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MODULATION OF ALPHA-METHYLDOPA BINDING TO THE ERYTHROCYTE MEMBRANE BY SUPEROXIDE DISMUTASE

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Summary

The anti-hypertensive drug alpha-methyldopa causes anti-erythrocyte antibodies in approximately 20% of patients. Although very little drug is bound to the membrane on incubation with the intact cell, this binding increases approximately fifty-fold when superoxide dismutase is inhibited by diethyldithiocarbamate. Solubilization of the membranes with sodium dodecyl sulfate and gel filtration in Bio-Gel A5M indicate that the major portion of the bound drug chromatographs with a peak of approximately 20,000 daltons. This peak contains three peptides, one of which is globin, but the other two contain the major portion of the radioactivity. This interaction may be the first step in the production of the drug-related erythrocyte auto-immunity.

Introduction

The occurrence of Coomb's positivity in patients on long-term treatment with alpha-methyldopa and the absence of any drug-related reactivity of the antibodies bound to the cells constitute an intriguing problem in the field of immunopathogenesis (1). We recently reported that alpha-methyldopa binds to erythrocyte membrane peptides when incubated with isolated membranes. This binding was found to be very tight, probably covalent,but not very selective, and was very significantly depressed in the presence of reducing agents. Hemoglobin, to which the drug was also bound, was found to compete with membrane peptides for alpha-methyldopa binding (2).

In the present study, we explored the interrelationship between alpha-methyldopa binding to the erythrocyte membrane and superoxide dismutase (SOD, EC 1.15.1.1.) all at the intact cell level at the time of incubation. We also pursued the interrelationship between superoxide dismutase and alpha-methyldopa auto-oxidation and found that auto-oxidation of the drug was inhibited in the presence of the enzyme in the cell-free state. When superoxide dismutase activity in the intact erythrocyte was inhibited, significant amounts of alpha-

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methyldopa bound. Evidence was found of selectivity of the membrane peptides which bound the drug under these circumstances.

Materials and Methods

The general methodology was as previously described (2). In the present studies, the drug was incubated in phosphate-buffered saline pH 7.4 with intact cells freshly obtained. All incubations were carried out at 37°C with oxygen slowly bubbling unless otherwise stated. Auto-oxidation and binding reactions were studied at 0.05 M phosphate pH 7.4 and 10^{-4}M EDTA at 25°C. Auto-oxidation of alpha-methyldopa was studied by first determining that a peak, representing the first oxidation product, developed at 310 nm and then following the appearance of this peak with time. The effect of bovine erythrocyte superoxide dismutase (Sigma Chemical Co.) on the auto-oxidation of the drug was monitored by measuring the absorption at 310 nm in the presence of enzyme at 250 ng/ml. [^{3}H]alpha-methyldopa binding to superoxide dismutase was studied by incubation of drug (1 µM, kindly donated by Merck, Sharp and Dohme and rechecked for radiochemical purity) with enzyme (2.8 µg/ml) for 2 hours. After dialysis to remove unbound drug, superoxide dismutase activity was measured according to the method of Heikkila and Cabbat (3).

Fresh erythrocytes were first incubated with diethyldithiocarbamate, a copper chelater and a known inhibitor of superoxide dismutase (4), washed thoroughly with phosphate-buffered saline and then incubated with [^{3}H]alpha-methyldopa (5 µM). After further washing of the cells, the latter were lysed and membranes separated by the method of Dodge et al (5), lyophilized and counted. Superoxide dismutase activity was measured after hemoglobin precipitation according to the method of Winterbourn et al (6). Catalase activity was measured by the method of Luck (7). Other inhibitors of both enzymes such as cyanide and azide at 5 mM final concentrations were similarly studied in the above system. Hemoglobin was determined using Drabeim's reagent (8). Solubilized membranes from diethyldithiocarbamate-treated intact cells were studied further using gel filtration Bio-Gel A5M (200-400 mesh) columns in sodium dodecyl sulfate together wtih appropriate molecular size markers as previously reported (2). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out by the method of Fairbanks et al (9).

Results and Discussion

Since the previous investigations had suggested a strong link between oxidation state and drug binding, it was decided to see whether the enzyme superoxide dismutase would affect the auto-oxidation of the drug in a cell-free system. The enzyme clearly suppressed the auto-oxidation of alpha-methyldopa (Figure 1). The rate of auto-oxidation of the drug was considerably slower than that of the substrate 6-hydroxy-dopamine used in the enzyme assay.

Experiments were undertaken to study the binding of alpha-methyldopa to the membrane peptides in the presence and absence of the superoxide dimutase inhibitor diethyldithiocarbamate at the intact cell level (Figure 2). With slightly more than 60% suppression of superoxide dismutase there was beginning increase in alpha-methyldopa binding. At over 80% inhibition there was marked increase

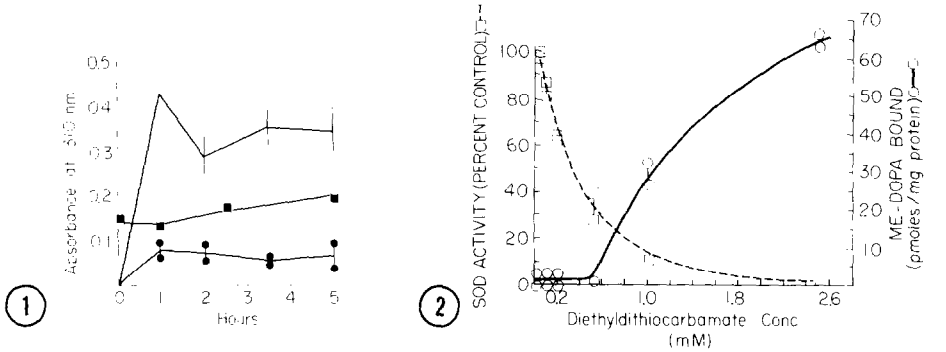

Figure 1. Suppression of auto-oxidation of alpha-methyldopa by bovine erythrocyte superoxide dismutase. The absorbance at 310 nm is a measure of auto-oxidation and in the presence of the enzyme (closed circles) is largely suppressed compared with the absence of enzyme (open circles). The diacetate derivative of the drug, in which auto-oxidation is prevented, shows no difference in absorbance in the presence (closed squares) and the absence (open squares) of the enzyme.

Figure 2. Inhibition of superoxide dismutase activity in the intact cell by diethyldiothiocarbamate together with increase in [^{3}H]alpha methyldopa bound per mg membrane protein.

in binding. There was also increase in drug binding to hemoglobin with the enzyme inhibition (data not shown). Catalase did not appear to have a significant role here and its activity increased after diethyldithiocarbamate treatment.

Conclusive evidence of the nature of the chemical species responsible for the increased drug binding could not be adduced using superoxide anion "scavengers." Penicillamine (2.5 mM) did reverse the increase but it could have operated as a non-specific reducing agent. To explore the use of subhuman primates having membrane antigens similar to the human Rh system (10) as possible models for methyldopa-induced auto-immune anemia, we examined drug binding to the erythrocytes of the baboon, rhesus monkey and chimpanzee. Although there was a dramatic increase in drug bound in all three when SOD was inhibited by diethyldithiocarbamate, the amount of drug bound without the inhibitor varied with the native SOD concentration. For example, the enzyme activity in the rhesus monkey was less than one-half that of the chimpanzee (or man) and over five times more drug bound to the membranes when incubated with the intact cells of the monkey.

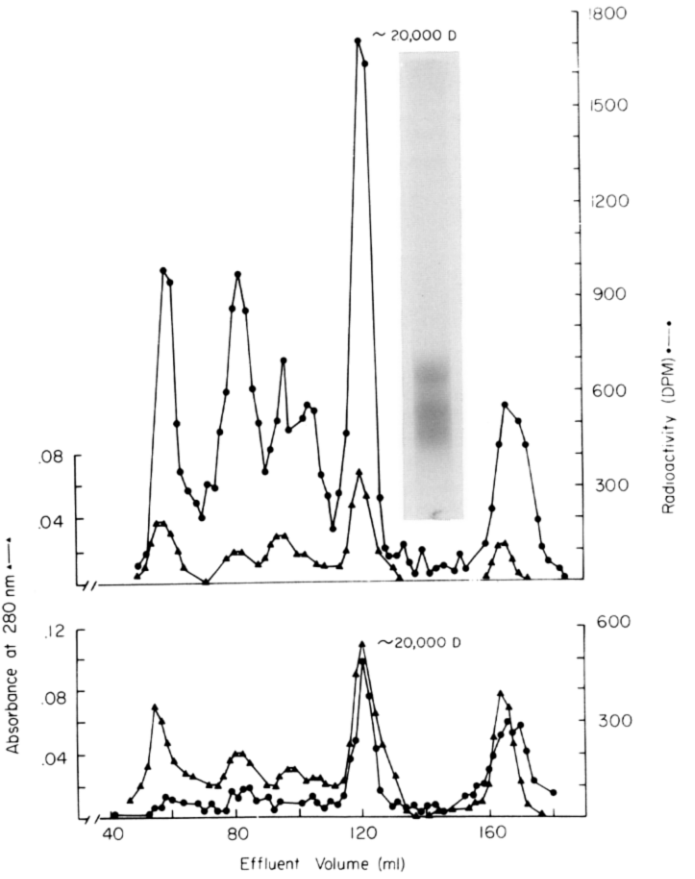

Figure 3. Gel filtration of erythrocyte membranes on Bio-Gel A5M. Upper panel: labelled methyldopa reacted with isolated membranes. Absorbance at 280 nm (closed triangles) and radioactivity (closed circles). Multiple labelled peptide peaks are seen. Lower panel: intact cells are reacted with the superoxide dismutase inhibitor, diethyldithiocarbamate (2.0 mM), washed, then labelled drug added. Membranes are prepared and solubilized. Absorbance at 280 nm (closed triangles), radioactivity (closed circles). A single labelled peptide peak predominates in the 20,000 molecular weight region. The peak at the extreme right is unbound drug. Insert: polyacrylamide gel electrophoresis showing that the peak contains three peptides.

When the labelled drug was incubated with intact human cells, the membranes recovered, solubilized and examined for distribution of proteins and radioactivity by gel filtration on Bio-Gel A5M columns, no significant radioactivity was observed above background level. However, when the cells had been previously treated with diethyldithiocarbamate, most of the peptide-associated radioactivity was observed in an apparently single peak of 20,000 dalton molecular weight range (Figure 3, lower panel). This differed from the non-select-

ivity of the binding of labelled alpha-methyldopa where the drug was incubated with isolated membranes. When the 20,000 dalton peak was examined by polyacrylamide gel electrophoresis, three bands were observed (insert to Figure 3). One of these was globin to which only a small amount of radioactivity was bound. The major portion of radioactivity was localized to the two unidentified bands of molecular weight 20,000 and 25,000.

Total inhibition of superoxide dismutase activity is a circumstance very unlikely to occur in vivo. However, it does point out the important role at the whole cell level for the enzyme in preventing auto-oxidation and binding of the drug. The superoxide anion or one of its related species formed during auto-oxidation somehow promoted the binding of the drug or one of its oxidative products to the erythrocyte membrane peptides. One possibility is a co-oxidation reaction as a result of hemoglobin auto-oxidation (11). It is likely that the hemoglobin acts as a "sink" protecting the membrane from drug interaction but it in turn as well as the membrane peptides are protected from drug interaction by the normal activity of superoxide dismutase. This important ubiquitous enzyme, which has received much attention since its initial description by McCord and Fridovich (12), is now known to have two forms, at least one of which is present in all human cells (13). Several different phenotypes have also been described (14). Although only limited fluctuation of the enzyme activity in diseased state has so far been found (15), the degree of suppression of activity probably need only be partial for increased binding of the drug to occur to the membrane peptides in the intact cell state especially over a prolonged period. Furthermore, as increasing complexities in superoxide anion chemistry continue to emerge, other drugs, normal metabolites, or disease states may be found to influence superoxide anion production and disposal, subsequent methyldopa binding, and the emergence of erythrocyte auto-sensitization.

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