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ORIGINAL ARTICLE

Wei Cai · Raymond E. Counsell · David E. Schteingart
Joseph E. Sinsheimer · Alfin D.N. Vaz ·
Linda L. Wotring

Adrenal proteins bound by a reactive intermediate of mitotane

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Abstract *Purpose*: Mitotane (o,p'-DDD), is the only adrenolytic agent available for the treatment of adrenocortical carcinoma. Previous studies have shown that mitotane covalently binds to adrenal proteins following its metabolism in adrenocortical tissue to a reactive acyl chloride intermediate. It was the objective of this study to compare the electrophoresis separation patterns of such adducts following activation of mitotane by various adrenocortical sources. *Methods*: With the use of a ^{125}I-labeled analog of mitotane, 1-(2-chlorophenyl)-1-(4-iodophenyl)-2,2-dichloroethane, gel electrophoresis patterns were obtained for homogenates from bovine, canine and human adrenocortical preparations as well as from a human adrenal preparation. Western immunoblotting analysis was used to test the resulting patterns for adducts of cytochrome P-450_{SCC} and adrenodoxin. *Results*: The electrophoresis separations were similar for all preparations, with bands at apparent molecular weights of 49.5 and 11.5 kDa being the most pronounced. Radiolabeling of the proteins of a human adrenal cancer cell line NCI H-295 was weak, but a band at 11.5 kDa was detected. Western immunoblotting analyses indicated that the band at 49.5 kDa corresponded in molecular weight to that of adrenal cytochrome P-450_{SCC}, but the band at 11.5 kDa did not correspond to adrenodoxin. *Conclusions*: The similarity of the results with canine and bovine adrenal preparations to that of human material offers useful systems for studying mitotane and its analogs. This should aid in understanding the mechanism of action of mitotane and in the design of compounds for the treatment of adrenocortical carcinoma.

Key words Mitotane · Adrenocortical protein binding · Gel electrophoresis · Adrenal carcinoma

W. Cai · J.E. Sinsheimer (✉) · L.L. Wotring
College of Pharmacy, University of Michigan, Ann Arbor, MI 48109-1065, USA
Tel. (313) 764-7351; Fax (313) 763-2022

R.E. Counsell
Department of Pharmacology, Medical School, University of Michigan, Ann Arbor, MI 48109-0632, USA

D.E. Schteingart
Department of Internal Medicine, Medical School, University of Michigan, Ann Arbor, MI 48109-0678, USA

A.D.N. Vaz
Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor, MI 48019-0606, USA

Introduction

Since the first report of its clinical utility in 1960 [2], mitotane [1-(2-chlorophenyl)-1-(4-chlorophenyl)-2, 2-dichloroethane; o,p'-DDD] remains the drug of choice in the treatment of adrenocortical cancer [15]. The adrenocorticolytic activity of mitotane in dogs is well established [8, 13, 20] and in selective cases it has dramatically increased the survival time of patients with adrenocortical cancer [25]. However, only about 35% of the patients treated with mitotane demonstrate any objective signs of effectiveness [25]. Moreover, especially at the large doses that may be needed to maintain serum levels for adequate treatment [7], mitotane exhibits severe side effects [7, 19]. These side effects and the partial efficacy raise the question of the use of mitotane for adjuvant treatment following surgery [17] and has restricted its use to inoperable or recurrent tumors [10, 24].

Thus, there is an obvious need for an improved chemotherapeutic agent to treat adrenocortical cancer [25]. As part of a program to develop such a drug, we have studied the mode of action of mitotane [4, 5, 16] as a model compound to develop structure–activity relationships for mitotane analogs. It has been previously established, during incubations with adrenal cortex homogenates from various species, that mitotane covalently binds to adrenal proteins through

a metabolic intermediate [5]. Thus mitotane, like other compounds containing a dihalogenated methyl moiety [1], undergoes enzymatic hydroxylation at its dichloromethine carbon followed by dehydrochlorination to form an acyl chloride derivative as the active intermediate. The previous investigation [5] employed the ^{125}I-labeled analog of DDD, 1-(2-chlorophenyl)-1-(4-iodophenyl)-2,2-dichloroethane, to detect protein binding. The metabolism of the iodinated analog was found to be similar to ^{14}C-labeled mitotane. Therefore, we continued to use this ^{125}I analog of mitotane in the present study with the objective of identifying specific proteins to which mitotane binds. These proteins may be important in the mechanism of action of mitotane.

Materials and methods

Gel electrophoresis

The preparation of adrenocortical subcellular fractions, their use in incubations to produce covalently bound adrenal proteins and the isolation of these bound proteins from bovine, canine and human adrenal preparations were as previously described [5]. For slab-gel electrophoresis based on the method of Laemmli [12], protein samples in 1.0% SDS (10 to 50 μl) were mixed with an equal volume of buffer (0.062 *M* Tris-HCl, pH 6.8), 1.0% SDS, 0.001% bromphenol blue as tracking dye, 10% glycerol and 5% β-mecaptoethanol, and placed in boiling water for 10 min. Aliquots of samples (12 to 200 μg protein in 15 to 90 μl of the buffer) were applied to an SDS-polyacrylamide gel (15 cm × 10 cm × 1 mm). Electrophoresis was carried out with a constant current of 12 mA for the stacking gel (4.5% acrylamide) and 25 mA for the separating gel (15% acrylamide) for about 3 h in a chamber containing an electrode buffer of 0.025 *M* Tris-HCl (pH 6.8), 0.192 *M* glycine and 0.1% SDS. Prestained molecular weight standards were phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme (Bio-Rad, Hercules, Calif.). The gel was fixed in a 25% methanol and 10% acetic acid solution overnight. To prevent cracking, 3% glycerol was then added to this solution and the gel allowed to stand for 24 h. The gel, covered with a cellophane membrane, was dried by a vacuum gel drier (Model 543, Bio-Rad) for 6 h at 60 °C using the gradient cycle and then exposed to Kodak AR X-ray film for 6 days. The autoradiogram was quantified using a soft laser scanning densitometer (SL-TRFF, Biomed Instruments, Fullerton, Calif.).

Immunoblotting analysis

For western immunoblotting analysis, the separated proteins were transferred electrophoretically from the gel to nitrocellulose paper (Hybond-ECL, Amersham, Arlington Heights, Ill.) by the method of Burnette [3] using a transfer buffer of 0.159 *M* glycine, 0.025 *M* Tris-HCl, 0.01% SDS and 20% methanol. The transfer was performed at 90 V for 1 h using a Trans-Blot cell (Bio-Rad). The nitrocellulose paper was then incubated for 1 h at room temperature with a solution of 1% Tween 20 and 5% nonfat dry milk in Tris-buffered saline solution (TBS; 20 m*M* Tris-HCl, pH 7.6, 0.14 m*M* NaCl) to reduce nonspecific binding of the antibody. The paper was then washed three times (1 × 15 min and then 2 × 5 min) with a solution of 1% Tween 20 in TBS, after which the nitrocellulose paper was incubated for 1 h at room temperature in antibody solutions against bovine adrenal cytochrome P-450$_{SCC}$ or adrenodoxin at dilutions of 1:10 000 or 1:8000, respectively. Both antibodies raised in rabbits, were kindly provided by Dr. J.K.M. Menon (University of Michigan). The paper was washed with 1% Tween 20 in TBS buffer three times and then incubated with a second antibody (antirabbit peroxidase) at a dilution of 1:14 000 for 1 h. The paper was washed with 1% Tween 20 in TBS buffer again and incubated with 2 ml of a 1:1 mixture of two ECL western blotting detection reagents (RPN 2109, Amersham) for 1 min. The nitrocellulose paper was covered with Saran wrap, mounted on a sheet of paper which was marked at two corners with 1 μCi of a ^{32}P-labeled marker and then the system was exposed to Hyperfilm-ECL (Amersham). The covered nitrocellulose paper was remounted on another piece of paper marked in the same position with ^{35}S ink and exposed to Kodak AR X-ray film for 6 to 21 days for detection of the ^{125}I-labeled protein bands.

Results and discussion

SDS-polyacrylamide gel electrophoresis followed by autoradiography of ^{125}I-labeled proteins demonstrated that radioactivity was associated with a few specific proteins among the numerous proteins in the canine adrenal cortex whole homogenate and its subcellular fractions as well as in the mitochondria from bovine adrenal cortex (Fig. 1). As shown in Table 1, the bands of apparent molecular weight 11.5 and 49.5 kDa were the most intensely labeled proteins in the whole homogenate from canine adrenal cortex. They represented 58% and 19%, respectively, of the total radioactivity on the gel. The radiolabeled protein bands of mitochondria from bovine adrenal cortex had a pattern similar to that of mitochondria from dog adrenal cortex except that an additional protein band of 34 kDa with 4% of the total radioactivity was found in bovine

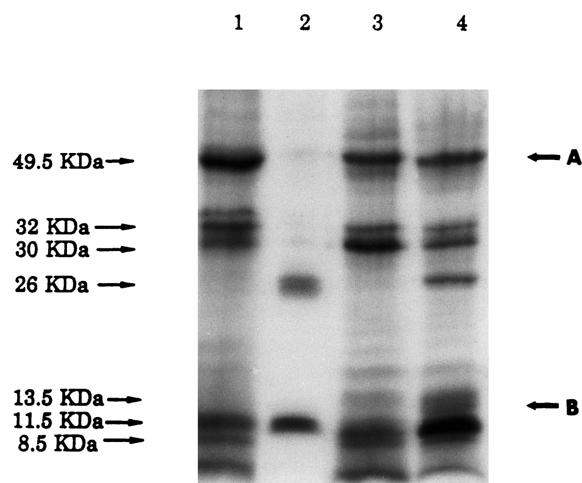

Fig. 1 Autoradiogram of labeled proteins from the following incubations (amount of labeled protein applied to the gel): *lane 1* bovine adrenal cortex mitochondria (54 μg); *lane 2* canine adrenal cortex cytosol (160 μg); *lane 3* canine adrenal cortex mitochondria (65 μg); *lane 4* dog adrenal cortex whole homogenate (180 μg); *A*, *B* positions of western immunoblotting analysis for cytochrome P-450$_{SCC}$ and adrenodoxin, respectively, run in combination with the autoradiogram of labeled proteins

Table 1 Percentage distribution[a] of the major labeled protein bands after incubation of the indicated adrenal preparations with [^{125}I]-1-(2-chlorophenyl)-1-(4-iodophenyl)-2,2-dichloroethane

Molecular weight (kDa)	Bovine	Canine			Human adrenal homogenate	
	Mitichondria	Cytosol	Mitochondria	Homogenate	Normal	Tumor
49.5	32	1	24	19	19	14
34	4	—	—	—	—	—
32	13	—	5	2	14	12
30	11	—	31	5	15	10
26	—	17	—	3	4	2
13.5	—	—	5	5	—	—
11.5	17	75	10	58	28	28
8.5	10	—	10	1	—	—

[a] Percentage for distinct bands of the total density in a given gel lane

mitochondria and a 13.5 kDa band was not detected in the bovine preparation. In the cytosol from canine adrenal cortex, the bands at 11.5 kDa and 26 kDa were the major protein adducts found.

Protein adducts previously reported [5] from incubations of the ^{125}I-labeled analog of DDD with whole homogenates of normal human adrenal cortex, adrenal tumors or with NCI H-295 human adrenal cancer cells [6] in culture for 7 days were also available. These proteins were separated by electrophoresis and the autoradiogram of the gel showed a pattern similar to that of canine adrenocortical whole homogenate (Fig. 2, Table 1). However, the total radioactivity bound to cellular proteins of NCI H-295 human adrenal cancer cells was limited and only the 11.5 kDa band was evident.

Based on previous reports of the effect of *o,p′*-DDD on adrenal P-450$_{SCC}$ [26] and adrenodoxin [14] and their molecular weight data [11, 21–23], it is possible that the 49.5 kDa and the 11.5 kDa bands might have been cytochrome P-450$_{SCC}$ and adrenodoxin, respectively. Immunoblotting assays were employed to examine these two protein bands. The bovine adrenal P-450$_{SCC}$ antibody used in this study had crossimmunoreactivity for the 49.5 kDa bands of human adrenal cortex and adrenal tumors as well as the band from canine adrenal cortex. This is consistent with a previous report that immunohistochemical distribution of the P-450$_{SCC}$ can be demonstrated in human normal adrenals, aldosteronoma and Cushing's adenoma using a specific antibody against bovine adrenal P-450$_{SCC}$ [18]. An autoradiograph showed that the radiolabeled protein bands of 49.5 kDa could be superimposed with the protein bands of immunoblotting (Fig. 1). Therefore, the 49.5-kDa protein band could be cytochrome P-450$_{SCC}$ acylated by the reactive intermediate of the ^{125}I-analog of mitotane.

It has been shown [9] that mitotane has an acute inhibitory effect on ACTH-promoted transformations of cholesterol to pregnenolone. Also, an adrenal P-450 system has been shown to support mitotane metabolism [13]. However, preliminary results from our laboratory suggest that P-450$_{SCC}$ is not involved in the metabolic activation of mitotane. That is, the metabolism of mitotane is not inhibited by the addition of cholesterol or by the P-450$_{SCC}$ enzyme inhibitor, aminoglutethimide, to our adrenocortical preparations. Thus, this radiolabeled protein could be a distinct P-450 with a molecular weight similar to that of P-450$_{SCC}$.

In a similar manner to the immunoblotting of cytochrome P-450$_{SCC}$, we found that the bovine adrenodoxin antibody also has crossimmunoreactivity to adrenodoxin of canine adrenal cortex as well as human adrenal cortex and adrenal tumor. However, the radiolabeled protein bands of 11.5 kDa could not be superimposed on the protein bands that reacted with the antibody. Also, a much more intense immunoblotting band was detected in canine adrenal mitochondria than any that was found in canine whole homogenates, while the 11.5 kDa radiolabeled protein was only seen in canine adrenal cytosol or whole homogenates.

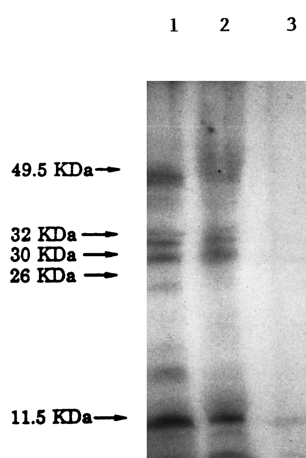

Fig. 2 Autoradiogram of labeled proteins from the following sources: *lane 1* normal human adrenal cortex, whole homogenate (180 µg); *lane 2* human adrenocortical tumor, whole homogenate (200 µg); *lane 3* NCI H-295 human adrenocortical carcinoma cells (200 µg)

Therefore, the 11.5 kDa protein band is not an adduct of adrenodoxin but of another low molecular weight adrenal protein or of a fragment of the 49.5 kDa band.

Identification of the major protein bands that reacted with the ^{125}I-analog of mitotane may aid in further elucidation of the mechanism of action of mitotane. The similarity of the patterns from canine and bovine adrenal material to that of human adrenal material offers useful model systems for studying mitotane and its analogs. Such studies should aid in the evaluation and design of new compounds that have potential for treating adrenocortical carcinoma.

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