CYTOCHROME P-450 AND 18-OXYGENASE SYSTEM FROM BEEF ADRENOCORTICAL MITOCHONDRIA - INHIBITORY EFFECT OF PUROMYCIN

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SUMMARY: Puromycin at a concentration of 5 X 10^{-4}M, inhibited 44% of the transformation of corticosterone to aldosterone in beef adrenocortical mitochondrial preparations. Puromycin also induced a difference spectra when added to mitochondrial cytochrome P-450, presenting a maximum at about 420 nm and a minimum at 390 nm. Competitive binding of puromycin with corticosterone on cytochrome P-450 was demonstrated. Attention therefore must be paid in using puromycin as a protein synthesis inhibitor in studying corticosteroid biosynthesis since it may also interfere directly with cytochrome P-450 which is involved in various hydroxylating systems.

Puromycin has been reported to inhibit the conversion of corticosterone to aldosterone in stimulated and unstimulated beef adrenal outer slices (1) and in rat adrenals (2, 3). It has been observed that chloramphenicol, a specific inhibitor of mitochondrial protein synthesis, had no effect on the conversion of corticosterone to aldosterone in the presence of NADPH. In mitochondrial fractions incubated with NADPH or with Krebs cycle intermediates, puromycin inhibited the conversion of corticosterone to aldosterone (2). These results suggest that the inhibitory effect of puromycin might be related at least in part to a direct action on the steroid 18-oxygenase system. The present article deals with *in vitro* experiments on mitochondrial preparations from beef adrenocortical glomerulosa rich fraction to study the effect of puromycin on the conversion of corticosterone to aldosterone; and on the binding of corticosterone to cytochrome P-450 which is known to be a component of the 18-oxygenase system (4,5,6).

Materials and Methods

Mitochondrial preparation and incubation

Mitochondrial preparations were obtained from a glomerulosa rich fraction of beef adrenals by the centrifugation technique of Schneider and Hogeboom (7). Mitochondrial fractions equivalent to 2.01 mg protein per ml were incubated with 1 uCi of tritiated corticosterone and varying amounts of non radioactive corticosterone at 37°C for 20 minutes in the presence of a NADPH generating system (4). The inhibitory effect of puromycin at a preselected concentration of 5×10^{-4}M was compared to controls. Incubation medium contained $MgCl_2$ (8.5×10^{-3}M), $CaCl_2$ (2.7×10^{-3}M), KCl (3.13×10^{-3}M), NaCl (7.59×10^{-3}M) and bovine serum albumin (2%). The pH was adjusted to 7.0 with 0.01 M potassium phosphate.

Isolation of ^{3}H-aldosterone formed

Known amounts of ^{14}C-aldosterone were added to incubation media prior to the extraction to monitor losses and the tritiated aldosterone formed was isolated by a previously described procedure (8, 9, 10). The identification criteria of Sandor and Idler (11) were used to confirm the identity of radioactive aldosterone formed during incubation i.e. when three consecutive identification steps (derivative formation and paper chromatography) yield (^{3}H/^{14}C) ratios with a coefficient of variation not greater than ± 5%.

Radioactivity

^{3}H-corticosterone (specific activity 50 Ci/mM) and ^{14}C-aldosterone (specific activity 55 mCi/mM) were purchased from New England Nuclear Co. and purified by paper partition chromatography before use. Simultaneous counting of ^{14}-carbon and tritium was performed in a liquid scintillation counter (Nuclear Chicago Mark I) equipped with an external standard. The counting error was kept at ± 1% by the accumulation of 1×10^{4} net counts.

Spectrophotometric studies

Spectrophotometric analysis of the binding of corticosterone and puromycin to cytochrome P-450 were performed as previously described (9) with a Unicam SP-800 recording spectrophotometer at 0°. Induced difference spectra were recorded by scanning between 370 and 500 nm.

Proteins were determined by the Lowry technique (12) using bovine serum albumin as a standard.

Trivial names used: aldosterone = 11β, 21-dihydroxy-4-pregne-3, 20-dione-18-al; corticosterone: 11β, 21-dihydroxy-4-pregne-3, 20 dione; puromycin: 3'-(α-amino-p-methoxyhydrocinnamamido)-3'-deoxy-N,N-dimethyl-adenosine.

Results

Transformation of ^{3}H-corticosterone to ^{3}H-aldosterone

The mitochondrial fractions from beef glomerulosa rich adrenals incubated in the presence of ^{3}H-corticosterone and a NADPH generating system synthetized ^{3}H-aldosterone. This product was identified by the criteria mentionned in the materials and methods section. As reported previously (13) and under the conditions used, aldosterone formation was linear with time for 60 minutes and with increasing concentrations of mitochondria to 2.75 mg protein per ml of incubation medium.

In a further series of experiments mitochondrial preparations were incubated in the presence of various corticosterone concentrations with or without puromycin (5×10^{-4}M). Figure 1 shows that puromycin, when present, partly inhibited the transformation of ^{3}H-corticosterone to ^{3}H-aldosterone. The double reciprocal plot of these data produced an upward curvature in both cases (curves not shown).

Spectral studies

Since it is thought that puromycin might have a direct inhibitory action at the 18-oxygenase level, its possible interaction with mitochondrial

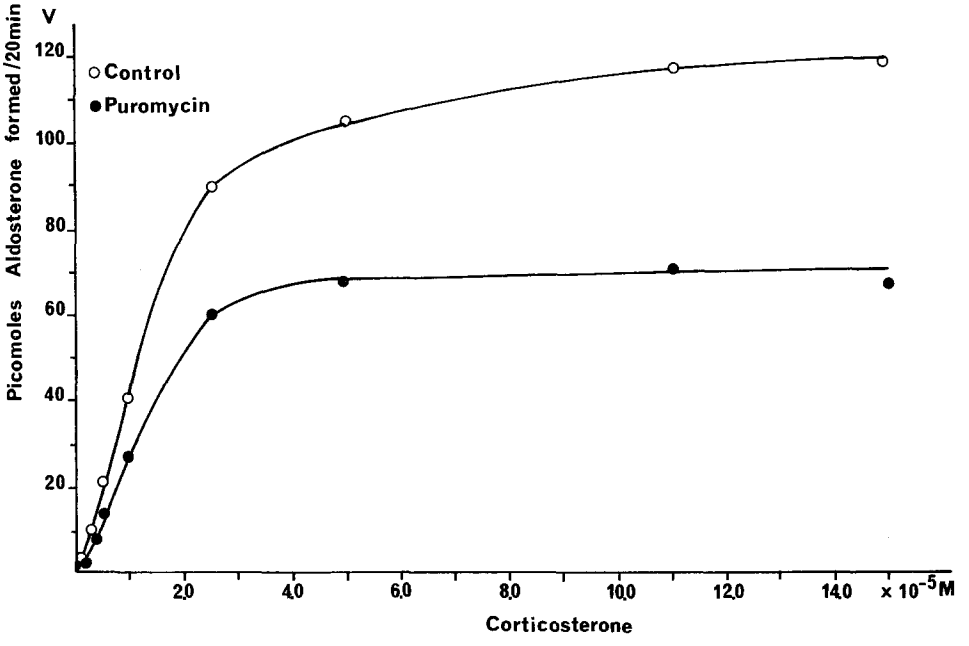

Figure 1

Effect of substrate (corticosterone) concentration on the formation of aldosterone in the presence or in the absence of 5 X 10^{-4}M puromycin. Mitochondria equivalent to 2.01 mg protein/ml were incubated with ^{3}H-corticosterone (1 μCi) and varying amounts of non radioactive corticosterone for 20 minutes in air with a NADPH generating system. PH was maintained at 7.0 with 0.01 M potassium phosphate.

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cytochrome P-450 was investigated. A mitochondrial suspension was equally distributed into two spectrophotometer cuvettes; 1.4 X 10^{-3}M puromycin was added to the sample cuvette, and an equal volume of buffer was added to the reference cuvette. Figure 2 shows the results obtained on scanning between 370 and 500 nm. The difference spectrum obtained has a trough at 390 nm and a maximum in the 420 nm area. Chloramphenicol and cycloheximide, two other protein synthesis inhibitors, did not induce a difference spectrum under similar conditions. Another series of experiments was per-

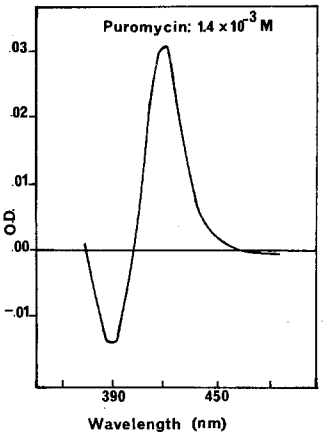

Figure 2

Puromycin difference spectra of cytochrome P-450. Mitochondria were suspended in Tris-HCl buffer 0.05 M, pH 7.0 at a protein concentration of 1.2 mg/ml and divided into two cuvettes. The sample cuvette contained 1.4 X 10^{-3}M puromycin.

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formed to study the saturation of cytochrome P-450 binding sites. Increasing amounts of puromycin were added to the sample cuvette containing a cytochrome P-450 preparation and difference spectra were recorded. The difference in optical density between 420 and 390 nm was used to evaluate the binding of puromycin to cytochrome P-450 (Fig. 3). The addition of corticosterone to mitochondrial cytochrome P-450 provoked a diminution in the total absorption induced by the presence of puromycin. Corticosterone (3.50 X 10^{-4}M and 6.25 X 10^{-4}M) added to cytochrome P-450 preparation in the sample and the reference cuvettes before the addition of puromycin provoked a diminution of the binding value. The spectrum induction effect produced by puromycin was completely inhibited by 14 X 10^{-4}M corticosterone (results not shown).

Discussion

The present study shows that puromycin significantly decreases the

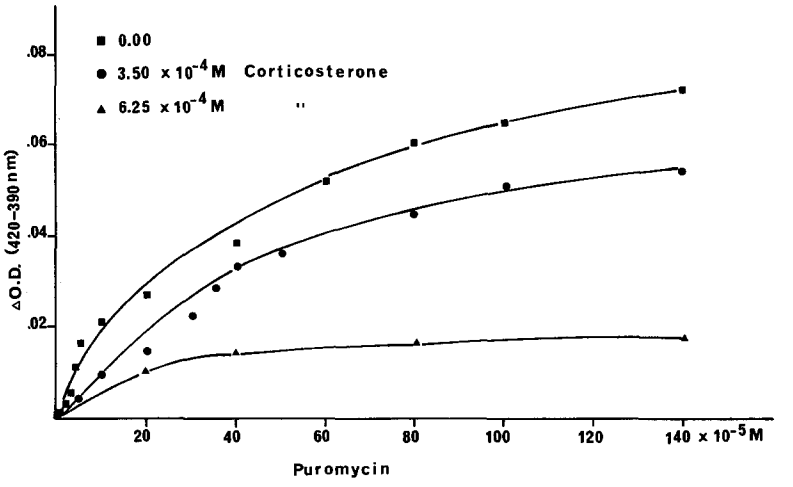

Figure 3

Inhibition of puromycin binding to cytochrome P-450 in the presence of corticosterone. Beef adrenal mitochondrial fraction was prepared in Tris-HCl buffer 0.05 M, pH 7.0 at a protein concentration of 1.2 mg/ml. Difference in optical density between 420 nm and 390 nm was recorded at 0°C and 5 minutes after each addition of puromycin.

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in vitro transformation of corticosterone to aldosterone by beef adrenal mitochondrial preparations. These results are in good agreement with reports of previous studies (1, 2, 3, 9).

Spectrophotometric studies revealed that puromycin can interact with mitochondrial cytochrome P-450, inducing a difference spectra similar to that produced by the binding of corticosterone to beef adrenocortical mitochondrial cytochrome P-450 (13). Corticosterone inhibited the spectral effect of puromycin as shown in Figure 3 and the degree of inhibition was directly proportional to the corticosterone concentration. At 14 X 10^{-4}M, corticosterone completely abolished the induction spectrum produced by the interaction of puromycin and cytochrome P-450. These results also indicate corticosterone bound to cytochrome P-450 is not readily displaced by puromycin.

The results obtained in this study show that puromycin may play a direct inhibitory role at the cytochrome P-450 level in interfering with the binding of corticosterone. Attention must therefore be paid in using puromycin as a protein synthesis inhibitor in the study of steroid biosynthesis since cytochrome P-450 appears to be involved in many steps of corticosteroid biosynthesis.

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References

1. Satura, T. and Kaplan, M. (1972) J. Clin. Invest., 51, 2246-2251.
2. Marusic, E.T. and Mulrow, P.J. (1972) Endocrinology, 91, 1125-1129.
3. Kittinger, G.W. (1964) Steroids, 4, 539-545.
4. Greengard, P., Psychoyos, S., Tallan, H.H., Cooper, D.Y., Rosenthal, O. and Estabrook, R.W. (1967) Arch. Biochem. Biophys., 121, 298-303.
5. Sandor, T. (1972) Steroid in Nonmammalian Vertebrates, pp. 253-327, (D.R. Idler, ed.) Academic Press, New York.
6. Sandor, T., Fazekas, A.G., Lehoux, J.-G., Leblanc, H. and Lanthier, A. (1972) J. Steroid Biochem., 3, 661-682.
7. Schneider, W.C. and Hogeboom, G.H. (1950) J. Biol. Chem., 183, 123-128.
8. Sandor, T. and Lanthier, A. (1963) Biochem. Biophys. Acta, 74, 756-762.
9. Lehoux, J.-G. and Forest, J.C. (1974) J. Steroid Biochem. 5, 00-00.
10. Lehoux, J.-G. (1974) Mol. Cell. Endocrinol., 2, 43-58.
11. Sandor, T. and Idler, D.R. (1972) Steroid in Nonmammalian Vertebrates, pp. 6-34 (Idler, D.R. ed.) Academic Press Inc., New York.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265-275.
13. Lehoux, J.G. and Forest, J.C. (1975 Biochem. Biophys. Res. Comm., 63, 84-91.