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BINDING OF SOME NON-STEROIDAL ANTI-INFLAMMATORY DRUGS TO GLUCOCORTICOID RECEPTORS *IN VITRO**†

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Abstract—The mechanism of action of non-steroidal anti-inflammatory drugs (NSAID) is felt to be via prostaglandin synthetase inhibition. Yet several clinical findings suggest these drugs may possess glucocorticoid agonist activity as well. To evaluate this possibility, a variety of non-steroidal anti-inflammatory drugs was examined to determine whether they compete for rat kidney glucocorticoid receptor sites *in vitro*. Meclofenamic acid, MK 410 (an indomethacin analogue) and indomethacin were the most potent competitors requiring 7×10^3-fold, 2×10^4-fold and 3×10^4-fold molar excess, respectively, to inhibit 50 per cent of the binding of 10 nM [^{3}H]dexamethasone to kidney cytosol binding sites. The same drugs also inhibited the binding of [^{3}H]dexamethasone in kidney slices requiring about 3-fold higher concentrations to achieve 50 per cent inhibition. At a 10^5-fold molar ratio, sulfinpyrazone reduced cytosol binding to 60 per cent, phenylbutazone to 85 per cent and ibuprofen to 89 per cent of control levels; aspirin and naproxen did not compete even at this concentration. Similarly, prostaglandins failed to compete with [^{3}H]dexamethasone for kidney receptors. Indomethacin inhibition of [^{3}H]dexamethasone binding appeared competitive when analyzed by a double reciprocal plot. As expected, inhibition of [^{3}H]dexamethasone cytosol binding by indomethacin prevented the appearance of the nuclear complex. Of the NSAID examined, none competed for plasma binding of [^{3}H]corticosterone except indomethacin which reduced the binding to 50 per cent of control at a 10^5-fold molar excess. In conclusion, these studies support the possibility that some NSAID may possess intrinsic glucocorticoid agonist activity. Furthermore, they indicate the glucocorticoid receptor site is not as specific as once thought and suggest that non-steroidal compounds may potentially serve as glucocorticoid agonists or antagonists.

Steroid hormone action is initiated by the binding of hormone to specific cytoplasmic receptors in target tissues [1]. Although each hormone receptor functions as a signal discriminator binding only ligands that possess a particular class of structural characteristics, it has become increasingly clear that non-steroidal anti-inflammatory drugs (NSAID) cluded from these steroid binding sites [2–4]. We have previously shown, for example, that some non-steroidal antiinflammatory drugs (NSAID) compete with aldosterone for binding sites in the kidney [5]. Since NSAID can bind to mineralocorticoid receptors, and since the major property of this class of drugs is an anti-inflammatory action, it was intriguing to consider whether some of the therapeutic efficacy of NSAID might be mediated by intrinsic glucocorticoid agonist activity in addition to the accepted mechanism—prostaglandin synthesis inhibition [6]. According to current concepts of hormone action, this would require binding of the NSAID to glucocorticoid receptors. To assess this possibility, we examined the ability of a series of NSAID to compete for [^{3}H]dexamethasone binding sites in kidney. As presented in this paper, despite non-steroidal structures and the varied configurations of the drugs tested, several NSAID appeared to have significant affinity for the glucocorticoid receptor *in vitro*.

MATERIALS AND METHODS

[1,2(n),^{3}H]dexamethasone, 26 Ci/m-mole, was purchased from Amersham/Searle (Arlington Heights, IL). Corticosterone was purchased from CalBiochem (La Jolla, CA). The following drugs were generously donated by the respective companies: indomethacin (IDM) [1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid], desmethyl-IDM [1-(*p*-chlorobenzoyl)-2-methyl-5-hydroxy-3-indolyl-acetic acid], desbenzoyl-IDM [2-methyl-(3-carboxymethyl)-5-methoxy indole], MK 410 (1-*p*-methylthiobenzyl-2-methyl-5-methoxy-3-indolyl-α-proprionate sodium salt), and dexamethasone (9-fluoro-11β,17,21-trihydroxy-16α-methyl-pregna-1,4-diene,3,20-dione) by Merck, Sharp & Dohme (West Point, PA); SC-19220 [1-acetyl-2-(8-chloro-10,11-dihydrodibenz- (b,f) (1,4)-oxazephine-10-carbonyl) hydrazine] by G. D. Searle (Chicago, IL); ibuprofen [2-(*p*-isobutylphenyl)-propionic acid] and prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$ by UpJohn (Kalamazoo, MI); phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) and sulfinpyrazone [1,2-diphenyl-4-(2′-phenylsulfinethyl)-3,5-pyrazolidinedione] by CIBA/Geigy (Summit, NJ); naproxen [(+)-6-methoxy-α-methyl-2-naphthalene acetic acid] by Syntex (Palo

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Alto, CA); meclofenamic acid (MCF) [*N*-(2,6-dichloro-*m*-tolyl) anthranilic acid] by Parke, Davis (Ann Arbor, MI); 5,8,11,14-eicosatetraynoic acid by Hoffmann–LaRoche (Nutley, NJ); and polyphloretin phosphate by A. B. Leo (Helsingborg, Sweden). Dr. Josef Fried (University of Chicago) kindly provided the 7-oxa-13-prostynoic acid. All drugs were used without further purification. The drugs were dissolved as described by the manufacturers either in alcohol or base. Final alcohol concentration was always less than 1 per cent and the pH of solutions was always titrated back to 7.4 before use. Solutions of drugs were made fresh daily.

Binding experiments. Male Sprague–Dawley rats of 100–200 g (Simonsen, Gilroy, CA) were adrenalectomized before use. The binding assay procedures have been described in detail previously [7–9]. In brief, after *in situ* perfusion with iced saline to wash out blood, kidneys were removed, rinsed and homogenized, and cytosol was prepared. Binding assays were performed with 10 nM [^{3}H]dexamethasone ± competitor incubated with cytosol for 3 hr at 0°. In kidney slice experiments, 275-μm slices were prepared using a McIlwain tissue chopper (Brinkman Instruments, Cantiague, NY). The slices were incubated at 25° for 40 min in a shaking water bath in incubating medium [7–9] containing 10 nM [^{3}H]dexamethasone ± competing drugs. At the conclusion of the incubation, the slices were rinsed, and cytosol was prepared. In both cytosol and slice experiments, protein-bound [^{3}H]-dexamethasone was determined by passing cytosol through G-50 fine Sephadex columns [7–9]. The protein peak was collected and aliquots were assayed for protein and radioactivity. In all binding studies, "non-specific" binding was defined as that binding resistant to incubation with 1000-fold excess unlabeled dexamethasone, and this value was subtracted from total binding to give "specific binding".

Nuclear binding. Kidney slices, prepared as described above, were incubated at 25° for 40 min in incubating medium with 10 nM [^{3}H]dexamethasone ± competing drugs. Pilot experiments showed that nuclear binding reached a plateau at 40 min. At the completion of the incubation, the slices were rinsed on nylon mesh, homogenized in a medium containing 0.25 M sucrose, 10 mM thioglycerol, 5 mM $MgCl_2$, 10 mM Tris, pH 7.4, and centrifuged at 700 *g* for 10 min to obtain crude nuclear pellets. The pellets were washed three times with homogenizing medium, extracted with cold ethanol at 0° for 30 min, and the extract was radioassayed for nuclear-bound steroid. DNA in the pellet was measured by the diphenylamine method [10].

RESULTS

To assess the ability of NSAID to bind to glucocorticoid receptors, assays were performed in which the ability of NSAID to compete for [^{3}H]dexamethasone binding sites was determined. Kidney cytosol or slices from adrenalectomized rats was employed as a source of glucocorticoid receptors. As shown in Fig. 1, several NSAID were successful in competing for [^{3}H]dexamethasone binding sites in both cytosol and slices. Of the drugs tested, meclofenamic acid was the most potent agent followed by MK-410, an indomethacin analogue, and indomethacin. In cytosol, these drugs required 7×10^3-fold, 2×10^4-fold and 3×10^4-fold molar excess, respectively, to achieve 50 per cent inhibition of [^{3}H]dexamethasine binding. For comparative purposes, about 2-fold dexamethasone or 2.5-fold corticosterone achieved 50 per cent reduction in [^{3}H]dexamethasone binding in the same system (data not shown).

Similar experiments were performed with kidney slices to determine whether the NSAID could penetrate cells and compete with [^{3}H]dexamethasone for receptor binding sites in intact cells. As shown in

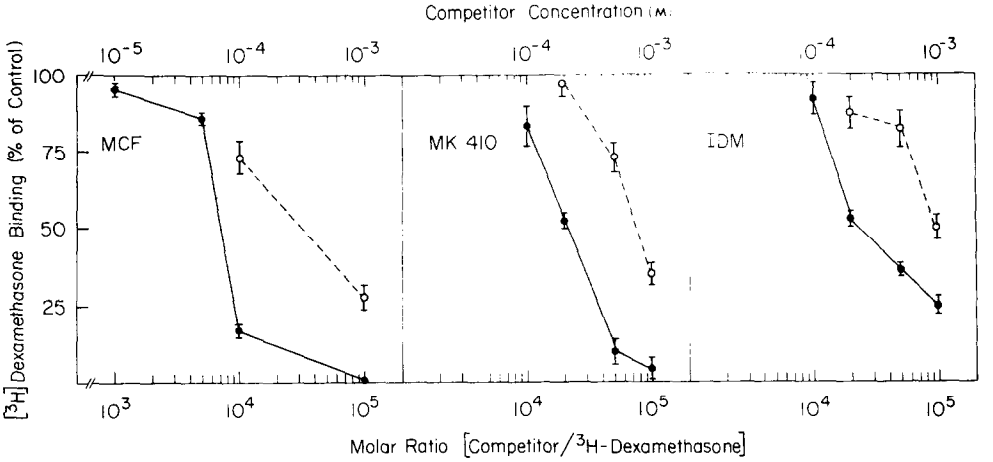

Fig. 1. NSAID competition for glucocorticoid receptors in kidney cytosol and slices. Kidney cytosol or slices were incubated with 10 nM [^{3}H]dexamethasone ± competitors. The cytosol (●) was incubated at 0° for 3 hr and specific binding determined. Total binding in the absence of competitor was 224 fmoles/mg of protein. The slices (○) were incubated at 25° for 40 min and specific binding was determined in the cytosol. Total binding in the absence of competitor was 104 fmoles/mg of protein. Symbols are MCF, meclofenamic acid; and IDM, indomethacin. Each point is the mean ± S.E. of four to six experiments.

Table 1. NSAID competition for specific [^{3}H]dexamethasone binding sites in rat kidney cytosol*

Drug	Per cent of control binding
Sulfinpyrazone	60 ± 4
Phenylbutazone	85 ± 3
Desmethyl-IDM	83 ± 13
Ibuprofen	89 ± 3
Aspirin	95 ± 2
Naproxen	98 ± 8
Desbenzoyl-IDM	103 ± 10

* Concentrations: [^{3}H]dexamethasone, 10 nM; NSAID 10^5-fold excess or 1 mM. Total binding in the absence of competitor was 224 fmoles/mg of cytosol protein. Values are means ± S. E. of four to six determinations.

Fig. 1, all three drugs tested were able to compete in slices, but in each case the concentration required to achieve 50 per cent reduction of [^{3}H]dexamethasone binding was approximately 3-fold higher than that required in cytosol. By way of comparison, the ability of unlabeled dexamethasone to compete remained relatively stable in slices whereas the corticosterone concentration required to achieve 50 per cent inhibition in slices was slightly higher than in cytosol (data not shown).

A series of additional NSAID were tested for competitive ability in cytosol and the results are listed in Table 1. It is apparent that these drugs are much less potent competitors, achieving less than 50 per cent inhibition of [^{3}H]dexamethasone binding at molar ratios of 10^5-fold. For comparative information, the relative anti-inflammatory potency of some of these drugs as judged by the available published ED_{50} required to inhibit carrageenan edema formation is as follows: IDM > MCF > naproxen > phenylbutazone > ibuprofen > aspirin [11, 12].

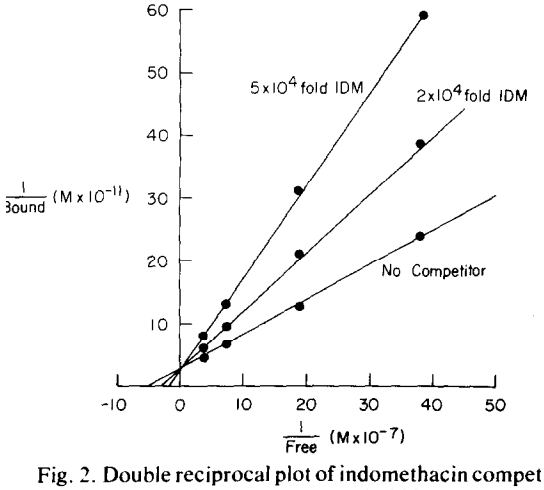

Fig. 2. Double reciprocal plot of indomethacin competition for [^{3}H]dexamethasone receptors in kidney cytosol. Kidney cytosol was incubated with [^{3}H]dexamethasone at various concentrations between 2.6 and 26 nM, either in the absence of indomethacin or in the presence of 2×10^4-fold or 5×10^4-fold indomethacin. Non-specific binding was subtracted from each point so that the plot represents specific binding. Each point is the mean of triplicate determinations.

Table 2. Inhibition of nuclear binding by NSAID*

Competitor	Molar ratio	Nuclear binding (fmoles/100 mg slices)
Control		217 ± 20
Dexamethasone	1	141 ± 18
Dexamethasone	10	44 ± 4
Indomethacin	10^5	67 ± 6
Meclofenamic acid	10^5	91 ± 8

* Slices were incubated 40 min at 25° in medium containing 10 nM [^{3}H]dexamethasone ± competitor. Values are means ± S. E. of four determinations.

To assess whether the decrease in [^{3}H]dexamethasone binding was the result of a competitive interaction, experiments were performed in kidney cytosol with various indomethacin and [^{3}H]dexamethasone concentrations and the data graphed as a double reciprocal plot. As shown in Fig. 2, the intercept on the vertical axis is unaffected by increasing the concentration of the indomethacin competitor, whereas the slope of the line is shifted. This pattern is consistent with competitive inhibition of the [^{3}H]dexamethasone binding.

Studies were then performed to confirm that indeed the decrease in [^{3}H]dexamethasone binding in cytoplasmic fractions of slices was associated with an inhibition of transfer of [^{3}H]dexamethasone–receptor complex to the nucleus. As shown in Table 2, nuclear binding was inhibited in roughly the proportion that cytoplasmic binding was decreased. Indomethacin and meclofenamic acid at a 10^5-fold molar ratio were better inhibitors than 1-fold dexamethasone but not as potent as 10-fold dexamethasone.

An additional property of NSAID which may be important in their interaction with the glucocorticoid

Table 3. Competition for [^{3}H]corticosterone binding sites in plasma*

Drug	Molar ratio [competitor/ [^{3}H]corticosterone]	Per cent of control binding
Corticosterone	1	49 ± 2
	10	6 ± 1
Dexamethasone	10	106 ± 3
Indomethacin	10^4	94 ± 4
	10^5	60 ± 4
Meclofenamic acid	10^5	91 ± 5
Phenylbutazone	10^5	102 ± 2
Ibuprofen	10^5	101 ± 4
Naproxen	10^5	101 ± 2

* [^{3}H]corticosterone, 26 nM, was incubated with plasma ± competitor for 15 min at 25° and binding determined. Non-specific binding has been subtracted from all values. Control binding in the absence of competitor was 87 nmoles/mg of protein. Values are means ± S. E. of three to four determinations.

Table 4. Competition for [^{3}H]dexamethasone binding sites in kidney cytosol*

Drug	Per cent of control binding (50 μg/ml drug)	(100 μg/ml drug)
Eicosatetraynoic acid	60 ± 4	26 ± 2
Polyphloretin phosphate	99 ± 5	61 ± 5
Dibenzoxazepine hydrazide	90 ± 6	85 ± 4
7-Oxa-13-prostynoic acid	115 ± 3	98 ± 8
Prostaglandin E_1	99 ± 2	97 ± 1
Prostaglandin E_2	107 ± 5	103 ± 4
Prostaglandin $F_{2\alpha}$	111 ± 2	112 ± 6

* Cytosol was incubated for 3 hr at 0° in the presence of 10 nM [^{3}H]dexamethasone ± competitor. At a concentration of 100 μg/ml, the competitor drugs are ~ 300 μM or a molar ratio of ~ 3×10^4 to [^{3}H]dexamethasone. Total specific binding in the absence of competitor was 224 fmoles/mg of cytosol protein. Values are means ± S. E. of at least four determinations.

system is the possibility that they may bind to transcortin, the corticosteroid binding globulin (CBG) in plasma. It is known that the NSAID bind extensively to plasma proteins but it is generally felt to be to non-specific sites on albumin [13]. To evaluate the possibility of binding to CBG and thus possibly displacing endogenous corticoids from specific plasma sites, competition studies were performed with [^{3}H]corticosterone bound to high affinity plasma sites [9]. As shown in Table 3, unlabeled corticosterone was a very potent competitor, inhibiting 50 per cent of the specific binding at a concentration equivalent to that of [^{3}H]corticosterone, that is a 1-fold molar ratio. Dexamethasone was a poor competitor for CBG binding sites, a fact which emphasizes the difference between these binding sites and the cytoplasmic receptors. Of the NSAID tested, all failed to reduce [^{3}H]corticosterone binding at 10^4-fold excess. Only indomethacin achieved significant competition at 10^5-fold excess.

In addition to NSAID, we were interested in the possibility that prostaglandins or prostaglandin antagonists [14] might also bind to glucocorticoid receptors. As shown in Table 4, at concentrations of 50–100 μg/ml, some of the prostaglandin antagonists successfully decreased specific [^{3}H]dexamethasone binding. Eicosatetraynoic acid was particularly active. Because of its structural resemblance to the prostaglandins, the latter were also examined for their competitive ability. None of the prostaglandins tested exhibited any potency for the binding sites.

DISCUSSION

The data presented indicate that some NSAID possess the structural requirements necessary to inhibit the binding of [^{3}H]dexamethasone to glucocorticoid receptors. The ability of NSAID to inhibit binding is dose dependent (Fig. 1), appears competitive (Fig. 2), and occurs in slices as well as cell-free cytosol (Fig. 1). As expected, inhibition of cytoplasmic binding blocks the formation of the [^{3}H]dexamethasone–nuclear complex (Table 2). In addition to NSAID, at least one prostaglandin antagonist, eicosatetraynoic acid, also exhibited some potency as a competitor; the prostaglandins themselves did not compete (Table 4). Whereas three drugs were potent competitors for cellular receptor sites (Fig. 1), only indomethacin exhibited affinity for the plasma binding sites presumably on CBG (Table 3).

Although it is clear that NSAID inhibit prostaglandin synthesis and that this is probably the major mechanism for their anti-inflammatory activity [6], all actions of these drugs may not be ascribable to this one mechanism. Since the present data indicate that some NSAID can bind to receptors, the possibility is raised that NSAID may possess intrinsic glucocorticoid activity. Furthermore, some of the activities previously described for various NSAID support the possibility of intrinsic glucocorticoid potency. For example, the following functions have been documented for aspirin, phenylbutazone or indomethacin, the drugs in use the longest: suppression of the pituitary–adrenal axis [15, 16], occasional improvement in the condition of patients with Addison's disease [7], suppression of lymphocyte transformation [18], induction of hepatic tyrosine amino transferase and other glucocorticoid inducible enzymes [19], and inhibition of cyclic AMP phosphodiesterase activity [20], an action reported for glucocorticoids [21]. Thus, there is some support for glucocorticoid agonist-like activity by various NSAID.

In order to better evaluate the possibility of intrinsic glucocorticoid agonist activity, compounds chemically related to IDM but lacking anti-inflammatory potency were studied. Of the four related compounds tested, IDM is the most potent anti-inflammatory agent and also the most potent inhibitor of prostaglandin synthetase [22]. Desmethyl-IDM and desbenzoyl-IDM are urinary metabolites. The former compound is modestly active as a synthetase inhibitor and has weak anti-inflammatory activity; the latter compound is essentially devoid of both activities [22]. The ability of these three compounds to bind to glucocorticoid receptors is in the same relative relationship as their anti-inflammatory activity (Table 1 and Fig. 1). MK-410, on the other hand, has only marginal synthetase-inhibiting activity *in vitro* which is much less than expected from its anti-inflammatory and analgesic efficacy in animal models and in man [22]. It is tempting to speculate that some of its anti-inflammatory activity may derive from glucocorticoid agonist activity, reflected by its high potency as a [^{3}H]dexamethasone competitor (Fig. 1). Accordingly, the very potent anti-inflammatory activity of IDM and MCF may derive from both prostaglandin synthetase and glucocorticoid agonist activity, the moderate anti-inflammatory activity of MK-410 may depend more on glucocorticoid agonist activity than synthetase inhibition, and the activity of naproxen, aspirin and ibuprofen may be mediated mainly by synthetase activity with some glucocorticoid contribution possible at high doses.

However, before extrapolations from the binding

data to estimates of functional activity for the various NSAID are accepted, several important points must be considered. First, the competitive binding experiments are an indirect approach. Since the NSAID are not available in radioactive form, their ability to bind to receptors has been measured by their ability to inhibit [^{3}H]dexamethasone binding. A positive result in a competitive assay may result from destruction of the receptor or binding to an allosteric site rather than the active site. Without direct binding experiments using radioactively labeled NSAID, it is difficult to resolve these possibilities. The competitive nature of the inhibition of [^{3}H]dexamethasone binding (Fig. 2) speaks against simple receptor destruction. Additional points which suggest specific binding are the differential inhibition of glucocorticoid and mineralocorticoid receptors [5] and the lack of competition for CBG.

The second important point requiring emphasis is the difference between binding and function. Even when receptor binding of a ligand can be directly demonstrated, it is not possible to predict whether this will result in agonist or antagonist function [1, 23, 24]. Since some NSAID appear to bind, it is clear that some activity will ensue. However, functional studies will be required to elucidate the nature of the end result of these binding interactions. For example, sulfinpyrazone competes for receptor sites but apparently lacks anti-inflammatory activity.

A third aspect to be stressed is that these experiments were performed *in vitro* and that extrapolation to the therapeutic situation is difficult. Relatively high concentration ratios were required to achieve significant dexamethasone competition. The likelihood of obtaining circulating concentrations high enough to occupy receptors *in vivo* differs for each drug and depends on many variables in addition to affinity for the receptor. At least one factor which reduces drug availability to the receptor, plasma binding to CBG [7], seems not to be important for most NSAID. To evaluate the possibility of achieving adequate concentrations of NSAID to occupy receptors, the following data are supplied. Doses of dexamethasone, which in normal patients cause suppression of the pituitary-adrenal axis and thus are presumably at an anti-inflammatory level, achieve plasma concentrations of ~5–10 nM [25]. In comparison, therapeutic doses of indomethacin in patients achieve concentrations in the range of ~10 μM [26]. Assays *in vivo* will be required to determine whether glucocorticoid receptor occupancy can be achieved with therapeutic doses of NSAID. However, studies *in vitro* employing high concentrations of NSAID as prostaglandin synthetase inhibitors must certainly be interpreted with caution.

In conclusion, the data demonstrate two important concepts. First is the thesis that the glucocorticoid receptor is not as specific as previously considered and that potential ligands are not restricted to those compounds possessing steroidal configurations. This supports the feasibility of designing steroidal agonist or antagonists with non-steroidal structures and thus provides more options and greater flexibility to the pharmaceutical chemists. Second is the consideration that NSAID may have multiple actions. Although a glucocorticoid function for NSAID has not been proven, the possibility has been raised. One must be careful, therefore, in attributing all actions of NSAID to prostaglandin inhibition. Variations in function, potency and specificity of the NSAID may be related, in part, to variations in non-prostaglandin types of activity.

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