal as well as intravenous injection. In that paper the ICI compound was shown to have a 2,300 fold higher affinity than tamoxifen for displacing 3H estradiol binding to brain tissue with an EC₅₀ of $\sim 10^{-10}M$. Based on a rat total brain weight of 2mg (since estradiol enters cells) and that ICI does not leave the brain readily since it doesn't get in, our dose of 10 μg gives an average concentration of 8.2 μM ($10^{-5}g/607(MW) \times 0.002L$) in the brain. It seems we should not use more since this is already 10,000 times its EC₅₀. The only problem we might consider is whether this dose is toxic, but we noticed no effects. We gave ICI 182,780, 15 min. before ischemia and tamoxifen was given i.v. at ischemia as it rapidly enters the brain, and the single i.v. dose of ICI 182,780 we used was $\sim 330 \mu g$ compared to Wade et al., (1993) of 250 $\mu g/day$. No other times were studied. Tamoxifen has

an IC₅₀ for displacement of ³H estradiol of ~1μM in the brain (Wade et al., 1993). This is similar to its effectiveness as an antioxidant as shown in Fig. 5 and therefore the only argument against an ER agonist effect is that the very potent pure ER antagonist ICI 182,780 has no effect on the neuroprotective action of tamoxifen.

ICI 182,780 has been shown to protect in intact females but Sawada et al., (2000) reported no effects in male mice and we also now show no protection in male rats. Also the ICI pretreatment in females was for 7 days, possible reflecting the time needed for inhibition of ER-induced synthesis of protective molecules. Our treatment was for 15 mins., as we used it only to test the possibility that tamoxifen's neuroprotective effect could be via its action as an estrogen agonist in the brain or the periphery.

Antioxidant activity and tamoxifen-induced neuroprotection

Oxidative damage to brain is a featured shared by several destructive and degenerative diseases (Milatovic et al, 2005; Gilgun-Sherki et al, 2002). Tamoxifen, or its immediate metabolite 4-hydroxytamoxifen, are effective antioxidants (Dubey et al, 1999; Wiseman et al, 1994), and antioxidants have been clearly shown to be protective in brain trauma and stroke (Hall et al, 1989; Chan, 1999). The major tamoxifen metabolite, 4-OH tamoxifen was shown to markedly reduce induced lipid peroxidation in brain mitochondria *in vitro* (Moreira et al, 2004), but the antioxidative effects of tamoxifen have never been directly tested in stroke models. During ischemia and especially during reperfusion there are high levels of free radicals generated, which would be expected to attack proteins and lipids of the cell membrane and mitochondria. Lipid peroxidation is initiated by a free radical-induced abstraction of a hydrogen from the polyunsaturated fatty acyl side chains of membrane lipids (phospholipids, glycolipids, glycerides and sterols), especially by hydroxyl radical and peroxynitrite radicals (Mattson, 1998). IsoPs are prostanooids produced independently of cyclooxygenase by free radical-catalysed peroxidation of arachidonic acid-containing lipids (Marin et al, 2000). Quantification of IsoPs has therefore been proposed as an accurate marker of oxidative stress in experimental models of free radical-induced injury and human diseases being deemed more reliable and specific than the more commonly measured thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) (Cherubini et al, 2005). An increased production of isoprostanes has been observed in human stroke (van Kooten et al, 1997). In addition, NeuroP, an analogous product generated from docosahexaenoic acid (DHA), offers a unique window into free radical damage to neuronal membranes *in vivo* (Montine et al, 2004) and therefore was measured for this purpose in this study by methods previously described (Milatovic et al, 2005).

In the present study, rat brain tissues were collected from vehicle or tamoxifen treated animals submitted to 2 hours rMCAo. IsoPs and NeuroPs were then measured to evaluate the role of antioxidative stress in tamoxifen's protection. The present study is the first report, to our knowledge, of measurements of IsoPs and NeuroPs in the rMCAo model in rats. Twenty-four hours after ischemia/reperfusion injury, significant rises in IsoPs and NeuroPs were observed in the infarcted hemisphere of ischemic rats relative to sham animals or the contralateral side. In our rMCAo model, treatment with tamoxifen at 3 hours after the initiation of ischemia reduced the IsoPs and NeuroPs to control levels. These control levels were high but the same for all the different conditions. They seem unlikely to represent normal levels of isoprostanes and may have been partially produced during the period between sacrifice of the animals and removal and dissection of the different regions before freezing these dissected samples in liquid N₂. Checking this possibility would require freezing the tissue *in situ* while the animal is anesthetized which precludes regional dissection of the brain, or dissecting the different regions from liquid N₂ frozen sections for which methods would have to be developed. However, the equivalent values for all the control levels implies that the increases specific to ipsilateral brain tissue is the true increase due to ischemia and reduction by tamoxifen to the control levels

means that tamoxifen completely inhibits the ischemia-induced increases. Marin et al. (2000) reported relatively much lower levels in contralateral brain tissue from rMCAo animals. In these studies the removed hemispheres were frozen in liquid N₂ and the isoprostane levels of the entire hemispheres measured with no dissection of the different regions. A significant increase in NeuroPs concentration (about 2-fold increase compared with sham animals, figure 4b), was seen in the ipsilateral cortex of ischemic animals, whereas a similar statistically significant increase of IsoPs (the overall free radical damage marker) was not observed in the contralateral side. However, the contralateral cortex levels were significantly decreased from the ipsilateral side perhaps indicating a tendency for an increase in the contralateral cortex.

Thus in total our data thus demonstrate that tamoxifen produces a significant reduction of IsoPs, and especially the increase in NeuroPs in rats submitted to transient cerebral ischemic injury. The inhibitory effect of tamoxifen could be due to a general scavenging of the free radicals required for the formation of isoprostanes from arachidonic acid or inhibition of Ca²⁺ binding proteins (lipocortins) that control phospholipase-dependent release of modified arachidonic acid from membrane phospholipids (Halliwell and Gutteridge, 1999). This possibility would be analogous to inhibition of calmodulin by tamoxifen (Kirk and Kirk, 1994). The data in figure 5 showing abolition of both IsoPs and NeuroPs in a brain homogenate where lipid peroxidation is chemically induced supports the free radical scavenging possibility. Since we also found decrease in both F2-IsoPs and F4-NeuroPs after tamoxifen exposure, tamoxifen should affect both arachidonic acid and docosahexaenoic acid in the same way if some other mechanisms are involved. In addition, F2-IsoPs and F4-NeuroPs are formed in situ on phospholipids and we have quantified esterified (bound) F2-IsoPs (only 5% are from free F2-IsoPs), thus there is no indication of an effect on the release of arachidonic or docosahexaenoic acid.

In summary, we have shown that the antioxidant activity of tamoxifen, measured here as inhibition of ischemia induced increased isoprostane levels, is found under the same conditions as were found to produce marked neuroprotection in focal cerebral ischemic injury in the rat, and the neuroprotective activity of tamoxifen is maintained when it is co-administered with the pure estrogen receptor antagonist ICI 182,780, given i.c. or i.p. These data are consistent with one mechanism of action for tamoxifen being its antioxidative activity, and conversely that action at estrogen receptors, both peripherally and centrally, are not part of the mechanisms of its neuroprotective actions.

The antioxidative action can therefore add to the inhibition of nNOS activity (Osuka et al, 2001) and inhibition of ischemia induced release of excitatory amino acids (Feustel et al, 2004), that we have previously shown in the rat rMCAo model, as being responsible for tamoxifen's remarkably effective neuroprotective effects. Since all these effects are included within our current knowledge of the damaging processes set in motion by cerebral ischemia (Chan, 1999; Dirnagl et al., 1999; Ginsberg, 2003; Hall and Braughler, 1989; Stroke therapy academic industry round table, 1999), we are tempted to ascribe a causal relation between these demonstrated effects of tamoxifen and its neuroprotection. Further studies on tamoxifen pharmacokinetics in order to relate dosing to blood and CNS levels and the concentrations and times at which different processes can be inhibited can be used to further support these associations and allow more rational design of any proposed clinical trials.

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Abbreviations

MCAo, middle cerebral artery occlusion; ICI 182,780, {7 α -[9-(4,4,5,5,5-pentafluoropentylsulfanyl)nonyl]estra-1,3,5-(10)-triene-3,17 β -diol}; EAAs, excitatory amino-acids; ER, estrogen receptor; SERM, selective estrogen receptor modulator; IsoP, isoprostanes; NeuroPs, neuroprostanes.

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Figure 1.

Box plots of the effects of treatment with tamoxifen on neurobehavioral scores with or without ICI 182,780. Treatment with 5 mg/kg tamoxifen alone, tamoxifen plus 1 mg/kg ICI 182,780 (intravenous injection, i.v.) and tamoxifen plus 10 μ g ICI 182,780 (intracisternal injection, i.c.) resulted in improved neurobehavioral scores at 24 hours ($p < 0.001$; ANOVA). There were no significant effects of treatment with 1 mg/kg ICI 182,780 (i.v.) and 10 μ g ICI 182,780 (i.c.) on neurobehavioral scores (see text). Eighteen is the score exhibited by normal animals and 3 is the minimum score. Each data point represents one animal. Vehicle, vehicle treated; ICI.v., intravenous injection of ICI 182,780; ICI.c., incisternal injection of ICI 182,780; tmx, tamoxifen - treated; tmx ICI.v., co-administration of tamoxifen and ICI 182,780 (i.v.); tmx ICI.c., co-administration of tamoxifen and ICI (i.c.).

Figure 2.

Box plots of the overall effects of treatment with tamoxifen and/or ICI 182,780 on brain infarct size. Tamoxifen (5 mg/kg) alone, tamoxifen plus 1 mg/kg ICI 182,780 (intravenous injection, i.v.) and tamoxifen plus 10 μ g ICI 182,780 (intracisternal injection, i.c.) reduced infarct volume significantly ($p < 0.001$). There was no significant effect of treatment with 1 mg/kg ICI 182,780 (i.v.) and 10 μ g ICI 182,780 (i.c.) on brain infarct size. Each data point represents one animal. vehicle: vehicle treated; ICI.v.: intravenous injection of ICI 182,780; ICI.c.: incisternal injection of ICI 182,780; tmx: tamoxifen - treated; tmxICI.v.: co-administration of tamoxifen and ICI 182,780 (i.v.); tmxICI.c.: co-administration of tamoxifen and ICI (i.c.). TTC: 2,3,5-triphenyltetrazolium chloride.

Figure 3.

Representative sections showing infarcted areas stained for TTC 24h after ischemia from vehicle treated animal (vehicle); intravenous (i.v.) ICI 182,780 (1 mg/kg) treated animal (ICIi.v.), intracisternal (i.c.) ICI 182,780 (10 μ g) treated animal (ICIi.c.); tamoxifen (5 mg/kg) treated animal (tmx); tamoxifen plus ICI 182,780 (1 mg/kg, i.v.) treated animal (tmx + ICIi.v.); and tamoxifen (5 mg/kg) plus ICI 182,780 (10 μ g, i.c.) treated animal (tmx + ICIi.c.). See figure 3 for the definitions of the different conditions.

Figure 4. Effects of tamoxifen on ischemia-induced F₂-isoprostane (a) and F₄-neuroprostane (b) levels. Tamoxifen (5mg/kg) or vehicle was administered intravenously to ischemic or sham animals 3 hours after onset of ischemia. F₂-isoprostanes and F₄-neuroprostanes were measured 24 hours after ischemia. All data are means $\pm$ SD; Numbers of animals were 3 for intact (non-

operated), sham/vehicle and sham/tamoxifen (5 mg/kg) group, 4 for ischemic vehicle and 5 for ischemic/tamoxifen group, one sample from all the regions per animal. * $p < 0.05$ for the comparison between vehicle and corresponding tamoxifen – treated groups for ischemic animals vehicle: vehicle treated; tmx: tamoxifen treated.

Figure 5.

Effect of different concentrations of tamoxifen on the formation of F₂-IsoPs (A) and F₄-NeuroPs (B) in peroxidizing rat brain homogenates. (n=3 samples per group±SEM). *p<0.05 versus control by one-way ANOVA followed by Bonferroni's multiple comparison tests. #p<0.05 versus AAPH by one-way ANOVA followed by Bonferroni's multiple comparison tests. Actual P values for effect of 1 μM tamoxifen is p=0.058 and for 50 μM tamoxifen is p=0.038.