Vol. 112, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1983 Pages 488-495

CHLOROQUINE STABILIZES HEPATIC GLUCOCORTICOID RECEPTORS

Mohammed Kalimi, John R. Hubbard, James R. Gaut, and Andrew Lord

Department of Physiology and Biophysics
Medical College of Virginia
Virginia Commonwealth University
Richmond, Virginia 23298 (U.S.A.)

Received March 14, 1983

Chloroquine (an antiarthritic, antimalarial, lysosomotropic amine) was found to significantly stabilize rat unbound hepatic glucocorticoid receptors in vitro for 2 h at 25°C. Chloroquine stabilization was concentration dependent with statistically significant protection at 0.3 mM concentration and optimal effectiveness at approximately 3 mM. KCl (0.3 M) induced unbound receptor inactivation at low temperature was also markedly reduced in the presence of 3 mM chloroquine. In addition, steroid prebound complexes were significantly stabilized at 4°C and 25°C by 3 mM chloroquine. Unlike molybdate (perhaps the most commonly used glucocorticoid receptor stabilizing reagent), chloroquine did not alter the sedimentation of glucocorticoid-receptor complexes in sucrose-density gradients. These results suggest that chloroquine may have useful application in glucocorticoid receptor quantitation, characterization and purification and may have interesting implications into the biological and pharmacological effects of chloroquine.

Chloroquine is a lysosomotropic amine that is well known for its antimalarial (1) and antiarthritic action (1). Recently chloroquine has been shown to have a number of biologically important effects such as (i) inhibiting epidermal growth factor induced DNA synthesis (2) and epidermal growth factor degradation (3) in human fibroblasts, (ii) reducing collagen production in isolated chick tendon fibroblasts (4), (iii) enhancing the release of glycoproteins from mouse 3T3 cells (5) and (iv) delaying insulin processing and clearence in rat liver tissue (6). Many of these effects appear to be due to the ability of chloroquine to enhance intracellular, lysosomal pH (3-5, 7). In addition, some of chloroquine's actions could be due to its DNA intercalating properties (1).

In this report, we present the novel observation that chloroquine significantly protects cytosolic glucocorticoid receptor binding from thermal

0006-291X/83 $1.50

and salt induced inactivation. The influence of chloroquine on glucocorticoid-receptor complex sedimentation in sucrose-density gradients is also reported.

Materials and Methods

MATERIALS

Steriods and Chemicals - [1,2,4-^{3}H]dexamethasone (31 Ci/mmole) was obtained from New England Nuclear Boston, Mass. Its purity was periodically checked by thin layer chromotography on precoated silica sheets (Eastman, Kodak) using a benzene: ethylacetate (60:40 v/v) solvent system. Sodium molybdate was purchased from Mallinckrodt Chemical Works (New York). Chloroquine and non-labelled dexamethasone were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other reagents were of analytical grade.

Animals - Male Sprague-Dawley rats (from Charles River Breeding Laboratories), 150-250 g, were adrenalectomized bilaterally 4 to 6 days before use and were maintained on standard Purina Rat Chow, 0.9% NaCl solution, and water *ad libitum*.

METHODS

Preparation of Cytosol - Rats were sacrificed by cervical dislocation. Livers were perfused *in situ* with ice-cold homogenization buffer (containing 10 mM Tris-HCl, 0.25 M Sucrose, pH 7.5) after which liver tissues were removed, cleaned of adhering tissue, and homogenized (1:3 W/V) in the perfusion buffer. The homogenates were passed through cheesecloth and centrifuged at 140,000 Xg for 1 h at 4°C. The upper fatty layer was discarded and the cytosol was carefully removed (adjusted to about 10 mg protein and a final pH of 7.5) and used for receptor studies. Protein content was determined by the method of Lowry et al. (8).

Stabilization of Unbound Glucocorticoid Receptors with Chemical Reagents - Chloroquine, sodium molydate, and KCl were prepared in concentrated stock solutions (100 mM-2 M, pH 7.5) in homogenization buffer prior to use.

In standard receptor stability experiments, up to 20 µl of chemical reagents were added per 1.0 ml of cytosol prior to incubation. High (3 mM) concentrations of chloroquine caused cytosol turbidity. However glucocorticoid receptors were not precipitated by chloroquine addition. In addition, in some experiments turbid cytosol was centrifuged for 10 min at 1,500 g and the clear supernatant was used for stability studies; samples gave the same results as uncentrifuged cytosol. In some experiments (Table I), KCl (0.3 M) or an equal volume of homogenization buffer was added to samples in addition to receptor stabilizing agents. Cytosolic samples were then incubated for the times and temperature indicated in figure and table legends. 200 µl of cytosol were removed (in duplicate) and incubated with 5 x 10^{-8} M [^{3}H]dexamethasone in the presence or absence of a 1000-fold excess of nonradioactive dexamethasone. After binding for 2 h at 4°C, the specific macromolecular-bound fraction of ^{3}H-labelled steroid was determined using the charcoal-dextration separation technique (9).

Stabilization of Prebound [^{3}H]Dexamethasone-Receptor Complexes - Cytosol was preincubated with 5 x 10^{-8} M [^{3}H]dexamethasone in the presence or absence of 1000-fold excess unlabelled steroid for 2 h at 4°C prior to addition of 3 mM chloroquine or an equal volume of homogenization buffer. After incubating for times and temperature indicated in Table II, the charcoal-dextran separation technique (9) was used to remove free [^{3}H]dexamethasone. Specifically bound [^{3}H]dexamethasone-receptor levels were determined in 200 µl cytosol samples (in duplicate) using a Beckman LS 100 C liquid scintillaiton counter.

Sucrose Gradient Centrifugation - Linear 5-20% sucrose gradients were prepared in 10 mM Tris buffer pH 7.5 containing 30 mM KCl (low salt gradients) or 300 mM KCl (high salt gradients).

Cytosol was first treated with saturating levels of [^{3}H] dexamethasone for 2 h at 4°C; cytosol was then incubated for 30 min at 4°C with homogenization buffer or for 30 min at 25°C with 3 mM chloroquine or with 10 mM sodium molybdate and treated with dextran-coated charcoal for 15 min at 4°C. After centrifuging the cytosol at 3,000 x g for 10 min, the resulting supernatant (0.4 ml) was layered on sucrose gradients. Myoglobin (2.0 S) bovine serum albumin (4.6 S) and human γ-globulin (7.1 S) were employed as references for estimation of receptor sedimentation coefficients by the method of Martin and Ames (10). Samples were centrifuged for 18 h at 4°C in a Spinco SW rotor at 40,000 rpm. Following centrifugation, starting at the top of each gradient, 8 drop fractions were collected with a Gilford Densiflow Apparatus. Charcoal resistant radioactivity of each fraction was measured following addition of 5 ml of Aquasol.

Statistical Analysis - Statistical analysis of results was performed according to the methods of Dunnett (11).

Results

Chloroquine was found to greatly stabilize unbound glucocorticoid receptors *in vitro* at 25°C. The effect of chloroquine was concentration dependent with significant stabilization at 0.3 mM, and optimal effectiveness at about 3 mM (data not shown). The influence of 3 mM chloroquine on the rate of unbound rat hepatic glucocorticoid receptor inactivation at 25°C is shown in Fig. 1. As previously reported (12-14), unbound glucocorticoid receptors were rapidly inactivated at 25°C with over 50% loss after 1 h incubation. However, in the presence of 3 mM chloroquine, receptor binding was almost totally protected after 1 h incubation at 25°C. After 2 h incubation control receptors were almost completely inactivated, while approximately 70% of receptor binding remained intact in samples containing 3 mM chloroquine. At the concentrations used chloroquine had little or no effect on cytosolic pH (even if not neutralized prior to addition) and had potent stabilizing action over a wide hydrogen ion concentration ranging from pH 6.5 to 8.0 (data not presented). Chloroquine was also found to significantly stabilize unbound glucocorticoid receptors at physiological temperature (data not shown).

Since purification techniques are often conducted at low temperature and utilize salt elution, we examined the effect of chloroquine on KCl induced receptor inactivation at 4°C. As shown in Table I, 0.3 M KCl significantly

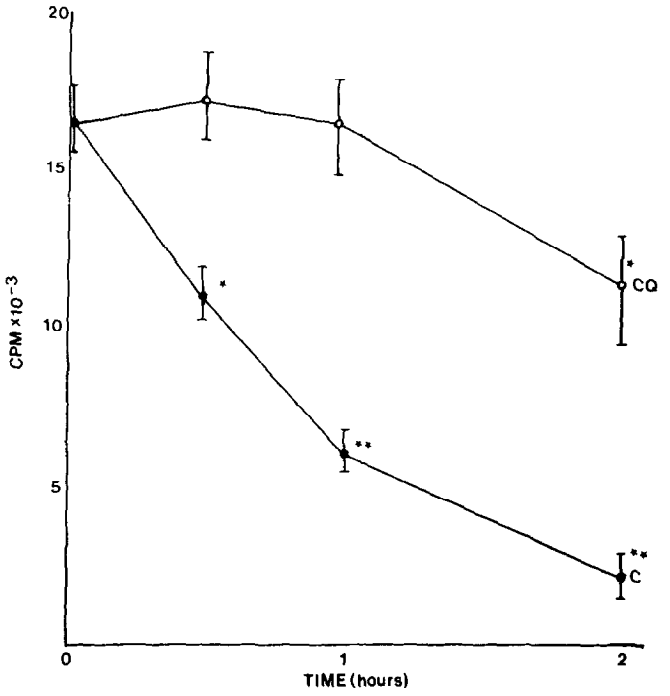

Fig. 1. Effect of chloroquine on liver unbound glucocorticoid receptor inactivation at 25°C. Cytosol was incubated at 25°C with homogenization buffer (●—●) or 3 mM chloroquine (o—o). Aliquots were removed (in duplicate) at times indicated and assayed for specific [^{3}H]dexamethasone binding as described in "Methods". Results are the mean ± SE of 5 experiments.

* Different from initial binding, P ≤ .05
** Different from initial binding, P ≤ .01

enhanced receptor inactivation. In the presence of 3 mM chloroquine (or 10 mM molybdate) KCl induced receptor inactivation was significantly reduced. A similar stabilizing effect of chloroquine was found in experiments where high

Table I

Effect of Chloroquine on KCl Inactivation of Glucocorticoid Receptors at 4°C

Experimental Group	CPM/0.2 ml Cytosol (after 2 h at 4°C)
Control	17266 ± 1601**
0.3M KCl	7760 ± 727
0.3M KCl + 10 mM molybdate	11787 ± 909**
0.3M KCl + 3 mM chloroquine	12220 ± 957**

Results are the mean ± SE of 5 experiments.

** Different from 0.3 M KCl samples, P ≤.01

concentrations of phosphate were used to promote receptor inactivation (data not shown). Thus chloroquine appears to protect unbound glucocorticoid receptors in vitro from either thermal or high salt induced inactivation.

The effect of chloroquine on prebound glucocorticoid-receptor complex stabilization was also investigated (Table II). As expected, prebound steroid-receptor complexes were much more stable than unbound receptors. Thus steroid-receptor complexes were inactivated about 16% after 24 h incubation at 4°C and about 34% after 3 h at 25°C. However in the presence of 3 mM chloroquine prebound glucocorticoid receptor complexes remained totally intact for 24 h at 4°C and only 16% inactivation was observed after 3 h at 25°C.

Since glucocorticoid receptor stabilization has been correlated with steroid-receptor complex aggregation (14-16), we investigated the effect of chloroquine (3 mM) on glucocorticoid-receptor complex sedimentation in sucrose-density gradients. As shown in Fig. 2, unlike 10 mM molybdate which enhanced the complex sedimentation coefficient from 7S to about 9S in low salt gradients, 3 mM chloroquine had no appearent effect on glucocorticoid-receptor complex sedimentation in low or high salt conditions.

Table II

Effect of Chloroquine on Prebound Glucocorticoid-Receptor Complex Inactivation at low and high temperature

Experimental Group	CPM/0.2 ml cytosol
A. Samples incubated 24 h at 4°C -	
control	16457 ± 948
chloroquine (3 mM)	19676 ± 717*
B. Samples incubated 3 h at 25°C -	
control	7720 ± 158
chloroquine (3 mM)	9771 ± 887*

Results are the mean ± SE of 6 samples.

Control binding in part A averaged 19586 CPM

Control binding in part B averaged 11665 CPM

*Different from control, $P \leq .05$

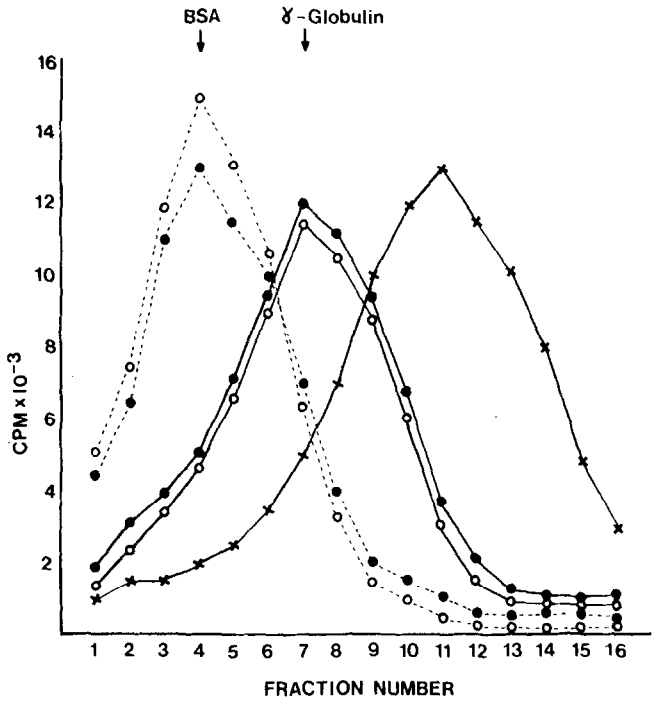

Fig. 2. Effect of chloroquine and molybdate on [^{3}H]dexamethasone-receptor complex sedimentation in sucrose-density gradients. 5-20% sucrose-density gradients were prepared as described in "Methods".

●---● Control samples in 300 mM KCl gradients
o---o Chloroquine samples in 300 mM KCl gradients
●—● Control samples 30 mM KCl gradients
o—o Chloroquine samples in 30 mM KCl gradients
×—× Molybdate samples in 30 mM KCl gradients

Discussion

Glucocorticoid receptors are rapidly inactivated *in vitro* making their quantitation, characterization and purification extremely difficult. Inactivation is most pronounced in high temperature and high salt conditions. Ligand-free receptors are particularly susceptible to inactivation although steroid-bound complexes are also inactivated in low and particularly high temperature. Methods to stabilize glucocorticoid receptors *in vitro* are therefore of considerable interest (12-14, 17). The results of this report clearly demonstrate that chloroquine acts as a very effective stabilizer of hepatic glucocorticoid receptors *in vitro*.

Since experiments reported herein were conducted in the absence of lysosomes and DNA, the mechanism of chloroquine stabilization of

glucocorticoid receptors could not be due to either its lysosomotropic or intercalating actions. Chloroquine must therefore alter receptor properties by direct effects on the receptor or by indirect effects on other receptor-modulating cytosolic components or enzymes. The possibility that chloroquine may influence receptor-regulating enzymes is supported by the direct inhibitory effect of chloroquine on cathepsin B (18). Steroid receptor aggregation has previously been proposed as one of the possible mechanisms of molybdate receptor stabilization (19). We therefore investigated the possibility that chloroquine stabilizing effects could be correlated with receptor aggregation. However as shown in Fig. 2, chloroquine had no effect on glucocorticoid-receptor complex sedimentation in sucrose-density gradients.

The results of this study suggest that chloroquine may have useful application in glucocorticoid receptor quantitation, characterization and purification. The influence of chloroquine on glucocorticoid receptors *in vivo* has not yet been determined. However as chloroquine is widely used pharmacologically, such studies may prove quite interesting particularly in light of the fact that both chloroquine and glucocorticoid hormones have potent antiarthritic actions.

Acknowledgements

Mohammed Kalimi is a recipient of a Research Career Development Award NIH AM00731.

References

1. Rollo, I.M. (1980) in the Pharmacological Basis of Therapeutics, eds. Gilman, A.G., Goodman, L.S., and Gilman, A. (MacMillan Pub. Co., New York) Chap. 45, pp. 1042-1045.
2. King, A.C., Hernaez-Davis, L. and Cuatrecasas, P. (1981) Proc. Nat., Acad. Sci. USA 78, 717-721.
3. King, A.C., Hernaez-Davis, L. and Cuatrecasas, P. (1980) Proc. Natl. Acad. Sci. USA 77, 3283-3287.
4. Neblock, D.S., and Berg, R.A. (1982) Biochem. Biophys. Res. Commun. 105, 902-908.
5. Nilsen-Hamilton, M., Hamilton, K.T., Allen, W.R., and Massoglia, S.L. (1981) Biochem. Biophys. Res. Commun. 101, 411-417.
6. Posner, B.I., Patel, B.A., Khan, M.N., and Bergeron, J.J.M. (1982) J. Biol. Chem. 257, 5789-5799.
7. Maxfield, F.R., Willingham, M.C., Davies, P.J.A. and Pastan, I. (1979) Nature 277, 661-663.

8. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
9. Beato, M. and Fergelson, P. (1972) J. Biol. Chem. 247, 7890-7896.
10. Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372-1379.
11. Dunnett, C.W. (1955) J. Am. Stat. Ass. 50, 1096-1121.
12. Nielsen, C.J., Sando, J.J., Vogel, W.M. and Pratt, W.B. (1977) J. Biol. Chem. 252, 7568-7578.
13. Hubbard, J. and Kalimi, M. (1983) Biochimica et Biophysica Acta 755, 178-185.
14. Hubbard, J. and Kalimi, M. (1982) J. Biol. Chem. 257, 14263-14270.
15. Toft, D. and Nishigori, H. (1979) J. Steroid. Biochem. 11, 413-416.
16. Sherman, M., Moran, M., Neal, R., Neiu, E. and Tuazon, F. (1982) in Progress in Research and Clinical Applications of Corticosteroids, Eds. Lee, H. and Fitzgerald, T. (Heyden Press, Philadelphis) pp. 45-65.
17. Hubbard, J. and Kalimi, M. (1983) Biochem. J. 210, 259-263.
18. Wibo, M. and Poole, B. (1974) J. Cell. Biol. 63, 430-440.
19. Depirro, R., Milani, C., Fusco, A. and Lauro, R. (1979) Clinical Chimica Acta 96, 9-14.