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Short communication

Effects of tamoxifen on oxyhemoglobin-induced cerebral vasoconstriction

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Abstract

The effect of tamoxifen on oxyhemoglobin-mediated cerebral vasoconstriction was examined. Tamoxifen caused a concentration-dependent relaxation of cerebral artery preparations contracted with oxyhemoglobin and phorbol myristate acetate with the IC_{50} values 0.66 ± 0.1 and 1.1 ± 0.1 μM, respectively. In cerebrovascular smooth muscle cells, oxyhemoglobin and phorbol myristate acetate induced protein kinase C activation, which was $220 \pm 7\%$ and $203 \pm 8\%$ of control, respectively. The increase in protein kinase C activity was prevented by tamoxifen. The results suggest that the ability of tamoxifen to reverse vasoconstriction is mediated, at least in part, via inhibition of protein kinase C.

Keywords: Cerebrovascular constriction; Tamoxifen; Oxyhemoglobin; Protein kinase C

1. Introduction

Tamoxifen, a non-steroidal anti-estrogen, is effective in the treatment of estrogen receptor positive, as well as ER negative breast cancers (Gibson et al., 1990). Although tamoxifen might be expected to act via the estrogen receptors, a variety of other potentially important actions of this agent are now recognized, including inhibition of protein kinase C (O'Brian et al., 1986; Couldwell et al., 1993) and antagonism of Ca^{2+} channels (Song et al., 1996). The effect of tamoxifen on protein kinase C has been of particular interest to us, especially in view of the possible involvement of this enzyme in the development of cerebrovascular spasm, which occurs after subarachnoid hemorrhage. Critical vasospasm is associated with cerebral ischemia and infarction, which account for approximately one-third of death and disability following cerebral hemorrhage (Findlay et al., 1991). The delay in the onset of vasospasm provides a potential therapeutic opportunity, which is lacking in other ischemic strokes. However, at present, there is no established pharmacotherapy, which would reverse vasospasm once it starts to develop. There is now evidence that oxyhemoglobin released from lysing erythrocytes in the subarachnoid clot is responsible for the problem (Findlay et al., 1991). The sustained phase of vasoconstriction induced by oxyhemoglobin is thought to involve protein kinase C activation (Cook and Vollrath, 1995; Vollrath et al., 1998). The observations that phorbol esters may induce cerebral vasospasm in animal models (Sako et al., 1993) are consistent with this suggestion. In this respect, the ability of tamoxifen to inhibit protein kinase C activity in cerebrovascular smooth muscle would be clinically useful. We therefore investigated the effects of tamoxifen on oxyhemoglobin- and phorbol myristate acetate-induced contractions of endothelium denuded rabbit cerebral artery preparations. In addition, we examined protein kinase C activity in cerebrovascular smooth muscle cells stimulated with oxyhemoglobin and phorbol myristate acetate.

2. Materials and methods

2.1. Materials

Hemoglobin, tamoxifen, and phorbol myristate acetate were from Sigma. All reagents for colorimetric protein

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kinase C assay were from Pierce. Other chemicals were commercial products of the highest grade available.

2.2. Recording of isometric tension

The experiments were carried out in rabbits (2.5–3.5 kg). Protocols for the humane treatment of animals according to the Declaration of Helsinki, and as approved by the University of Alberta Animal and Ethics review were followed in all experiments. The animals were killed with an intravenous overdose of pentobarbitone, and the cerebral arteries were removed and placed in oxygenated Krebs–Henseleit solution (in mM: NaCl 130, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and dextrose 11). Basilar arteries (2–3 mm rings) were denuded of endothelium and set up in organ baths containing Krebs–Henseleit buffer at 37°C, gassed with 95% O_2 and 5% CO_2. The arterial rings were equilibrated for 1 h. Contractions to oxyhemoglobin, phorbol myristate acetate, and potassium chloride were recorded isometrically using force-displacement transducers and a Grass 7D polygraph. Tamoxifen was administered in increasing, cumulative concentrations (0.01–10 μM) to preparations in which a tonic contraction to the spasmogens had developed. The relaxation of the preparations exposed to tamoxifen was expressed as the percentage of maximum tension produced by the vasoconstrictors. The IC_{50} values for tamoxifen were determined using Excel by fitting an exponential curve to the data.

2.3. Cerebrovascular smooth muscle cell culture

Cells were prepared as described previously (Vollrath et al., 1998). Briefly, basilar arteries were isolated under sterile conditions and the sections of the arteries were placed in a Petri dish containing Dulbecco's modified Eagle medium (DMEM). The explants (1–2 mm segments) were transferred to 25-cm culture flasks containing 1 ml of DMEM supplemented with 10% foetal calf serum, penicillin (10.000 u/ml), and streptomycin (10 μg/ml). When the primary cultures were almost confluent, the cells were transferred to 75-cm^2 flasks and then routinely subcultured at a split ratio of 1:3.

2.4. Protein kinase C activity

Protein kinase C activity was measured in the membrane and cytosolic fractions of cerebrovascular smooth muscle cells treated with oxyhemoglobin (10 μM) and phorbol myristate acetate (160 nM) for 5 min, in the presence or absence of tamoxifen (7 μM). After incubation, the cells were scraped in ice-cold phosphate buffered saline (145 mM NaCl/10 mM sodium phosphate; pH 7.4), and centrifuged at $200 \times g$ for 10 min. The pelleted cells were homogenized in ice-cold buffer containing Tris–HCl (25 mM), EGTA (0.5–4 mM), EDTA (2 mM), dithiotreitol (2.5 mM) and leupeptin (20 μM). Homogenized cells were separated into cytosolic and membrane fractions by centrifugation at $15\,000 \times g$ for 60 min at 4°C. The membrane pellets were resuspended in the ice-cold homogenization buffer and the protein kinase C was solubilized via sonication. Samples were adjusted to equal protein concentration (estimated by the Bradford method) using bovine serum albumin as a standard. The standard reaction mixture (15 μl) contained 100 mM Tris at pH 7.4, 10 mM ATP, 50 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.01% Triton X-100, phosphatidylserine (1 mg/ml), the peptide substrate (pseudosubstrate peptide labeled with a fluorescent dye), and the endogenous protein kinase C (10 μl). The samples were incubated at 30°C for 30 min. The reaction mixture was then applied to the separation units containing the affinity membranes (Pierce), which specifically bind the phosphorylated peptide. The bound substrate was eluted from the affinity membranes using a buffer containing 15% formic acid, and its absorbance was measured at 570 nm.

2.5. Statistical methods

All results are reported as the mean ± S.E.M., with the number of preparations used in parentheses. Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnet's test when significant probability was reached. Values of $P < 0.05$ were considered to be significant.

3. Results

Both oxyhemoglobin (10 μM) and phorbol myristate acetate (160 nM) induced a slowly developing contraction of basilar artery preparations, which reached a plateau at about 5–15 min and was maintained for several hours. To

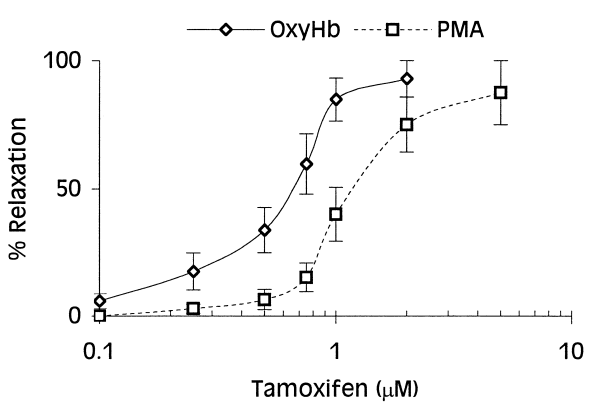

Fig. 1. Cumulative concentration–response curves for the relaxation produced by tamoxifen in basilar artery preparations. Arterial rings denuded of endothelium were pre-contracted with oxyhemoglobin (10 μM) or phorbol myristate acetate (160 nM), and each concentration of tamoxifen applied for 5 min. Data are expressed as the mean ± S.E.M. for experiments conducted with seven or more ring preparations from five animals.

mimic the situation in vasospasm, in which therapeutic agents are administered after vasospasm had been initiated, tamoxifen was added to basilar artery preparations in which a tonic contraction to oxyhemoglobin and phorbol myristate acetate had developed (Fig. 1). The IC_{50} values for tamoxifen against oxyhemoglobin- and phorbol myristate acetate-induced contractions were 0.66 ± 0.09 μM ($n = 7$) and 1.1 ± 0.105 μM ($n = 7$), respectively. A continuing slow decrease in tension was observed at the lowest concentrations of tamoxifen after longer exposure times (10–15 min), indicating that the effects of this agent were time-dependent. To determine whether the ability of tamoxifen to relax the arterial preparations contracted with oxyhemoglobin could arise from an effect mediated by the endothelial cells, responses to bradykinin (10^{-12}–10^{-7} M), administered to the preparations pre-contracted with 5-hydroxytryptamine (10^{-6} M), were recorded as an indicator of the preservation of the endothelium. These experiments have shown that bradykinin had no relaxant effect on contractions, while tamoxifen retained its relaxant activity. To determine whether oxyhemoglobin- and phorbol myrtistate acetate-induced cerebrovascular contractions were associated with the activation of protein kinase C, the cultured cells were treated with these agents for 5 min, in the presence or absence of tamoxifen (7 μM). As shown in Fig. 2, oxyhemoglobin and phorbol myristate acetate caused an increase in protein kinase C activity in the membrane fractions which was $220 \pm 6.6\%$ and $203 \pm 7.7\%$, respectively. The 30-min pre-treatment with tamoxifen (7 μM) reduced protein kinase C activity to the level not different from the controls (Fig. 2). The rise in the protein kinase C activity in membrane fractions obtained from cells treated with either oxyhemoglobin or phorbol ester was associated with a concomitant decrease in the activity of the enzyme in the cytosolic fractions (61.7% and 68.3% of the control for oxyhemoglobin and phorbol myristate acetate, respectively). However, the changes in the cytosolic fractions did not reach statistical significance.

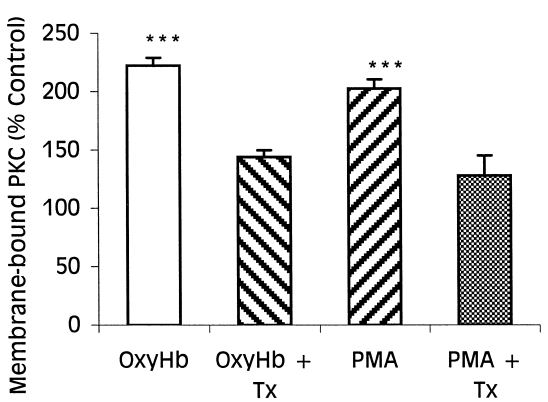

Fig. 2. Effects of tamoxifen on protein kinase C activity in the membrane fractions from cerebrovascular smooth muscle cells exposed to oxyhemoglobin (10 μM) and phorbol myristate acetate (160 nM) for 5 min. Tamoxifen (7 μM) was administered 30 min before application of oxyhemoglobin or phorbol myristate acetate. Data are expressed as the mean ± S.E.M. of 10 experiments. *** $P < 0.001$ when compared to control value.

4. Discussion

This is the first demonstration that tamoxifen, an antiestrogen compound widely used in the treatment of breast and other cancers, can reverse a sustained cerebral vasoconstriction induced by oxyhemoglobin, a spasmogen implicated in the pathogenesis of cerebral vasospasm. The IC_{50} value for tamoxifen against oxyhemoglobin-mediated contraction appears to be in line with the therapeutic dose of tamoxifen for breast cancer patients, which is about 40 mg/day for the time period of up to 5 years, and which typically results in 0.5–1 μM concentration of this agent in plasma (Bratherton et al., 1984). In addition to the inhibition of cerebrovascular responses induced by oxyhemoglobin, tamoxifen also inhibited sustained contraction to phorbol myristate acetate, a direct stimulator of protein kinase C (Zhang et al., 1995). Thus, our observation suggests that the effects of tamoxifen on cerebrovascular contraction may be mediated, at least in part, via inhibition of protein kinase C. This suggestion is further supported by the observation that activation of this enzyme by oxyhemoglobin, was reversed by tamoxifen in a concentration corresponding to the IC_{50} values for inhibition of protein kinase C by this agent in cancer cells (O'Brian et al., 1986). It is well-documented that upon stimulation, the classical (α, β, γ) and novel (δ, ε, η, θ) protein kinase C isoforms undergo translocation to the plasma membrane, a hallmark demonstrating activation (Walsh et al., 1996). Thus, it is conceivable that tamoxifen interferes with protein kinase C translocation, a suggestion for which there is some experimental evidence (Cheng et al., 1998). Since tamoxifen is an amphiphilic cation with a hydrophobic region containing phenyl and amine groups (O'Brian et al., 1986; MacGregor and Jordan, 1998), it is possible that it interferes with the hydrophobic interactions between the protein kinase C and phosphatidylserine, a lipid co-factor necessary for protein kinase C activity (Walsh et al., 1996).

The present studies have also shown that tamoxifen was more effective when tested against the oxyhemoglobin-mediated vasoconstriction than the phorbol ester-mediated action. This suggests that other sites of action mediated by tamoxifen should be considered, a view that is supported by the reports indicating that tamoxifen exhibits a number of effects including antagonism of a variety of ion channels and calmodulin, and inhibition of myosin light chain kinase (MacGregor and Jordan, 1998), which may be the potential sites of action of this agent in cerebrovascular smooth muscle. Thus, it is conceivable that the effectiveness of tamoxifen in attenuating the oxyhemoglobin-

mediated vasoconstriction arises from multiple effects of this agent.

In conclusion, the results of the present studies demonstrate that tamoxifen is able to prevent the contractile action of oxyhemoglobin and suggest that this effect may be mediated, at least in part, by the inhibition of protein kinase C activity. The beneficial cerebrovascular effects mediated by tamoxifen could lead to the development of effective pharmacotherapy in cerebral vasospasm.

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