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RELATION OF STEROID STRUCTURE TO ENZYME INDUCTION IN THE INDUCTION OF TYROSINE AMINOTRANSFERASE AND TRYPTOPHAN PYRROLASE

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SUMMARY

The structural requirements for the induction of tyrosine aminotransferase and tryptophan pyrrolase have been defined by comparing the relative induction of these 2 enzymes in male adrenalectomized rats. It has been demonstrated that the receptor for each induction discriminates between steroid inducing agents possessing only minor structural variations to the extent that it can be stated that intranuclear induction of TAT and TP is occurring via two different mechanisms. The partial structural requirements for each induction are described. The chemotherapeutic significance of these findings, with respect to drug design, are discussed.

INTRODUCTION

THE ability of glucocorticoids to induce the synthesis of certain enzymes has been ascribed to the interaction of the steroid with repressors or activators at or near the transcriptional level in mammalian cells[1]. Two enzymes, tyrosine aminotransferase (TAT) (ED2.6.1.5) and tryptophan pyrrolase (TP) (EC1.13.1.12) are induced within a very short time upon administration of glucocorticoids to adrenalectomized animals and this rapid rise in enzyme activity is known to be due to increased rates of protein[2–6] and mRNA synthesis[7–9]. Although TAT and TP induction appear to be closely related events there has not yet been any clear indication as to whether a glucocorticoid acts on one or more receptors in stimulating the synthesis of these two enzymes.

We have investigated this problem by measuring the induction of TAT and TP in adrenalectomized rats using twenty-one steroids as inducing agents.

EXPERIMENTAL

Materials

The following commercial steroid preparations were used: Cortone 50 mg/ml and Hydrocortone 25 mg/ml from Merck, Sharp & Dohme, Meticortelone (prednisolone acetate) 25 mg/ml from Schering, and Depo-Medrol (6α-methylprednisolone) 40 mg/ml from Upjohn. Fluorometholone was a gift from British Drug House (Toronto). All other steroids were obtained from the Mann Research Laboratories.

L-tyrosine, A grade, α-ketoglutaric acid, A grade, and L-tryptophan, A grade were obtained from Calbiochemical Corporation. Sodium diethyldithiocarbamate was obtained from Fisher Scientific Company, pyridoxal phosphate from General Biochemicals and sodium ascorbate (U.S.P.) from Pfizer Co. Ltd.

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The methemoglobin used was commercial twice crystallized horse hemoglobin from Nutritional Biochemicals, which consists largely of methemoglobin by spectral analysis, and was made up using 0·2 M phosphate buffer, pH 7·0.

The protein standard used was from Hartman–Leddon Co. and had a total protein content of 7·0%.

Unless otherwise noted, all solutions were prepared using triple distilled water.

Treatment of animals

Male adrenalectomized Sprague–Dawley rats weighing 150–174 g were obtained from the Canadian Breeding Laboratories (St. Constant, Quebec) and were housed in conditions of regulated light (12 hr light and 12 hr darkness). The animals were maintained on Purina rat chow up to 12 hr before the tests, with free access to physiological saline (0·9% NaCl) at all times. They were used within 3 to 10 days following bilateral adrenalectomy. Completeness of adrenalectomy was determined at autopsy and if residual adrenal tissue was found, the animal was not included in the experiment. In view of possible circadian rhythm [10], injections of glucocorticoids were given between 8–10 a.m. and the animals were sacrificed 4 hr after treatment, between 12–2 p.m.

The steroids were given intraperitoneally, on a molar basis ($6{\cdot}21 \times 10^{-6}$ mol/100 g rat) as a solution or as a finely dispersed suspension in propylene glycol: 0·9% NaCl (1 : 1 v/v) in a volume of 0·5 ml or less/100 g of body weight. Control animals received an equivalent volume of vehicle, assuming a dosage of $6{\cdot}21 \times 10^{-6}$ mol/100 g body weight.

The rats were killed by decapitation and were exsanguinated. Livers were removed and placed in ice-cold 0·25 M sucrose containing Medium M (0·02 M Tris buffer, pH 7·6, 0·01 M magnesium acetate, 0·1 M KCl, 0·04 M NaCl and 0·006 M mercaptoethanol) (Sucrose-Medium M), cut up with scissors, rinsed free of blood with fresh medium and homogenized lightly in 3 vol. of Sucrose-Medium M using a Teflon-glass homogenizer (Canlab, 'Caframo' stirrer-type RZRl-64). The homogenates were centrifuged at $23{,}500 \times g$ in a Sorvall RC-2B centrifuge for 10 min at 0°C and the supernatant was removed and centrifuged at $100{,}000 \times g$ for 120 min in a Beckman preparative ultracentrifuge, Model L-2. The supernatant material (cell sap) was used for the enzyme assays[11]. All preparations were maintained at 0° ± 5°C.

Assay of tyrosine aminotransferase

Tyrosine aminotransferase was assayed by a slight modification of two previously described methods[11, 12]. To 2·0 ml of 0·71 M sodium borate, pH 8·0, containing 9·6 μmol of L-tyrosine, were added successively: 0·2 ml of 0·35 M α-ketoglutarate, 0·1 ml of 0·1 M sodium diethyldithiocarbamate, 0·1 ml of 1·2 mM pyridoxal phosphate and 0·5 ml of the cell sap in the appropriate dilution. The blank contained all components except L-tyrosine.

The initial rate of formation of the complex of enol-borate and p-hydroxyphenylpyruvate was followed in a Zeiss PMQ II spectrophotometer at 25° ± 1°C. The results are expressed as millimicromoles of p-hydroxyphenylpyruvate ($\epsilon = 9830$) per hour per mg of protein in the cell sap[11].

Assay of tryptophan pyrrolase

Tryptophan pyrrolase activity was assayed after incubation of the cell sap

with an equal volume of the homogenizing medium containing a mixture of 2·5 mM L-tryptophan, 0·5 mg of methemoglobin per ml and 30 mM sodium ascorbate at 37°C for 1 hr[13].

A slight modification of the standard assay of tryptophan pyrrolase activity of Piras and Knox[13] was made. The assay mixture had a total volume of 3·0 ml and contained 0·4 ml of 12·5% liver extract, 0·7 ml of 0·2 M sodium phosphate, pH 7·0, 0·2 ml of methemoglobin, 1·4 ml of glass-distilled water, 0·2 ml of 0·05 M L-tryptophan and 0·1 ml of 0·3 M sodium ascorbate, added in the order given. The activity was calculated from the initial increase in absorbancy over 20 min at 360 nm, using $\epsilon = 4530$ for kynurenine in 3-cm curvettes at 25° ± 1°C against a water blank. Activities are expressed as millimicromoles of kynurenine formed per hour per mg of protein[11].

Protein content

The protein content of the cell sap was measured by a slight modification of a previcusly described method[14].

One millilitre of copper solution (0·5% $CuSO_4 \cdot 5H_2O$ in 1% sodium tartrate) was added to 50 ml of 2% anhydrous sodium carbonate in 0·1 N sodium hydroxide. The solution was mixed well and used immediately.

Five millilitres of copper-sodium carbonate mixture was added to a 1:100 dilution of the cell sap in a test-tube. Water was added to make up the volume to 1 ml if necessary. The solutions were left at room temperature for at least 10 min.

0·5 ml of 1 N Folin-Phenol reagent (prepared from 2 N stock solution, Fisher Scientific Co.) was added rapidly. The solutions were shaken vigorously and left at room temperature for at least 60 min.

Readings were taken in a Zeiss spectrophotometer PMQ II at 500 nm and the corresponding protein concentrations were read from a standard protein curve in the range of 10–400 μg of protein.

Statistics

Each compound was tested on 5 similarly treated adrenalectomized rats. Q rejection tests[15, 16] were performed on the results which are expressed as the mean value ± the standard error for five animals. It is reasonable in this type of experiment to expect rather large deviations in assay results; however, it is a general view that values of the standard error no greater than ± 10% of the mean are acceptable. Of the 42 assays carried out, 13 fall outside this range, but only one (cortisol and TP) sufficiently so as to cast doubt on its validity. Each supernatant was assayed once for both TAT and TP. No supernatants were pooled.

RESULTS

As expected, treatment of adrenalectomized Sprague–Dawley rats with cortisone raised the level of TAT and TP approximately five-fold (Table 1). Surprisingly, however, cortisone was the most active of the steroids used whereas one would have expected the more potent glucocorticoids such as prednisolone and hydrocortisone to have a much greater effect. We have no explanation for this anomalous behaviour; however, one could argue that the rate limiting step in this particular test system involves factors other than the intrinsic activity of the steroids, the most likely of these being uptake and transport to the receptor site.

Table 1. Effect of various steroids on the induction of tyrosine aminotransferase (TAT) and tryptophan pyrrolase (TP) in adrenalectomized male Sprague–Dawley rats

Compound*	Vehicle†	Tyrosine aminotransferase activity (mean ± S.E.) nmole/hr/mg protein	% Activity of cortisone	Tryptophan pyrrolase activity (mean ± S.E.) nmol/hr/mg protein	% Activity of cortisone	Ratio of effect $\frac{TAT}{TP}$
Cortisone acetate	S	1803·8 ± 57·1	100·0	117·8 ± 9·6	100·0	1·0
Cortisol acetate	S	1513·0 ± 120·9	84·0	101·0 ± 29·0	86·0	0·98
Meticortelone (prednisolone acetate)	S	1519·2 ± 168·5	84·0	48·8 ± 7·7	41·5	2·0
6α-Methylprednisolone	A	1209·3 ± 38·3	67·0	25·5 ± 4·4	22·0	3·1
Prednisone	SPG	1321·4 ± 28·9	73·0	24·4 ± 2·5	21·0	3·5
Betamethasone	SPG	1469·4 ± 117·2	81·5	42·1 ± 4·5	36·0	2·3
Fluorometholone	SPG	1509·6 ± 4·5	84·0	92·4 ± 12·4	78·0	1·1
Cortexolone	SPG	273·3 ± 8·8	15·0	6·8 ± 0·6	5·8	2·6
16-Dehydropregnenolone	SPG	333·2 ± 10·5	18·5	9·7 ± 0·7	8·2	2·3
5α-Dihydrocortisol	SPG	468·3 ± 32·9	26·0	31·1 ± 2·8	26·0	1·0
5β-Dihydrocortisol	SPG	339·0 ± 11·6	19·0	14·2 ± 1·4	12·0	1·6
Allopregnanolone	SPG	294·3 ± 25·8	16·0	10·8 ± 0·7	9·2	1·7
11β-Hydroxyprogesterone	SPG	572·8 ± 47·1	32·0	39·1 ± 6·4	33·0	0·97
11α-Hydroxyprogesterone	SPG	313·6 ± 21·6	17·0	39·9 ± 5·0	34·0	0·50
Corticosterone	SPG	1676·6 ± 79·3	93·0	117·3 ± 23·9	99·0	0·94
Deoxycorticosterone	SPG	463·6 ± 12·2	26·0	22·9 ± 1·2	19·0	1·4
11-Dehydrocorticosterone	SPG	915·2 ± 95·3	51·0	50·9 ± 4·5	43·0	1·2
21-Deoxycortisol	SPG	723·8 ± 70·7	40·0	37·0 ± 4·3	31·0	1·3
3β-Tetrahydrocortisol	SPG	411·5 ± 45·7	23·0	28·6 ± 5·7	24·0	0·96
Testosterone	SPG	259·8 ± 22·4	14·0	9·2 ± 1·8	7·8	1·8
Progesterone	SPG	268·3 ± 17·1	15·0	12·1 ± 0·9	10·2	1·5
None	SPG‡	339·7 ± 22·6	18·8	19·9 ± 3·5	17·0	

*List of abbreviations used: *Meticortelone (prednisolone acetate)* 11β,17α-dihydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione; *6α-methylprednisolone* 6α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione; *prednisone* 17α,21-dihydroxy-1,4-pregnadiene-3,11,20-trione; *betamethasone* 9α-fluoro-16βmethyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione; *fluorometholone* 9α-fluoro-6α-methyl-11β,17α-dihydroxy-1,4-pregnadiene-3,20-dione; *cortexolone* 17α,21-dihydroxy-4-pregnene-3,20-dione; *16-dehydropregnenolone* 3β-hydroxy-5,16-pregnadien-20-one; *5α-dihydrocortisol* 11β,17α,21-trihydroxy-5α-pregnane-3,20-dione; *5β-dihydrocortisol* 11β,17α,21-trihydroxy-5β-pregnane-3,20-dione; *allopregnanolone* 3β-hydroxy-5β-pregnan-20-one; *3β-tetrahydrocortisol* 3β,11β,17α,21-tetrahydroxy-5β-pregnan-20-one.

†A – aqueous. S – saline. SPG – saline: propylene glycol 1 : 1 (v/v).

‡Demonstrates resting level of TP and TAT in adrenalectomized animals.

In spite of this behaviour, certain of the steroids tested were almost equal in activity* with cortisone, namely, hydrocortisone (84%), prednisolone (84%), betamethasone (82%), fluorometholone (84%), and corticosterone (93%) in TAT induction. The compounds able to induce TP almost to the levels induced by cortisone were hydrocortisone (86%), fluorometholone (78%) and corticosterone (99%). With one exception (fluorometholone) the only steroids able to induce TP to levels near cortisone were the naturally occurring ones.

Of the inactive glucocorticoids tested, none behaved in an unusual fashion, e.g. cortexolone gave values close to control values as did the dihydrocortisols, deoxycorticosterone and tetrahydrocortisol. Five compounds gave values below that of the control – 16-dehydropregnenolone, allopregnanolone, testosterone, progesterone and cortexolone.

DISCUSSION

The most popular explanation of the mechanism of induction of TAT and TP by glucocorticoids is that because the induction occurs concomitant with a rise in mRNA and protein synthesis [2–9], it can be inferred that the primary event is one which occurs intranuclearly and involves either a derepression and/or an activation mechanism. Based on this assumption, our results become rather enlightening for it is apparent that some of the compounds tested partition themselves in a different manner *in the nucleus*. One answer for this behaviour is that there are two different receptors involved in the induction of each enzyme. To illustrate this point (see Table 1), it is apparent that the naturally occurring glucocorticoids (cortisol ($R = 0{\cdot}98$) and corticosterone ($R = 0{\cdot}94$)) fit both receptors equally well (TAT and TP ratios in brackets). If, however, one makes structural modifications of these natural compounds, the receptor 'fit' is not always the same for both inductions. The TP receptor cannot apparently tolerate the introduction of a double bond at position 1 in the parent molecule. The effect is apparent for prednisolone ($R = 2{\cdot}0$), prednisone ($R = 3{\cdot}5$), 6α-methylprednisolone ($R = 3{\cdot}1$), and betamethasone ($R = 2{\cdot}3$). When both a 6α-methyl and a 9α-fluoro (fluorometholone) are present, however, the adverse effect of the double bond is overcome ($R = 1{\cdot}1$).

Inspection of some of the weakly active compounds reveals other differences in the receptors. Although the dihydrocortisols are weak inducers, TAT and TP respond equally to 5α-dihydrocortisol ($R = 1{\cdot}0$) whereas TP response for the 5β is weaker than that of the TAT response ($R = 1{\cdot}6$). The TP receptor can clearly not tolerate a change in planarity of rings A and B while the TAT receptor can.

A similar argument holds for the 11-hydroxyprogesterones: with 11β-hydroxyprogesterone both enzymes are induced to the same extent ($R = 0{\cdot}97$) but the 11-epi-compounds differ by a factor of two. The TAT receptor obviously prefers an 11β configuration over an 11α- while the TP receptor is indifferent to changes in configuration at this position.

The oxygen at 11 is obviously required for the induction of both enzymes since when it is lacking, activity falls off drastically. Since this oxygen is probably involved in binding at a receptor site its absence decreases the binding constant of a steroid to very low values. The compounds cortexolone and 16-dehydropreg-

*Activity as used here refers to the inductive capacity of each steroid tested at the given dose and time interval.

nenolone validate this hypothesis since both lack oxygen at 11 and both fail to induce either TAT or TP above control levels.

Another explanation for the differences noted here is that the levels of possible activators or repressors for the enzymes could be affected. If this is true, the mechanism of induction of both TAT and TP could be identical whilst the effect of the steroid on the presence or absence of an activator or repressor would be at a different site and involve a different receptor and mechanism. What is apparent from this study, regardless of the actual mechanism, is that any given steroid molecule should not be expected to produce a similar response in even closely related test systems since the receptors *are* different and will thus impose different limitations on the structure of the interacting molecule.

While this report was being written, Samuels and Tomkins[17] published their results on the induction of TAT in HTC cells. They classified steroids as inactive, optimal, sub-optimal, and anti-inducers and stated that the only absolute structural requirement for an anti-inducer was a 17β-OH group. Our results partially confirm their findings, i.e. testosterone values are below the control value. However, we found this also to be the case for progesterone, allopregnanolone, 16-dehydropregnenolone and cortexolone. Perhaps these substances, in our experiments, are being metabolized to 17β-OH compounds which possess androgenic activity like testosterone and therefore become anti-inducers.

We have not attempted to interpret our findings with respect to the classical structure–activity relations for either of the enzymes since the degree of induction produced from compound to compound will vary with compound solubility, particle size, transport and metabolizing enzymes, to name a few factors. In spite of these limiting factors, the well established structure activity pattern for glucocorticoids does emerge in part, but alone has little value.

From a biochemical standpoint our findings are not surprising, indeed, they lend support to what we would intuitively expect to be the case, i.e. that for each primary action of a steroid there is a structurally unique target or receptor the interaction with which, controls the magnitude of the primary event.

It may be argued that there is little value in comparing the ability of steroids to induce two particular enzymes since comparisons have already been made for other effects, e.g. salt retention vs. anti-inflammatory behaviour. However, the anti-inflammatory capacity of a steroid has little molecular significance since the net effect is secondary to the primary effects and represents the sum of all primary and secondary effects of the steroid on the animal, the responses involved being complex and multiple.

Since steroids of the glucocorticoid type induce and/or cause multiple effects in an animal and since the glucocorticoid activity of a steroid is measured by the magnitude of such a complex response (gluconeogenesis, anti-inflammatory, lymphocyte involution, etc.) it is conceivable that a steroid, although capable of inducing *one* of the primary events but none of the others, could be passed over as an effective chemotherapeutic agent. Hydrocortisone obviously fits all glucocorticoid receptors but other suitably chosen steroids may not fit all glucocorticoid receptors (e.g. prednisolone in our study). However, use of such steroids might lead to a considerable chemotherapeutic advantage being achieved, e.g. TP induction as the sole response. The acute and chronic effects of such a compound in an intact animal should be studied. A similar approach should be

feasible with other naturally occurring regulators such as progestins, androgens and cyclic AMP.

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