Alteration of hepatic glucocorticoid receptor stability and nuclear binding *in vitro* by citrate

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Citrate greatly stabilized rat hepatic unbound glucocorticoid receptors in cell-free conditions at 4°C with optimal effectiveness at 5–15 mM. Control receptors were inactivated at 4°C with a half-life of less than 12 h. However, in the presence of 10 mM-citrate, unbound receptors were almost completely stabilized for 48 h at 4°C. Citrate at a concentration of 1–2 mM yielded half-maximal stabilization. The stabilizing effect of citrate was rather specific, as succinate, α -oxoglutarate, oxaloacetate, malate and pyruvate had no apparent stabilizing action. Citrate stabilized receptors over a wide range of H⁺ concentrations, with complete protection between pH 6.5 and 8.5. In addition, citrate appeared to have a significant effect on glucocorticoid–receptor complex activation into a nuclear binding form. Thus 5–10 mM-citrate enhanced nuclear binding, with optimal activation achieved at 10 mM concentration. As analysed by sucrose-density-gradient centrifugation and DEAE-cellulose chromatography, no apparent change was observed in the physical characteristics of the glucocorticoid receptor in the presence of citrate.

Citrate, in addition to being a key intermediate of cell metabolism, exerts a regulatory influence on certain intracellular enzymes. For example, citrate inhibits the activity of phosphofructokinase by potentiating the inhibitory action of ATP (McGilvery, 1970; Stryer, 1981). In addition, citrate stimulates fatty acid synthesis by promoting the association of inactive acetyl-CoA carboxylase monomers into active polymers (McGilvery, 1970). Citrate also has been shown to significantly stabilize antithrombin III if added in high concentrations (0.5–1.0 M) *in vitro* (Busby *et al.*, 1981). The regulatory versatility of citrate suggests that this molecule could conceivably regulate certain other enzymes or non-enzymic proteins in such a manner as to alter their biochemical (and possibly biological) properties either *in vivo* or *in vitro*.

In the present paper, we have investigated the effect of citrate on glucocorticoid receptor properties. Our observations show that citrate significantly stabilizes unbound glucocorticoid receptors *in vitro* and alters the nuclear binding properties of the steroid–receptor complexes.

Experimental procedures

Materials

[1,2,4-³H]Dexamethasone (sp. radioactivity 31 Ci/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A. Its purity was

periodically checked by t.l.c. on precoated silica sheets (Eastman–Kodak) with a benzene/ethyl acetate (3:2, v/v) solvent system. Sodium molybdate was purchased from Mallinckrodt Chemical Works (New York, NY, U.S.A.). Unlabelled steroids, citrate, succinate, malate, pyruvate, oxaloacetate and α -oxoglutarate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Animals

Male Sprague–Dawley rats (from Charles River Breeding Laboratories), weighing 150–250 g, were adrenalectomized bilaterally 3–6 days before use. Rats were maintained on standard Purina Rat Chow, 0.9% NaCl solution (saline) and water *ad libitum*.

Methods

Preparation of cytosol. Rats were killed by cervical dislocation. Liver were perfused *in situ* with ice-cold Tris buffer (10 mM-Tris/HCl/0.25 M-sucrose, pH 7.5, at 4°C). The liver tissue was then removed, cleaned of adhering tissue and homogenized (1:3, w/v) in the perfusion buffer. The homogenates were passed through cheesecloth and centrifuged at 3000 g in a Sorvall SS 34 rotor at 4°C for 10 min, to sediment a crude pellet. The cytosol portion was prepared by centrifuging the supernatant at 270 000 g for 30 min at 4°C. The

upper fatty layer was discarded and the cytosol was carefully removed, adjusted to about 10mg of protein/ml and a final pH of 7.5 at 4°C (with dilute NaOH or HCl), and used. Protein content was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Determination of glucocorticoid receptor specific binding. The cytosol was incubated at 4°C for 90min with [³H]dexamethasone at a final concentration of 50nM. The specific macromolecular bound fraction was determined by using the dextran-coated charcoal technique (Beato & Feigelson, 1972) in the presence or absence of 1000-fold excess of non-radioactive dexamethasone. The radioactivity was assayed by using Aquasol (New England Nuclear) in a Beckman LS 100C counter with 65% efficiency for ³H. Correction for sample quenching was made by use of the external standard of the instrument.

Effect of chemical reagents on unbound glucocorticoid receptor stability *in vitro*. Citrate, succinate, α -oxoglutarate, oxaloacetate, malate and pyruvate were prepared in concentrated stock solutions (100–500mM-Tris/HCl, pH 7.5, at 4°C) before use.

In standard receptor stability experiments, 1–20 μ l of chemical reagents (alone or in combination) was added per 1.0ml of cytosol before incubation. After incubation (times and temperatures are indicated in Figure legends), 200 μ l of cytosol was removed (in duplicate) and assayed for specific glucocorticoid receptor binding as described above.

Sucrose-density-gradient centrifugation. Linear 10–30% sucrose density gradients were prepared in 10mM-Tris buffer, pH 7.5 (measured at 4°C), containing 10mM-KCl (low salt gradients). (a) In one group cytosol was first treated with 10mM-citrate for 24h at 4°C. Cytosol was then incubated with saturating levels (50nM) of [³H]dexamethasone and incubated for 2h at 4°C. (b) In a second group cytosol was pretreated with saturating levels of [³H]dexamethasone for 2h at 4°C. Citrate (10mM) was added to cytosol and incubated for 45min at 25°C. Samples from both groups were then treated with dextran-coated charcoal for 15min at 4°C. After centrifuging the cytosol at 3000g for 10min the resulting supernatant (0.4ml) was layered on sucrose gradients. Myoglobin (2.0S), bovine serum albumin (4.6S), and human γ -globulin (7.1S) were employed as references for estimation of receptor sedimentation coefficients (Martin & Ames, 1961). Samples were centrifuged for 18h at 4°C in a Spinco SW rotor at 40000 rev./min. After centrifugation, starting at the top of each gradient, eight-drop fractions were collected with a Gilford Densiflow Apparatus. Charcoal-resistant radioactivity of each fraction was measured after addition of 5ml of Aquasol.

Nuclear binding assay. Nuclei were purified from a 2000g pellet of liver homogenate using 1.8M-sucrose in 10mM-Tris/HCl buffer, pH 7.5, containing 0.025M-KCl as previously described (Kalimi *et al.*, 1973). The washed nuclei were suspended in homogenization buffer. Portions of the nuclear suspension containing 50 μ g of DNA were centrifuged at 2000g for 5min at 4°C and the supernatant fractions were discarded. [³H]Dexamethasone-labelled cytosol (0.2ml) was added and a final volume of 1ml of reaction mixture was made by the addition of homogenization buffer.

Nuclei were resuspended by gentle agitation with a vortex mixer, and the suspension was incubated for 1h at 4°C. After incubation the nuclei were removed by centrifugation at 2000g for 5min. The nuclear pellet was washed four times with 2ml of homogenization buffer. Nuclear-bound [³H]dexamethasone was extracted for 30min at 4°C with 0.5ml of 0.4M-NaSCN. [About 95% of the total radioactivity in the cell nuclei was extractable by 0.4M-NaSCN (J. Hubbard & M. Kalimi, unpublished work).] The mixtures were centrifuged at 2000g for 5min and 200 μ l of supernatant was counted for radioactivity as described above. NaSCN extraction of nuclear bound radioactivity gave essentially the same results as direct measurement of radioactivity in nuclear pellets. A correction was made for non-specific binding by adding a 1000-fold excess of unlabelled dexamethasone to cytosol. The non-specific binding was approx. 5% of total binding in these experimental conditions. DNA was estimated by the diphenylamine method of Burton (1956) with calf thymus DNA as standard.

DEAE-Cellulose chromatography. This was carried out as described previously by Sakaue & Thompson (1977).

Statistical analysis. Statistical analysis of results was performed by the method of Dunnett (1955).

Results

Citrate stabilization of unbound hepatic glucocorticoid receptors

The effect of 10mM-citrate on unbound hepatic glucocorticoid receptor stability during incubation for 6–48h at low temperature is shown in Fig. 1. Control specific receptor binding decreased with a half-life of less than 12h at 4°C. However, when 10mM-citrate was added to liver samples before incubation, receptor specific binding remained completely stabilized for at least 48h at 4°C.

The concentration-dependence of citrate-mediated glucocorticoid receptor stabilization is shown in Fig. 2. Citrate (0.5mM) provided a small protective effect on receptors incubated for 24h at 4°C. However, significant stabilization was obtained with 1mM-citrate, and optimal effectiveness was observed with

Fig. 1. Kinetics of unbound glucocorticoid receptor inactivation at 4°C in the presence and absence of citrate

Cytosol was incubated at 4°C with homogenization buffer (●) or with 10 mM-citrate (○). Portions were removed in duplicate at times indicated and assayed for specific [³H]dexamethasone binding as described under 'Methods'. Results are means ± S.E.M. for four or more experiments. * indicates a difference from the initial binding level ($P \leq 0.05$).

Fig. 2. Dependence of citrate concentration on glucocorticoid receptor stabilization

Cytosol was incubated at 4°C with 0–15 mM-citrate as shown above. After 24 h incubation samples were assayed for specific [³H]dexamethasone binding as described under 'Methods'. Results are means ± S.E.M. of three experiments. * indicates a difference from 24 h incubated control binding ($P \leq 0.01$).

5–15 mM-citrate. Half-maximal stabilization was achieved with 1–2 mM-citrate. Scatchard analysis indicated that the difference in [³H]dexamethasone binding in control and citrate-treated samples after incubation for 6 h at 4°C was due to a stabilization of receptor concentration rather than a change in receptor affinity for the ³H-labelled steroid [control sample specific binding (pmol/mg of protein): 0.28 ± 0.04 and K_d 2.6 ± 0.18 nM and 10 mM-citrate-treated sample specific binding (pmol/mg of protein) 0.42 ± 0.06 and K_d 3.2 ± 0.12 nM; values are means of three experiments].

The glucocorticoid receptor stabilizing effect of citrate was studied over a wide range of H⁺ concentrations (results not shown). At each pH value examined (between pH 6.5 and 8.5) 10 mM-citrate completely stabilized receptor binding for 24 h at 4°C.

The specificity of citrate-mediated receptor stabilization was tested by determining the effect of several other carboxylic acids on receptor inactivation *in vitro*; 10 mM amounts of succinate, α -oxoglutarate, oxaloacetate, malate and pyruvate had no apparent stabilizing effect on the glucocorticoid receptor incubated for 24 h at 4°C (results not shown).

Effect of citrate on glucocorticoid–receptor complex binding to nuclei

The effect of citrate (5–20 mM) on glucocorticoid–receptor complex binding to isolated nuclei is shown in Fig. 3. Cytosol was pre-incubated for 2 h at 4°C with a saturating level of [³H]dexamethasone to form steroid–receptor complexes. Samples were then incubated for 45 min at 4°C (Fig. 3a) or at 25°C (Figs. 3b and 3c) before binding to isolated nuclei for 1 h at 4°C. As expected elevation of temperature from 4°C to 25°C greatly promoted steroid–receptor complex binding to nuclei in control samples with an approx. 5-fold increase. Citrate (5–10 mM) had little or no effect when added to samples before incubation at 4°C for 45 min (Fig. 3a). Interestingly, however, complexes that were incubated in the presence of citrate at 25°C for 45 min were significantly altered with respect to their ability to bind to nuclei (Fig. 3b). Concentrations of 5 and 10 mM-citrate significantly enhanced nuclear binding about 25% and 35% respectively. Citrate (10 mM) had no significant effect on ³H-labelled steroid dissociation from prebound glucocorticoid–receptor complexes (as determined by the dextran-coated charcoal assay) after 45 min incubation at 25°C and at 1 h incubation at 4°C (the length of time used for thermal activation and nuclear binding assays). Therefore the effects of citrate on nuclear binding were not due to alteration of the concentration of input complex. When 10 mM-citrate was added after activation at 25°C, little or no change was observed in nuclear binding, suggesting

Fig. 3. Effect of citrate on glucocorticoid-receptor complex binding to isolated nuclei

Cytosolic receptors were pre-incubated with excess [^3H]dexamethasone for 2 h at 4°C (as described under 'Methods'). Samples were then incubated for 45 min at 4°C (a) or 45 min at 25°C (b and c) before nuclear binding determinations as described under 'Methods'. Samples in (a) and (b) were incubated with citrate during the 45 min incubation before nuclear binding. On the other hand, citrate was added after the 45 min incubation (just before nuclear binding determinations) to samples in (c). Experimental groups represent samples treated with homogenization buffer (group 1), 5 mM-citrate (group 2), 10 mM-citrate (group 3) and 20 mM-citrate (group 4). Results are expressed as percentages of the 25°C incubated control samples (b, group 1) and are means $\pm$ S.E.M. of five or more experiments. * indicates a difference from 25°C incubated control samples (group 1 in b) ($P \leq 0.05$).

that citrate may influence thermal activation itself rather than nuclear binding (Fig. 3c).

Effect of citrate on glucocorticoid receptor physical properties

The DEAE-cellulose elution profiles of both unactivated and heat-activated samples were unaltered by the presence of 10 mM-citrate, suggesting that citrate does not greatly alter the net surface charge of either activated or unactivated glucocorticoid-receptor complexes (results not shown). In addition, both unactivated and heat-activated ^3H -labelled steroid-receptor complexes sedimented at 7–8 S in 10–30% sucrose density gradients in the

presence or absence of 10 mM-citrate (results not shown). Thus citrate did not appear to significantly alter the size or aggregation properties of the receptor.

Discussion

Inactivation of glucocorticoid receptors *in vitro* is of considerable interest since it (i) makes purification and characterization of glucocorticoid receptors exceedingly more difficult, (ii) may hinder accurate quantification of glucocorticoid receptor levels in experimental and clinical assays and (iii) may lend insight into receptor regulation.

In the present study we have made the novel observation that citrate greatly stabilizes glucocorticoid receptor binding *in vitro* (Fig. 1) with optimal effectiveness at 5–10 mM concentrations (Fig. 2). Scatchard analysis indicated that citrate maintained receptor concentration during incubation rather than altering receptor affinity for the ^3H -labelled steroid. Interestingly, the stabilizing effect of citrate was rather specific as chemically and metabolically related carboxylic acids such as succinate, α -oxoglutarate, oxaloacetate, malate and pyruvate had no apparent receptor stabilizing action. These results not only suggest that citrate may have rather unique interactions with the unbound receptor or receptor-modulating factors, but also that citrate probably acts directly rather than being metabolized to one of the other carboxylic acids. It is noteworthy that citrate has been shown by others to block thermal degradation of antithrombin III *in vitro* (Busby *et al.*, 1981). However, in their system much higher concentrations of citrate (0.5–1.0 M) were required. The use of citrate in unbound glucocorticoid receptor stabilization may afford certain advantages over other commonly used receptor stabilizers. For example, we found that receptor stability at 4°C in the presence of citrate (Fig. 1) was greater than when the cytosolic receptor was incubated in the presence of reducing agents such as dithiothreitol or chelating agents such as EDTA and EGTA (results not shown). This difference between citrate and metal chelating agents also indicated that the stabilizing effect of citrate was probably not merely due to Ca^{2+} chelating action.

In addition to the stabilizing effect of citrate on unbound glucocorticoid receptors *in vitro*, citrate also had a significant influence on glucocorticoid-receptor complex binding to isolated nuclei (Fig. 3), with enhancement of nuclear binding between 5 and 10 mM concentrations. Citrate appeared to facilitate the thermal activation process, as identical concentrations of citrate had no effect at low temperature (Fig. 3). When 5–10 mM-citrate was added after activation, little or no change in complex nuclear binding occurred, suggesting that 5–10 mM-

citrate was probably altering the activation process itself rather than the ability of the activated complexes to bind to nuclei (Fig. 3).

To determine if citrate had a significant effect on the physical properties of the glucocorticoid receptor, both DEAE-cellulose chromatography and sucrose-density-gradient analysis were performed. These studies indicated that no apparent alteration occurred in the receptors' physical properties in the presence of citrate. Thus, although other stabilizers such as molybdate are often associated with steroid-receptor complex aggregation (DePirro *et al.*, 1979; Chen *et al.*, 1981), such a relationship was not found with citrate.

The effect of endogenous citrate on glucocorticoid receptors *in vitro* and *in vivo* is yet to be explored. Although half-maximal stabilization of receptors was achieved with 1–2 mM-citrate, subcellular concentration of rat liver (hepatocyte) citrate ranges from 0.2 to 5.2 mM (William & Corkey, 1979; Soboll *et al.*, 1980). Hence it is possible that citrate could have significant effects on the receptor under physiological conditions. This possibility is of great interest, since citrate is an important metabolite that is closely regulated and could influence many cellular processes including hormone action.

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