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Evidence that 6-hydroxydopamine is an inhibitor of catechol-*O*-methyltransferase in intact tissue

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The effect of in-vitro sympathetic denervation on the *O*-methylation of 2-hydroxyoestradiol was examined using the rabbit thoracic aorta. Aortic segments were exposed to 6-hydroxydopamine (400 mg litre^{-1}, 10 min), incubated in 2-hydroxyoestradiol (5 μM, 60 min), and the 2-methoxyoestradiol formed was quantified using HPLC with electrochemical detection. Pre-exposure to 6-hydroxydopamine reduced *O*-methylation by 90% in intact aortic strips. However, *O*-methylation was also reduced (81%) in rabbit aorta that had been surgically denervated before exposure to 6-hydroxydopamine. Furthermore, the *O*-methylation of 2-hydroxyoestradiol (20 μM, 15 min) by partially purified soluble catechol-*O*-methyltransferase was inhibited by 6-hydroxydopamine (400 mg litre^{-1}) by 95% and 51% in the absence and presence of antioxidant, respectively. These results suggest that the inhibition of catechol-*O*-methyltransferase by 6-hydroxydopamine reported for the purified enzyme, applies to the intact tissue and that the inhibition is dependent on oxidation of the 6-hydroxydopamine. Subsequent experiments using dialysis techniques demonstrated that the inhibition of soluble catechol-*O*-methyltransferase by 6-hydroxydopamine was irreversible in part, but the degree of irreversibility was influenced by pH and by the extent of in-vitro oxidation of 6-hydroxydopamine.

Since the discovery of its unique ability to destroy selectively adrenergic neurons (Tranzer & Thoenen 1967, 1968), 6-hydroxydopamine (6-OHDA) has become a widely used experimental tool for the in-vivo (Malmfors & Sachs 1968; Jonsson et al 1972; Finch et al 1973; Levin & Wilson 1981) and in-vitro (Aprigliano & Hermsmeyer 1976; Purdy et al 1981; O'Donnell & Reid 1984) destruction of sympathetic neurons. In the present study 6-OHDA was used in-vitro to examine the effect of sympathetic denervation on the *O*-methylation of catechol oestrogens (the major metabolites of oestrogens) by rabbit isolated thoracic aorta.

We found initially that the in-vitro exposure of the tissue to 6-OHDA caused a decrease in the *O*-methylation of catechol oestrogens, which appeared inconsistent with previous reports since the enzyme responsible for *O*-methylation, catechol-*O*-methyltransferase (EC 2.1.1.6) (COMT), is considered to be located exclusively in extraneuronal sites (rabbit aorta: Levin & Wilson 1977, 1981; Henseling et al 1978; rat heart: Fiebig & Trendelenburg 1978; dog saphenous vein: Paiva & Guimaraes 1978; Brandao et al 1980). In view of these reports, it was considered unlikely that the *O*-methylation of the catechol oestrogens was occurring in adrenergic nerves in the rabbit aorta.

Two possible explanations were proposed for the inhibition of *O*-methylation by 6-OHDA. Firstly, 6-OHDA could have prevented the entry of catechol oestrogens into cells, thereby inhibiting subsequent *O*-methylation of the catechol oestrogens. This seems unlikely, however, since the uptake of catechol oestrogens into extraneuronal cells is independent of known inhibitors of catecholamine uptake (Reid et al 1984a, b). This observation suggests that catechol oestrogens enter cells by passive diffusion. Furthermore, 6-OHDA has been shown to have no influence on the uptake of catecholamines into smooth muscle cells (O'Donnell & Reid 1984). Therefore, it seemed unlikely that 6-OHDA would prevent catechol oestrogens from gaining access to extraneuronal sites of *O*-methylation.

The second explanation considered was that 6-OHDA could produce a direct inhibition of COMT. This alternative seemed more likely since 6-OHDA has been reported to be a COMT inhibitor in studies conducted with partially purified enzyme (Borchardt 1975). Moreover, 6-OHDA has been reported to accumulate in extraneuronal sites, as demonstrated in the mouse atria (Jonsson & Sachs 1971), and therefore could interact with COMT

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located in extraneuronal cells. The experiments described in this paper explored the latter possibility.

METHODS

Tissue experiments

Male, new Zealand white rabbits (3–4 kg) were killed by stunning with a blow to the head followed by exsanguination. The entire length of the descending thoracic aorta was removed and cleared of blood and adhering lipids. The aorta was cut longitudinally and the endothelial cells removed by rubbing the intimal surface of the artery with a wooden applicator stick (Furchgott & Zawadzki 1980). The vessel was then cut into the appropriate number of segments. When required, isolated media or isolated adventitia was obtained by the peeling technique described by Maxwell et al (1968). Each segment was placed in a physiological solution (mM: NaCl 117, KCl 4·8, KH_2PO_4 1·2, $MgSO_4$ 1·2, $CaCl_2$ 2·5, $NaHCO_3$ 25, glucose 5·5, EDTA 0·03). All solutions were aerated with 5% CO_2 in O_2 at 37 °C throughout the incubation procedure, except during the incubation with 6-OHDA.

Tissues were exposed to 6-OHDA in-vitro by the procedure described by Aprigliano & Hermsmeyer (1976) and modified by O'Donnell & Reid (1984). Aortic segments were incubated in 400 mg litre^{-1} 6-OHDA for 10 min at 37 °C. To prevent oxidation of the 6-OHDA, the Krebs solution was not aerated and was slightly modified in that it contained the antioxidant, ascorbic acid 0·57 mM, and no $NaHCO_3$. Tissues were then transferred to two changes of normal aerated Krebs solution for a total time of 180 min.

Following the denervation procedure with 6-OHDA, tissue segments were preincubated in Krebs solution for 30 min, and then incubated in Krebs solution in the absence (controls) or presence of 2-hydroxyoestradiol (2-OHE_2) 5 μM for 60 min. Both the preincubation and incubation solutions contained ascorbic acid (0·57 mM) as an antioxidant.

Experiments with soluble COMT

Soluble COMT was isolated and partially purified from rat liver according to Sole & Hussian (1977). The specified amount of soluble COMT was incubated in 0·005 M K_2HPO_4 (pH 7·4) containing 20 μM 2-OHE_2 in the absence or presence of 400 mg litre^{-1} 6-OHDA at 37 °C for 15 min. In addition, COMT was incubated in K_2HPO_4 buffer in the absence of the substrate, 2-OHE_2. The incubation solution also contained 50 mM $MgCl_2$ and 100 μM *S*-adenosylmethionine, and in some experiments the antioxidant, dithiothreitol (1 mM).

Dialysis experiments

Partially purified soluble COMT isolated from rat liver (approximately 0·14 mg protein) was incubated in modified Krebs solution (see previous section, Tissue experiments) at 37 °C for 10 min in the absence or presence of 400 mg litre^{-1} 6-OHDA. This solution was then dialysed against two changes, each of 4 litres, of 0·005 M K_2HPO_4 for a total time of approximately 20 h at 4 °C. The original K_2HPO_4 was at either pH 7·4 or 9·0 and was substituted with fresh 0·005 M K_2HPO_4 (pH 7·4) after either 7 or 17 h. Following dialysis, the dialysate was assayed for COMT activity using 2-OHE_2 as substrate, i.e., 500 μl of dialysate was added to 0·005 M K_2HPO_4 (pH 7·4) containing 50 mM $MgCl_2$, 100 M *S*-adenosylmethionine and 1 mM dithiothreitol, in the absence or presence of 20 μM 2-OHE_2, and incubated for 15 min at 37 °C.

Post-incubation extraction and HPLC assay

The extraction and assay procedures outlined by Reid et al (1985) for *O*-methylated catechol oestrogens were followed. Briefly, 2-methoxyoestradiol (2-$MeOE_2$) was extracted from the incubation solution with chloroform–ethyl acetate (75:25) and, where appropriate, from tissue with chloroform. The organic solvents were evaporated to dryness under N_2, and the residue was redissolved in acidified ethanol (95% ethanol containing 0·01 M HCl). [^{3}H]Oestradiol was used as internal standard. Samples were assayed by high performance liquid chromatography with electrochemical detection, using a 5 μM reverse phase C_{18} radial compression module cartridge. An isocratic mobile phase of 0·1 M ammonium phosphate combined with 50-70% acetonitrile was used and delivered at a flow rate of 1·5 ml min^{-1}. The working electrode potential was set at 0·74 V.

Protein measurement

The protein content of the partially purified COMT was measured by the method of Bradford (1976) with bovine serum albumin as standard.

Materials

S-Adenosyl-1-methionine chloride, 6-hydroxydopamine hydrobromide, 2-hydroxyoestradiol and 2-methoxyoestradiol were obtained from Sigma Chemical Co. (St Louis, MO, USA). Oestradiol [6,7-^{3}H(N)] (40–60 Ci mmol^{-1}) was purchased from New England Nuclear (Boston, MA, USA). The

dialyser tubing was No. 3787-D22 from Arthur H. Thomas Co. (Philadelphia, PA, USA) and had a molecular weight cutoff of 12 000.

RESULTS

Tissue experiments

The pre-exposure of tissues to 6-OHDA resulted in a large reduction in the *O*-methylation of 2-OHE_2 by the intact rabbit aorta. When either the media or adventitia was isolated from the aorta, the amount of *O*-methylation occurring in these tissues was also reduced by 6-OHDA pretreatment (Fig. 1).

Experiments with soluble COMT

Before the effect of 6-OHDA on the *O*-methylation of 2-OHE_2 by partially purified soluble COMT could be examined, it was necessary to establish the appropriate amount of soluble COMT (or amount of protein) to use in the experiments. This must be linearly related to COMT activity (or the amount of product formed). When varying amounts of COMT were incubated with 20 μM 2-OHE_2 for 15 min, it was found that the amount of 2-$MeOE_2$ formed was linearly related to protein weight up to at least 0·14 mg protein (unpublished results). An amount of 0·07 mg protein was selected for use in the subsequent experiments with soluble COMT.

Fig. 2 illustrates that the exposure of partially purified soluble COMT to 400 mg $litre^{-1}$ 6-OHDA

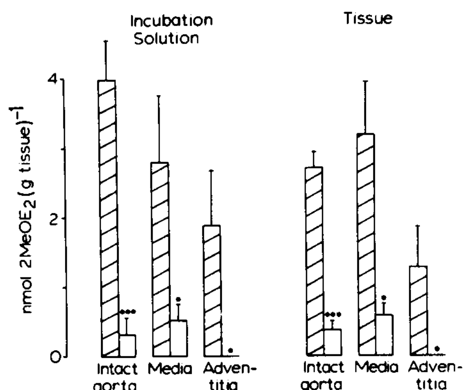

FIG. 1. The effect of 6-OHDA on the *O*-methylation of 2-OHE_2 by the intact aorta, the isolated media and the isolated adventitia from rabbit thoracic aorta. Tissues were pre-exposed to a modified Krebs solution in the absence (shaded bars) or presence (open bars) of 400 mg $litre^{-1}$ 6-OHDA for 10 min at 37 °C. Tissues were then placed in Krebs solution for 3 h, and incubated in 2-OHE_2 5 μM for 1 h at 37 °C. The abcissa represents nmol of 2-$MeOE_2$ formed g^{-1} of tissue in the Krebs solution and in the tissue. Values are mean ± s.e. from tissues from 6 rabbits. Values from tissues exposed to 6-OHDA which are significantly less than those for NO 6-OHDA are indicated by asterisks (paired *t*-test): * $0.05 > P > 0.01$; ***$P < 0.001$.

in the presence of the antioxidant, dithiothreitol, caused a significant reduction in the *O*-methylation of 2-OHE_2. The reduction was more marked in the absence of dithiothreitol (Fig. 2).

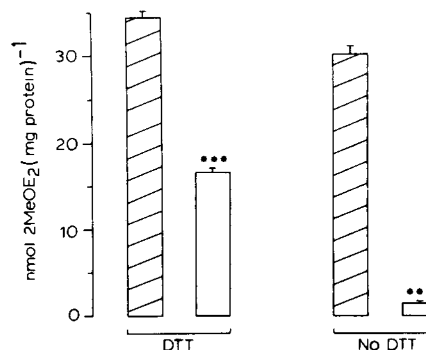

FIG. 2. The effect of 6-OHDA on the *O*-methylation of 2-OHE_2 by partially purified soluble COMT. A constant volume of soluble COMT was incubated in 0·005 M K_2HPO_4 containing 2-OHE_2 20 μM for 15 min at 37 °C in the absence (shaded bars) or presence (open bars) of 400 mg $litre^{-1}$ 6-OHDA. Some experiments were carried out in the presence of 1 mM dithiothreitol (DTT). The abcissa represents nmol of 2-$MeOE_2$ formed mg^{-1} of protein. Values are mean ± s.e. from 5 or 8 experiments. Values for 6-OHDA which are significantly less than those for NO 6-OHDA are indicated by asterisks: ***$P < 0.001$.

Dialysis experiments

Partially purified soluble COMT was exposed to 6-OHDA, separated from the 6-OHDA by dialysis, and then assayed for COMT activity using 2-OHE_2 as substrate. The pre-exposure of soluble COMT to 6-OHDA caused a significant decrease in the *O*-methylation of 2-OHE_2 (Fig. 3). The reduction in *O*-methylation was influenced by two variables (Fig. 3). Firstly, the inhibition increased when the pH of the first dialyzing solution (see Experimental Procedures for details) was raised from 7·4 (21·8% inhibition) to 9·0 (33·6% inhibition). Secondly, the reduction in *O*-methylation was markedly greater when the first dialysing solution was replaced after 17 h (81·8% inhibition) rather than after only 7 h (33·6% inhibition).

To confirm that the concentration of 6-OHDA was diluted to negligible proportions during dialysis in K_2HPO_4, the dialysing solutions were incubated with 2-OHE_2 (20 μM) and soluble COMT for 15 min following dialysis. The *O*-methylation of 2-OHE_2 was not influenced by the presence of any of the dialysing solutions.

DISCUSSION

It remains undisputed that 6-OHDA is a very selective agent for the production of experimentally

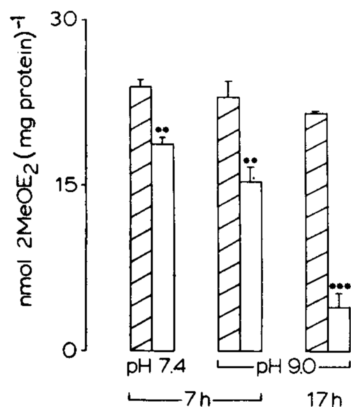

FIG. 3. The effect of pre-exposure to 6-OHDA on the O-methylation of 2-OHE_2 by partially purified soluble COMT. Soluble COMT was incubated in modified Krebs solution in the absence (shaded bars) or presence (open bars) of 400 mg $litre^{-1}$ 6-OHDA for 10 min at 37 °C. The solution was dialysed against 2 changes of 0·005 M K_2HPO_4 for a total time of 20 h at 4 °C. The original K_2HPO_4 was at either pH 7·4 or 9·0, and was substituted with fresh 0·005 M K_2HPO_4 after 7 h or after 17 h, as specified. The dialysate was then incubated with 20 μM 2-OHE_2 for 15 min at 37 °C, and the amount of 2-$MeOE_2$ present per mg of protein was determined. Values are mean ± s.e. from 4 experiments. Values for 6-OHDA which are significantly different from those for NO 6-OHDA are indicated by asterisks: ** $0{\cdot}01 > P > 0{\cdot}001$; ***$P < 0{\cdot}001$.

induced degeneration of adrenergic neurons. The high degree of selectivity it displays is based on the principle that it is concentrated in adrenergic nerves by the specific transport mechanism for catecholamines, neuronal uptake (Tranzer & Thoenen 1967, 1968; Thoenen & Tranzer 1968; Jonsson 1971; Ljungdahl et al 1971). On the other hand, 6-OHDA is generally a highly cytotoxic substance (Sachs & Jonsson 1975), and the present study uncovers a toxic effect of 6-OHDA in intact tissue unrelated to denervation—that of the inhibition of COMT, an enzyme required for the metabolism of the neurotransmitter, noradrenaline, as well as many other catechol-containing compounds.

We found initially that the pre-exposure of tissues to 6-OHDA in-vitro inhibited the O-methylation of the catechol oestrogen, 2-OHE_2, by the rabbit aorta. However, the inhibition was equally as marked in the isolated media from rabbit aorta, which does not contain adrenergic nerves (Maxwell et al 1968). Thus, the observed reduction in O-methylation did not appear to be the result of sympathetic denervation by 6-OHDA. In subsequent experiments, using partially purified soluble COMT, the O-methylation of 2-OHE_2 was still reduced by 6-OHDA, demonstrating that the enzyme was directly inhibited. The latter confirm the original findings of Borchardt (1975) which also strongly indicate that 6-OHDA inactivates partially purified COMT. Since 6-OHDA is highly susceptible to oxidation, the antioxidant, dithiothreitol, was present in most experiments. When the antioxidant was not included, however, the inhibition of O-methylation was even more marked, suggesting that the oxidized form of 6-OHDA was responsible for the inhibition.

The hypothesis that oxidized 6-OHDA is the active inhibitory compound is a feasible proposition, in light of the fact that the two main theories on the degenerative neuronal action are both associated with the susceptibility of 6-OHDA to oxidation (Sachs & Jonsson 1975). Firstly, the oxidation results in the formation of highly reactive quinones, which may undergo covalent, irreversible binding to nucleophilic groups of proteins. Such a binding would cause denaturation of molecules of vital importance to the integrity of the neuron (Adams et al 1972; Jonsson 1976). Alternatively, hydrogen peroxide and superoxide and hydroxyl radicals formed during oxidation of 6-OHDA may be responsible for nerve degeneration (Heikkila & Cohen 1972, 1973). The finding that 6-hydroxydopamine p-quinone was as effective an inhibitor of COMT as was 6-OHDA strongly suggests that the quinone or its cyclization product is responsible for the inhibition (Borchardt 1975).

Dialysis techniques were used to investigate whether the inhibition of COMT by 6-OHDA was irreversible. In these experiments, soluble COMT was exposed to 6-OHDA, separated from it by dialysis, and then incubated with 2-OHE_2. Since this pre-exposure still caused a decrease in the O-methylation of 2-OHE_2, the mechanism of inhibition was shown to be irreversible, at least in part. The percentage inhibition, and therefore the degree of irreversibility, increased when the dialysing solution was replaced after 17 h rather than only 7 h. It is possible that this increase was due to a loss in effectiveness of the antioxidant, dithiothreitol, in the first dialysing solution somewhere between 7 and 17 h. This postulation supports the previous suggestion that the inhibition of O-methylation is dependent on the oxidation of the 6-OHDA none of which would occur in the reduced antioxidant concentration. Since more oxidized 6-OHDA would now be present, the inhibition would be more marked.

The degree of irreversibility was marginally increased when the pH of the first dialysing solution was raised from 7·4 to 9·0, indicating a slight dependence of the inhibition on pH. Once again, this

could be related to the state of oxidation of the 6-OHDA, since it oxidizes much more rapidly in alkaline conditions (Aprigliano & Hermsmeyer 1976). Thus, at pH 9·0, 6-OHDA would be oxidized at a greater rate than at pH 7·4, and accordingly would have a higher capacity to inhibit COMT. Our results with intact tissue are in accord with the findings of previous studies conducted with partially purified COMT which suggest that 6-OHDA irreversibly inactivates the enzyme and that this inactivation could be prevented with antioxidants.

Finally, our demonstration that acute treatment of an intact tissue with 6-OHDA produces an inhibition of COMT is of significance. Noradrenergic denervation procedures including the use of 6-OHDA, are often used to establish either the neuronal or extraneuronal location of enzymes involved in catecholamine synthesis and degradation. It follows that the use of a chemical denervating agent may have effects beyond neuronal destruction, that is, at extraneuronal enzymatic sites. It must be realized that the non-neuronal effects of 6-OHDA may, under appropriate conditions, markedly influence the interpretation of data derived from studies employing chemically-induced sympathectomy.

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