Vol. 92, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1980 Pages 155-162

5'-DEOXYPYRIDOXAL INHIBITION OF GLUCOCORTICOID RECEPTOR BINDING IN HeLa S_3 CELLS AND RAT THYMOCYTES

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Received November 19,1979

Summary

Recent evidence suggests a possible role for pyridoxal 5'-phosphate, the active physiological form of vitamin B_6, in steroid hormone action (1-3). We now report that 5'-deoxypyridoxal, a synthetic vitamin B_6 antagonist, causes a rapid and complete loss of dexamethasone receptor binding in cytosol preparations in whole HeLa S_3 cells or in rat thymocytes. This effect is concentration and time dependent, and is specific for 5'-deoxypyridoxal. In whole cell incubations of either HeLa S_3 cells or rat thymocytes at 37°C, a 50% reduction in [^{3}H]dexamethasone binding was observed in the presence of 0.25 mM 5'-deoxypyridoxal. Cytotoxicity was not evident in rat thymocytes or HeLa S_3 cells incubated with 3.0mM or 1.0mM 5'-deoxypyridoxal respectively. Pyridoxal 5'-phosphate, 5'-deoxypyridoxine and 5'-deoxypyridoxamine were ineffective in causing a loss of steroid receptor binding. These studies suggest that 5'-deoxypyridoxal may be an effective non-steroidal compound which can effect the binding of glucocorticoids to specific receptor proteins in whole cells.

Introduction

Steroid antagonists provide invaluable tools for probing the basic mechanism of hormone-receptor binding. In addition, they are of interest as potential chemotherapeutic agents, particularly in the treatment of hormone-dependent cancers. Clinically useful antagonists must exhibit high potency at low concentrations, little hormonal activity, and low toxicity (4). Non-steroidal antagonists are of particular interest, providing an alternative between large pharmacological doses of hormones and major ablative surgical procedures (5). Non-steroidal antagonists are known for estrogens, androgens and mineralocorticoids (6-9). Analogous non-steroidal glucocorticoid antagonists have not been fully developed.

0006-291X/80/010155-08$01.00/0

Evidence suggests that some hormone antagonists act by blocking the action of hormone at its receptor (7,10,11). We have demonstrated that 5'-deoxypyridoxal causes a rapid and complete loss of [^{3}H]dexamethasone from specific receptor proteins from two different cell types. Rat thymocytes were chosen, as they have well-characterized glucocorticoid receptors and have been previously employed in studies with cortexolone, a steroidal glucocorticoid antagonist (12,13). HeLa S_3 cells derived from a cervical carcinoma were chosen as a mammalian cell line, which contain high affinity saturable receptors which are specific for glucocorticoids (14). The effect of 5'-deoxypyridoxal was observed for cytoplasmic receptor in cytosol preparations at 2°C, and for nuclear receptor binding in whole cell preparations at 37°C. A possible role of 5'-deoxypyridoxal as a glucocorticoid antagonist is suggested.

Materials and Methods

Thymus tissue was obtained from male Sprague-Dawley rats which were adrenalectomized 4-6 days prior to sacrifice. Thymus cell suspensions were prepared in Krebs-Ringer bicarbonate buffer with 10 mM glucose, pH 7.4 (KRBG), equilibrated with 95% O_2/5% CO_2 (15). Cells were used at a cytocrit of 0.1-0.2 ml of packed cells per ml of cell suspension. The cytocrit was determined by the microhematocrit method.

HeLa S_3 cells were grown to confluency as monolayer cultures in Joklik's Minimum Essential Medium (Grand Island Biological Company) supplemented with 7 1/2% fetal calf serum and 2 mM glutamine in a humidified 5% oxygen/95% air atmosphere at 37°C. Cells were harvested by treatment with versene, washed with 2-3 volumes of unsupplemented Joklik's medium and resuspended to a final concentration of 1-3 x 10^7 viable cells/ml. Cell viability was determined in a hemacytometer using exclusion of Trypan Blue as a criterion.

[6,7-^{3}H]Dexamethasone (36.0 Ci/mmol) was purchased from New England Nuclear and stored at 4°C in benzene:ethanol (9:1, v:v). Solvent was evaporated prior to the addition of cell suspensions. Stock solution of unlabeled dexamethasone (Steraloids) was prepared at a concentration of 1 x 10^{-4} M in KRBG buffer. 5'-Deoxypyridoxal was prepared by the method of Mühlradt and Snell (16). 5'-Deoxypyridoxine and 5'-deoxypyridoxamine were prepared by catalytic hydrogenolysis of phosphorylated precursors (17). Stock solutions (50 mM) were prepared in 1.5 mM $MgCl_2$, adjusted to pH 7.0 with KOH, and frozen until use. Dextran-coated charcoal (1.0% Norit, 0.1% dextran) was prepared in 1.5 mM $MgCl_2$ and stored at 4°C. Other chemicals were reagent grade and were obtained from Sigma or Fisher.

Aliquots of thymus cell or HeLa S_3 cell suspension were incubated with 2 x 10^{-8} M [^{3}H]dexamethasone alone or in the presence of a 100-fold molar

excess of unlabeled dexamethasone for 1 1/2-2 h at 2°C. The difference in binding values obtained for cells incubated in the presence and absence of unlabeled dexamethasone represented the saturable receptor binding fraction. Thymocyte nuclei were prepared and nuclear receptor binding was measured using the hypotonic lysis technique of Munck and Wira (18). HeLa S_3 cell nuclear receptor binding was measured by the freeze-thaw hypotonic lysis procedure described by Sibley and Tomkins (19). Thymocyte cytoplasmic fractions were prepared by hypotonic lysis in 1.5 mM $MgCl_2$ (18). HeLa S_3 cytoplasmic fractions were prepared by suspending the cells in 1-3 ml of 1.5 mM $MgCl_2$, and homogenizing with three-10 sec bursts of a chilled Tekmar Ultra-Turrax homogenizer apparatus. Cellular debris was removed by a 1 min centrifugation at 10,000 x g. The supernatant represented the cytoplasmic receptor fraction or "cytosol".

Results

Fig. 1 shows the effect of 5'-deoxypyridoxal on [^{3}H]dexamethasone binding to nuclear receptor in whole HeLa S_3 cells (A) and whole rat thymocytes (B). Incubation of cells in the presence of millimolar concentrations of 5'-deoxypyridoxal for 30 min at 37°C caused a decrease of saturable [^{3}H]dexamethasone binding to nuclear receptor in both HeLa S_3 cells and thymus cells. 5'-Deoxypyridoxal concentrations of 0.2 mM caused a 50% reduction in nuclear dexamethasone receptor binding in both HeLa S_3 cells and thymocytes. At 5'-

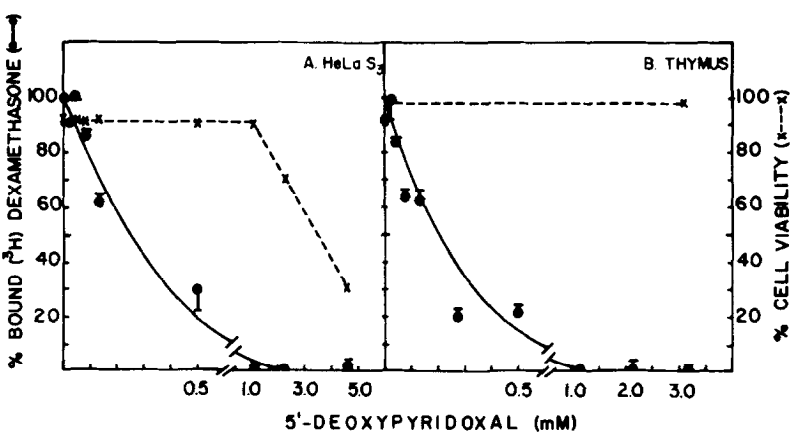

Figure #1. The influence of 5'-deoxypyridoxal treatment of whole cells on nuclear dexamethasone receptor binding. HeLa S_3 cells or rat thymocytes were incubated with either 2 x 10^{-8}M [^{3}H]dexamethasone alone or [^{3}H]dexamethasone plus 2 x 10^{-6}M unlabeled dexamethasone for 30 min at 37°C. The cells were then treated with the concentrations of 5'-deoxypyridoxal indicated for an additional 30 min at 37°C. Nuclear binding was measured using a hypotonic lysis assay (see Methods). The data shown represent the saturable binding fractions (mean ± S.E.; 2 determinations) calculated by subtracting binding values obtained in the presence of 2 x 10^{-6} M unlabeled dexamethasone. 5'-Deoxypyridoxal did not alter the level of nonsaturable binding. Cells were cooled to 2°C immediately after incubation and viability determined one-half hour after incubation was completed.

deoxypyridoxal concentrations of ≈1 mM, saturable dexamethasone receptor binding was reduced to less than 2% of the binding found for both HeLa S_3 cells and thymocyte controls. Incubation of HeLa S_3 cells or rat thymocytes with similar concentrations of 5'-deoxypyridoxal prior to equilibration with [^{3}H]-dexamethasone gave similar results (data not shown).

In an effort to determine whether the decreased glucocorticoid receptor binding observed following 5'-deoxypyridoxal treatment was a consequence of cell death, cell viability was determined. HeLa S_3 and thymus cell viabilities were not affected by incubation with 5'-deoxypyridoxal at concentrations of the compound which reduced glucocorticoid receptor binding. No decrease in thymus cell viability was observed at 5'-deoxypyridoxal concentrations as high as 3.2 mM (Fig 1B). HeLa S_3 cell viability was similarly unaffected at 5'-deoxypyridoxal concentrations of ≈1 mM. High concentrations of 5'-deoxy-pyridoxal (4.5 mM) decreased HeLa S_3 cell viability by 70%. Thus a ≈ 98% reduction in nuclear glucocorticoid receptor binding occurs in whole cells incubated with 1 mM 5'-deoxypyridoxal, a concentration which does not affect cell viability.

The effect of time of incubation on the 5'-deoxypyridoxal-induced loss of [^{3}H]dexamethasone binding to isolated cytoplasmic receptor from HeLa S_3 and thymus cytosol preparations is shown in Fig 2. Cytoplasmic receptor preparations were incubated for 60 min at 2°C in the presence of 5 mM 5'-deoxypyridoxal. A first-order exponential decrease in bound [^{3}H]dexametha-sone was observed, with k_{-1}= 2.24 x 10^{-2} min^{-1} for HeLa S_3 cytoplasmic dexa-methasone receptor complexes (Fig 2A) and k_{-1}=2.31 x 10^{-2} min^{-1} for thymocyte receptor complexes (Fig 2B). The half-life of loss of [^{3}H]dexamethasone binding in the presence of 5'-deoxypyridoxal was $t_{1/2}$= 31 min and $t_{1/2}$=30 min for HeLa S_3 and thymus cytoplasmic receptors, respectively. Values have been corrected for non-saturable binding and for loss of [^{3}H]dexamethasone bind-ing which occurs in the absence of added 5'-deoxypyridoxal. After 60 min, loss of [^{3}H]dexamethasone binding in the absence of added 5'-deoxypyridoxal

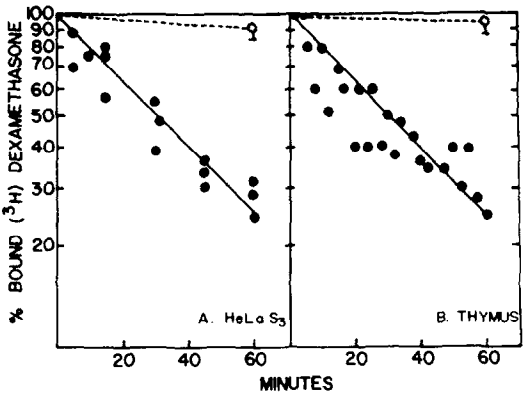

Figure #2. The influence of 5'-deoxypyridoxal on dexamethasone receptor binding in isolated cytosols. Isolated HeLa S_3 cells or thymocytes were incubated with 10^{-8} M [^{3}H]dexamethasone for 2 h at 2°C. Aliquots (0.9 ml) of cytosol were brought to 5 mM 5'-deoxypyridoxal with the addition of 0.1 ml of 50 mM stock solution (pH 7.6). Controls received an equivalent amount of 1.5 mM $MgCl_2$. At intervals thereafter 100 µl aliquots of cytosol were removed, treated with 100 µl of dextran-coated charcoal for 5 min and centrifuged at 10,000 x g for 1 min. Samples of the supernates were placed into scintillation vials for the determination of radioactivity. The data was plotted as the % of the control [^{3}H]dexamethasone binding observed in 5'-deoxypyridoxal-treated cytosol. [^{3}H]Dexamethasone binding in control cytosols is indicated by open circles. Data from three separate experiments are plotted.

represented only 5-10% of the total initial binding in control HeLa S_3 and thymus cytosols. These data, in conjunction with those represented in Fig 1, show that 5'-deoxypyridoxal can decrease glucocorticoid receptor binding in whole cells at physiological temperatures (37°C) and in isolated cytoplasmic fractions at 2°C.

Several 5'-deoxypyridoxal analogs were next examined for their effect on [^{3}H]dexamethasone binding to rat thymocyte cytoplasmic receptors (Fig 3). Pyridoxal 5'-phosphate, 5'-deoxypyridoxine, 5'-deoxypyridoxamine or 5'-deoxypyridoxal at a final concentration of 5 mM were added to thymus cytosol for 2 hr at 2°C. Pyridoxal 5'-phosphate, 5'-deoxypyridoxine, and 5'-deoxypyridoxamine had little effect on the amount of saturable bound [^{3}H]dexamethasone in cytosol preparations. In cytosol incubated with 5 mM 5'-deoxypyridoxal, a six-fold decrease in the amount of bound [^{3}H]dexamethasone was observed after 2 h. The importance of the C4'-carboxaldehyde group of 5'-deoxypyridoxal is indicated by the failure of the corresponding alcohol or amine to effect a similar loss of steroid-receptor binding. In addition, the presence of the

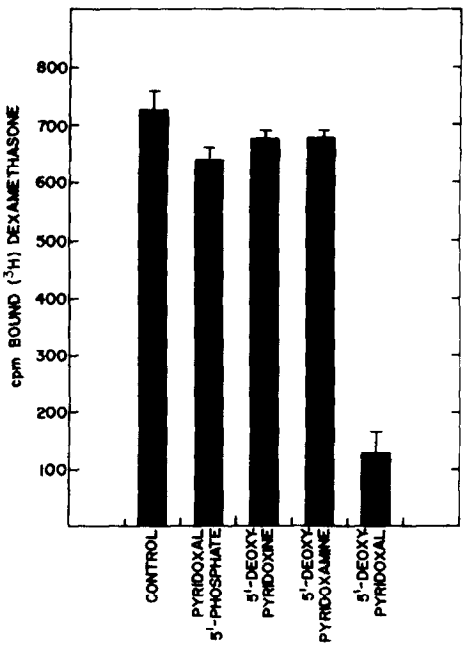

Figure #3. The specificity of 5'-deoxypyridoxal action on cytoplasmic dexamethasone receptor. Thymocytes were incubated at $2^{0}C$ for 2 h with either 2 x 10^{-8} M [^{3}H]dexamethasone alone or [^{3}H]dexamethasone plus 2 x 10^{-6} M unlabeled dexamethasone. Cytosols were prepared and incubated with 5 mM of the indicated compounds at $2^{0}C$ for an additional 2 h. Receptor binding was measured with the dextran-coated charcoal assay and saturable binding values (mean ± S.E., 2 determinations) are plotted.

apolar methyl substituent at C5' appears to be essential for the reduction in glucocorticoid receptor binding. Pyridoxal 5'-phosphate with a highly charged C5' phosphate group, is ineffective in causing a loss of steroid receptor binding. These data indicate that 5'-deoxypyridoxal acts in a highly specific manner to reduce glucocorticoid receptor binding in these preparations.

Discussion

We have demonstrated that 5'-deoxypyridoxal, a potent synthetic vitamin B_6 analog, decreases the binding of dexamethasone to nuclear glucocorticoid receptor complexes in HeLa S_3 cells and rat thymocytes in vitro at 37°C and in isolated cytoplasmic fractions at 2°C. A rapid and nearly complete loss of [^{3}H]dexamethasone binding was found for nuclear receptor complexes in whole

HeLa S_3 cells and rat thymocytes incubated at 37°C in the presence of 1 mM 5'-deoxypyridoxal, with no alteration in cell viability. In HeLa S_3 and thymocyte cytosol preparations at 2°C, similar dissociation rates of bound [^{3}H]-dexamethasone from receptor complexes were calculated, approximately 20-fold greater than those found in control cytosols. 5'-Deoxypyridoxal appears to be quite specific for producing reductions in glucocorticoid receptor binding, as pyridoxal 5'-phosphate, 5'-deoxypyridoxal and 5'-deoxypyridoxamine were ineffective.

5'-Deoxypyridoxal appears to be a non-steroidal compound which can decrease the binding of glucocorticoids to specific receptor proteins in whole cells. Whether the effects of 5'-deoxypyridoxal are the result of its inherent vitamin B_6 antagonist action or due to a direct interaction with steroid receptor remains to be established. Nevertheless, its potential as a non-steroidal glucocorticoid antagonist is suggested.

Acknowledgements

Supported in part by NIH grants AM 20892 and AM 20762.

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