Dexamethasone Megadoses Stabilize Rat Liver Lysosomal Membranes by Non-Genomic and Genomic Effects

Burkhard Hinz[1,3] and Rolf Hirschelmann[2,†]

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Purpose. Membrane-stabilizing effects may be part of glucocorticoid action during high-dose glucocorticoid therapy. The present study investigates the mode of action of dexamethasone megadoses on rat liver lysosomal membranes.

Methods. Following intravenous administration of dexamethasone in rats, the release of β-glucuronidase from liver lysosomes was assessed *ex vivo* as a marker for lysosomal membrane integrity.

Results. Dexamethasone megadoses significantly inhibited β-glucuronidase release 10 min post-administration by 38% (3 mg/kg dexamethasone) and 33% (10 mg/kg dexamethasone) at corresponding dexamethasone liver concentrations of 3.9×10^{-5} mol/kg and 15.1×10^{-5} mol/kg, respectively. Comparable inhibition of β-glucuronidase release (34% for 3 mg/kg and 38% for 10 mg/kg) was observed 24 h after administration of dexamethasone, although dexamethasone liver concentrations had already declined to 0.09×10^{-5} mol/kg and 0.19×10^{-5} mol/kg, respectively. A 2-h oral pretreatment of rats with the glucocorticoid receptor antagonist RU 486 (10 mg/kg) did not alter immediate (10 min) stabilization by dexamethasone (3 mg/kg), but almost completely prevented lysosomal membrane protection 24 h after dexamethasone injection.

Conclusions. Dexamethasone megadoses may preserve lysosomal membrane integrity by a dual action involving both rapid nongenomic effects occurring instantaneously after administration and long-term receptor-dependent genomic events.

KEY WORDS: lysosomal membrane stabilization; dexamethasone megadoses; nongenomic steroid action; genomic steroid action.

INTRODUCTION

Glucocorticoids have been shown to exert most of their effects genomically, i.e., via occupation of cytosolic glucocorticoid receptors, translocation of the glucocorticoid-receptor complex into the nucleus, and subsequent activation or repression of *de novo* synthesis of mRNA and protein (1–3). Genomic effects are characterized by a lag period ranging from at least 30 min to several hours or even days between the entry of the glucocorticoid into the cell and the manifest action of its hormonal response. However, compelling evidence suggests that, in addition to genomic effects, glucocorticoids may exert nongenomic actions occurring instantaneously after glucocorticoid exposure, or with a very short latency period (4–6). Nongenomic glucocorticoid effects are mediated by membrane-bound receptors, or by physicochemical interactions with cellular membranes, and are insensitive to competitive inhibitors of intracellular glucocorticoid receptors.

Glucocorticoid pulse therapy involves intravenous administration of high doses of prednisolone (0.5–2 g) or its equivalents (7). The goal of pulse therapy is to avoid the complications and side effects of long-term steroid therapy, and to achieve antiinflammatory and immunosuppressive effects similar to those obtained with high daily doses of glucocorticoids (7). Introduced for the treatment of kidney rejection episodes about 30 years ago, pulse therapy has been successfully used for various severe pathological conditions such as rheumatoid arthritis (8), systemic lupus erythematosus (9), shock (10), acute spinal cord trauma (11), brain disorders where edema and elevated intracranial pressure are involved (12), and more recently for multiple sclerosis (13). As the glucocorticoid plasma and tissue concentrations achieved under treatment with glucocorticoid megadoses are far beyond those required for cytosolic receptor saturation, additional nongenomic effects have been discussed to account for their therapeutic action (6). Accordingly, glucocorticoids have been shown to stabilize lysosomal membranes in the 10^{-6}–10^{-4} M range, thereby inhibiting the release of lysosomal enzymes (4, 6, 14, 15). Lysosomal enzymes have been implicated as mediators of acute and chronic inflammatory states, e.g., articular tissue degradation in several rheumatic diseases (16). However, the mechanism underlying the membrane-stabilizing action of glucocorticoids is still a subject of debate. In the *in vivo* studies published so far, effects of glucocorticoids on lysosomes have been investigated under experimental conditions that make it difficult to distinguish between genomic and nongenomic steroid action (17,18).

To assess the mode of action of high-dose glucocorticoid treatment on the integrity of rat liver lysosomal membranes under conditions as close as possible to the situation *in vivo,* the present study investigates the effect of dexamethasone megadoses on the integrity of liver lysosomal membranes of rats treated with the steroid *in vivo* before the isolation of lysosomes. We show herein that dexamethasone megadoses may preserve lysosomal membrane integrity by a dual action involving both receptor-independent nongenomic effects occurring instantenously after administration as well as long-term receptor-dependent genomic events.

MATERIALS AND METHODS

Animals

Female Wistar rats weighing 140–220 g were housed in cages with free access to standard food and tap water. They were maintained at a room temperature of 23 ± 1°C. All experiments were in accordance with the "Principles of Laboratory Animal Care."

Experimental Protocol

Dexamethasone disodium phosphate (KRKA Pharmaceuticals, Novo mesto) was administered intravenously at doses equivalent to 0.1, 3, and 10 mg/kg dexamethasone. To assess whether the observed effects of dexamethasone were mediated via intracellular glucocorticoid receptors, rats were

[1] Department of Experimental and Clinical Pharmacology and Toxicology, Friedrich Alexander University Erlangen-Nürnberg, Fahrstrasse 17, 91054 Erlangen, Germany.
[2] Department of Pharmacology and Toxicology, School of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Strasse 4, 06120 Halle (Saale), Germany.
[3] To whom correspondence should be addressed. (e-mail: hinz@pharmakologie.uni-erlangen.de)
[†] Deceased in March 2000.

ABBREVIATIONS: cyclic AMP, cyclic 3′,5′-adenosine monophosphate; cyclic GMP, cyclic 3′,5′-guanosine monophosphate; i.v., intravenous.

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pretreated with the glucocorticoid receptor antagonist RU 486 (generously supplied by Roussel Uclaf, Romainville, France) 2 h prior to i.v. administration of dexamethasone. RU 486 (10 mg/kg) was administered orally as a suspension (vehicle: 3 drops Tween 80/10 ml 0.9% saline). Control animals received the respective vehicle. Animals were killed by cervical dislocation 10 min or 24 h after i.v. administration of dexamethasone and livers were obtained for subsequent studies.

Determination of the Integrity of Rat Liver Lysosomes

The integrity of rat liver lysosomes was assessed by determining the release of the specific marker enzyme β-glucuronidase (EC 3.2.1.31; β-D-glucuronide-glucuronohydrolase) according to Ignarro (17). Following cervical dislocation, the liver was excised, rinsed quickly, minced in ice-cold 0.25 M sucrose/0.05 M Tris acetate pH 7.4, and two portions of the largest liver lobe (0.7 g each) were weighed. For homogenization procedures, a Potter homogenizer with a motor driven glass pestle (600 rmp) was used. One portion of liver was homogenized on ice in 6.3 ml of 0.07 M sucrose/0.05 M Tris acetate pH 7.4. The remaining portion of liver was homogenized on ice in 6.3 ml of 0.1% v/v Triton X-100/0.05 M Tris acetate pH 7.4. These two preparations will be referred to as sucrose homogenates and Triton X-100 homogenates, respectively. The homogenates were kept at 4–8°C for 30 min and centrifuged thereafter at 27,000 g for 20 min. Supernatant fractions (0.1 ml) were removed and assayed for β-glucuronidase activity.

The activity of β-glucuronidase was determined according to Fishman (19). This assay is based on the colorimetric determination of phenolphthalein liberated from its substrate phenolphthalein-β-D-glucuronide by the action of β-glucuronidase. 27,000 g supernatants (0.1 ml) were incubated with 0.8 ml 0.1 M acetate buffer pH 7.4 and 0.1 ml 5 mM phenolphthalein-β-D-glucuronide for 1 h at 38°C. For blank values, incubations of supernatants were performed with 0.9 ml 0.1 M acetate buffer. The reaction was stopped by a 1-min boiling of the samples. After addition of 1.5 ml water all solutions were centrifuged at 5000 g for 10 min. For colorimetric determination of phenolphthalein, 2 ml 5000-g supernatant was added to a solution consisting of 2.5 ml 0.22 M glycine/0.22 M sodium chloride, 1 ml 5% (m/V) trichloroacetic acid, 0.35 ml 1 M sodium hydroxide and 0.15 ml water. The liberated phenolphthalein was determined colorimetrically at 540 nm. The lysosomal enzyme activity in supernatant fractions derived from Triton X-100 homogenates was used as an index of total activity. Lysosomal enzyme activity in supernatant fractions derived from sucrose homogenates reflects that portion of total activity which was redistributed to the free form as a result of the mechanical (homogenization) and osmotic (hypotonic sucrose buffer) forces applied to the liver lysosomes. Results were expressed as changes in the ratio between free and total β-glucuronidase activity according to the following formula: β-glucuronidase release (%) = (free activity × 100) / total activity. The calculated ratio was used as an index of the integrity of rat liver lysosomal membranes. Accordingly, agents that stabilize lysosomal membranes should retard the redistribution of β-glucuronidase into the free form thereby decreasing the percentual β-glucuronidase release.

HPLC Determination of Dexamethasone in Rat Liver

For determination of dexamethasone in rat liver, portions of liver were homogenized using a Potter homogenizer and extracted with methanol. Dexamethasone was determined by high performance liquid chromatography (HPLC) technique (Merck-Hitachi; UV detection: 242 nm) by means of a C18 reversed-phase column (5 μm, 125 mm × 4 mm; Merck, Darmstadt, Germany) and a mobile phase of methanol/water (60/40% by volume) at a flow rate of 0.8 ml/min. Prednisolone was used as an internal standard. The limit for determination of dexamethasone was 25 nM.

Statistics

Comparisons between groups were performed using Student's unpaired t test. $P < 0.05$ was considered to be statistically significant.

RESULTS

To investigate the effect of dexamethasone megadoses on the integrity of lysosomal membranes, the release of β-glucuronidase from rat liver lysosomes was determined *ex vivo* as a marker for lysosomal membrane integrity. According to Fig. 1A, treatment of rats with dexamethasone megadoses significantly inhibited β-glucuronidase release 10 min post-administration by 38% (3 mg/kg) and 33% (10 mg/kg), respectively. Corresponding dexamethasone liver concentrations were 3.9×10^{-5} mol/kg (3 mg/kg dexamethasone) and 15.1×10^{-5} mol/kg (10 mg/kg dexamethasone) (Table I). Ad-

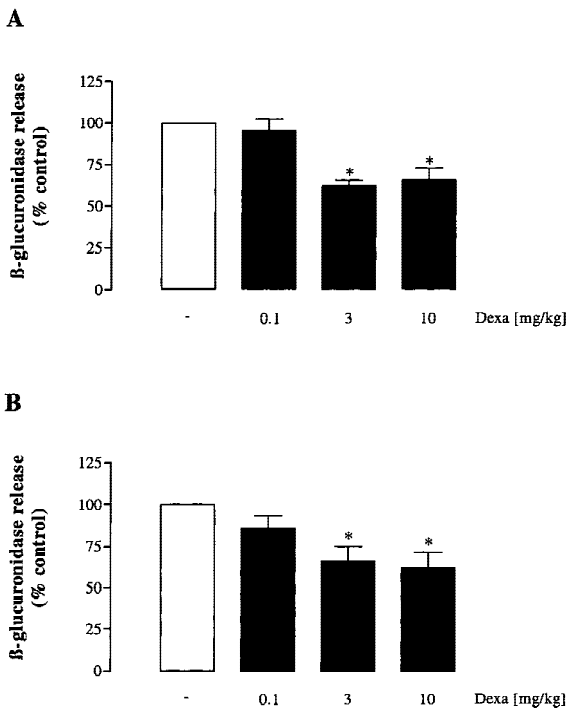

Fig. 1. Influence of dexamethasone (0.1–10 mg/kg) on the release of β-glucuronidase from rat liver lysosomes 10 min (panel A) and 24 h (panel B) after i.v. administration. Results were expressed as ratios between free and total β-glucuronidase activity. β-glucuronidase release from liver lysosomes from rats treated with vehicle served as controls and were set as 100%. Values are means ± SEM of n = 6 animals per group. *$P < 0.05$ treatment vs. control (open column), Student's t test.

Table 1. Dexamethasone Concentrations in Rat Liver after i.v. Administration of Dexamethasone[a]

Group	Dexamethasone liver concentration [10^{-5} mol/kg wet tissue]	
	10 min post-administration	24 h post-administration
Dexamethasone 0.1 mg/kg i.v.	0.29 ± 0.05	n.d.[b]
Dexamethasone 3 mg/kg i.v.	3.90 ± 0.33	0.09 ± 0.01
Dexamethasone 10 mg/kg i.v.	15.10 ± 1.37	0.19 ± 0.07

[a] Following homogenization of livers, dexamethasone concentrations were determined by HPLC technique. Values are means ± SEM of n = 6 animals per group.
[b] (n.d. = not detectable)

ministration of dexamethasone at a dose of 0.1 mg/kg leading to a liver concentration of 0.29×10^{-5} mol/kg left lysosomal enzyme release virtually unaltered (Fig. 1A).

When lysosomal membrane integrity was determined 24 h after administration of dexamethasone megadoses, comparable inhibitions of β-glucuronidase release (34% for 3 mg/kg and 38% for 10 mg/kg) were observed (Fig. 1B). At this time dexamethasone liver concentrations had declined to 0.09×10^{-5} mol/kg and 0.19×10^{-5} mol/kg, respectively (Table 1). Administration of 0.1 mg/kg dexamethasone led to a 15% inhibition of β-glucuronidase release 24 h post-injection (Fig. 1B).

To assess a possible involvement of intracellular glucocorticoid receptors in mediating lysosomal membrane stabilization, additional experiments were performed using the glucocorticoid receptor antagonist RU 486. A 2-h oral pretreatment of rats with RU 486 (10 mg/kg) left stabilization of lysosomal membranes 10 min after administration of dexamethasone (3 mg/kg i.v.) unaltered, but almost completely prevented lysosomal membrane stabilization by dexamethasone 24 h after injection (Fig. 2A,B). Pretreatment of rats with RU 486 did not influence dexamethasone concentrations in rat liver as compared to respective levels determined after administration of dexamethasone alone (data not shown). Furthermore, administration of RU 486 alone displayed no significant influence on lysosomal membrane integrity at both time points studied (Fig. 2A,B).

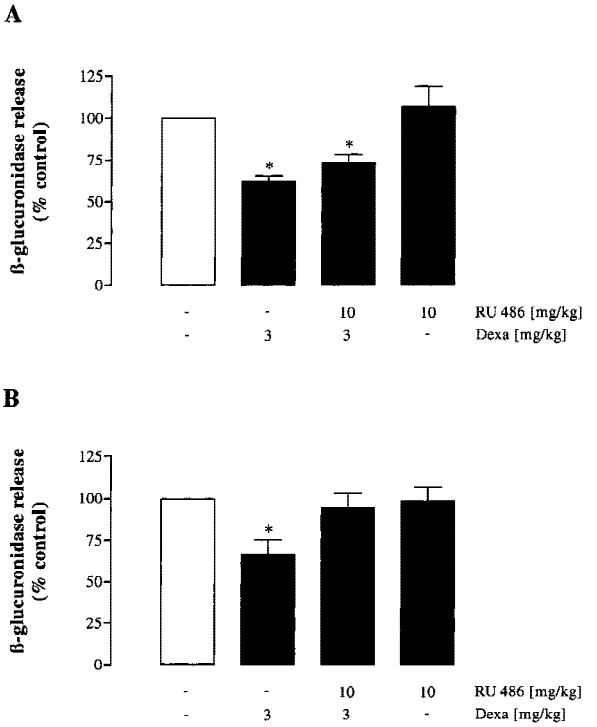

Fig. 2. Influence of RU 486 (10 mg/kg) on the inhibitory effect of dexamethasone (3 mg/kg) on β-glucuronidase release from rat liver lysosomes 10 min (panel A) and 24 h (panel B) after i.v. administration of dexamethasone. RU 486 (10 mg/kg) was administered orally 2 h prior to i.v. administration of dexamethasone. Results were expressed as ratios between free and total β-glucuronidase activity. β-glucuronidase release from liver lysosomes from rats treated with vehicle served as controls and were set as 100%. Values are means ± SEM of n = 6 animals per group. $*P < 0.05$ treatment vs. control (open column), Student's t test.

DISCUSSION

The present study demonstrates a stabilizing action of dexamethasone megadoses on liver lysosomes of rats. Furthermore, our data point towards a sequential protective action of dexamethasone on the integrity of lysosomal membranes with an immediate nongenomic effect and a delayed genomically-mediated process.

Glucocorticoids have been reported to stabilize lysosomal membranes since the early 1960s (14). However, previous studies investigating the influence of glucocorticoids on lysosomal membrane integrity of rats treated with these agents *in vivo* before the isolation of lysosomes (17,18) were performed under experimental conditions (i.e., administration of steroids at 18 h and again 3 h prior to sacrifice and liver removal) that make it impossible to distinguish between genomic and nongenomic steroid action. In the present study, the release of β-glucuronidase from rat liver was determined *ex vivo* before and after decline of the typical lag time necessary for receptor-dependent genomic effects of glucocorticoids, thus allowing a discrimination of nongenomic and genomic effects. Moreover, to ensure a short time interval between glucocorticoid administration and first liver removal, dexamethasone was administered intravenously as the disodium phosphate salt that has been shown to release its alcohol more rapidly than hemisucinates being used for intravenous administration of prednisolone and methylprednisolone (20,21).

In the present study, inhibitory effects of dexamethasone megadoses on β-glucuronidase release from rat liver lysosomes were demonstrated as early as 10 min after administration of the steroid. This rapid onset of action stands in marked contrast to the widely reported latency of action of glucocorticoids that is attributed to the time required to synthesize or to repress *de novo* synthesis of mRNA and protein (1–3). Further support for nongenomic events underlying fast membrane stabilization may be derived from our observation that pretreatment of rats with the glucocorticoid receptor antagonist RU 486 did not alter the stabilizing action of dexamethasone. For the same dose and route of administration used in our experiments, RU 486 has previously been demonstrated to be effective in antagonizing antiinflammatory

effects of dexamethasone in rats (22,23) and to interact with cytosolic glucocorticoid receptors in various rat tissues (24).

Nongenomic effects of glucocorticoids occurring within seconds to minutes may be divided into specific nongenomic actions mediated by membrane-bound receptors and unspecific nongenomic events initiated by physicochemical interactions with cellular membranes (5,6). In comparison to the concentrations necessary for specific nongenomic effects (i.e., $>10^{-9}$ M), the glucocorticoid concentrations reported for direct interaction with biological membranes are in the micromolar range (i.e., 10^{-6}–10^{-4} M) (4,6,14,15). In our experiments, dexamethasone liver concentrations were even higher than 10^{-5} mol/kg at 10 min after i.v. administration of 3 mg/kg and 10 mg/kg dexamethasone, respectively. By contrast, dexamethasone at a dose of 0.1 mg/kg that has been shown to exert receptor-dependent antiinflammatory effects in rats and mice (22,23) failed to stabilize liver lysosomal membranes at corresponding liver concentrations below 10^{-5} mol/kg. Thus, to provide inhibition of lysosomal enzyme release, dexamethasone doses had to be used that produce plasma and tissue levels beyond those required for receptor saturation. Therefore, it is conceivable that nonspecific, nongenomic effects may underlie the rapid dexamethasone effects described here. It has been suggested in this context that glucocorticoids cause a direct membrane stabilization by condensing or restricting the movement of phospholipid acyl chains that extend into the hydrophobic inner layers of the membrane bilayer (25).

Most surprisingly, the inhibitory effects of dexamethasone megadoses on β-glucuronidase release observed 24 h after administration of the steroid were comparable to those occurring 10 min post-injection. As dexamethasone liver concentrations determined 24 h after megadose treatment had already declined to 0.09×10^{-5} mol/kg (3 mg/kg dexamethasone) and 0.19×10^{-5} mol/kg (10 mg/kg dexamethasone), respectively, membrane stabilization could not be a result of a long-lasting accumulation of dexamethasone in rat liver. Moreover, if the remaining dexamethasone concentrations were responsible for direct nongenomic membrane effects, administration of 0.1 mg/kg dexamethasone producing tissue levels of 0.29×10^{-5} mol/kg at 10 min after injection should be expected to exert a fast stabilizing effect. The late inhibitory effect of dexamethasone on β-glucuronidase release was almost completely prevented by RU 486, suggesting that dexamethasone, in addition to its immediate receptor-independent stabilizing action, may exert a long-term protective effect on lysosomal membranes via receptor-dependent events. Further evidence for this assumption may be derived from the finding that dexamethasone at a dose of 0.1 mg/kg caused a moderate but reproducible decrease in β-glucuronidase release from liver lysosomes 24 h after injection. In contrast to the expected dose dependency in genomic steroid action, both dexamethasone megadoses preserved membrane integrity to a comparable degree. This plateau effect may be explained by the fact that the available glucocorticoid receptors are saturated following administration of a dose as high as 3 mg/kg. A lack of dose dependency beyond a critical glucocorticoid concentration has also been reported in a previous study investigating the influence of several glucocorticoids on homologous passive cutaneous anaphylaxis in rats (26) with dexamethasone acetate and hydrocortisone acetate reaching a plateau effect at dosages exceeding 1 mg/kg and 10 mg/kg, respectively.

An involvement of genomic events in the stabilization of lysosomal membranes may also explain earlier findings showing increased fragility of liver lysosomes isolated from adrenalectomized rats, in comparison to lysosomes isolated from intact animals (27). It is tempting to speculate that dexamethasone displays long-term effects on the composition and function of lysosomal membranes. Indeed, most of the steroid effects on membrane properties reported so far have been demonstrated to be receptor-mediated rather than direct receptor-independent actions (4). Moreover, *in vitro* lysosomal enzyme release from rat liver and human neutrophils was inhibited by cyclic 3′, 5′-adenosine monophosphate (cyclic AMP) elevating agents (e.g., catecholamines) and enhanced by cyclic 3′, 5′-guanosine monophosphate (cyclic GMP)-generating compounds (28,29). As glucocorticoids are required under certain conditions for the full expression of cyclic AMP-mediated hormonal activities (4), it is also conceivable that dexamethasone may regulate the stability of lysosomal membranes indirectly via receptor-mediated "permissive" effects (e.g., regulation of β-adrenergic receptors, increase in intracellular cAMP levels by inhibition of cAMP-dependent phosphodiesterase enzymes).

The results of the present study support the recently suggested hypothesis that the mechanisms underlying the therapeutic effect of high glucocorticoid doses may include both nongenomic and genomic effects that may act in an additive manner (6). Accumulation or labilization of lysosomes in injured sites has long been known to be one of the manifestations of inflammatory processes. In particular, lysosomal enzymes have been shown to play a crucial role in rheumatoid arthritis as they may degrade components of the connective tissue, such as collagen, protein–mucopolysaccharide complexes, glycoproteins, elastin, and fibrin. Accordingly, enhanced activity of lysosomal enzymes has been observed in the synovial fluid of patients with rheumatoid arthritis (16). Moreover, pretreatment of kidney donors with large intravenous doses of methylprednisolone has been demonstrated to prevent the release of lysosomal enzymes from ischemic kidney cells, thereby reducing the incidence of acute tubular necrosis (30).

Altogether, our data strongly suggest that, in addition to its fast receptor-independent stabilizing effect, dexamethasone megadoses may exert long-term actions on the lysosomal membrane via a receptor-dependent mechanism. In conclusion, genomic corticosteroid effects on lysosomal properties in connection with the early onset nongenomic membrane stabilization may contribute to the antiinflammatory and immunosuppressive activity of glucocorticoid megadoses in the therapy of various clinically important disorders.

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