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The Subcellular Distribution of Glucocorticoid–Receptor Complexes as Studied by Chemical Crosslinking of Intact HTC Cells

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Treatment of intact HTC cells with glutaraldehyde results in redistribution of glucocorticoid binding sites between cytosolic and nuclear fractions. The decrease in cytosolic receptors and their accumulation at the nuclear level were found to be directly related to the glutaraldehyde concentrations employed in our procedure and inversely related to the cell density of samples. When the data from eleven separate experiments were combined, and analyzed by linear regression of cytosolic and nuclear levels of receptor complexes vs the ratios between the DNA and glutaraldehyde concentration of our samples, two lines were obtained whose intercepts on the ordinate yielded values of cytosolic and nuclear receptors corresponding to 37.5 and 62.5% of the total cellular pool, respectively. When we compared the subcellular redistribution of glucocorticoid receptor to that of the cytosolic enzyme lactate dehydrogenase upon HTC cell crosslinking with glutaraldehyde, we found that the cytosolic and nuclear levels of the enzyme were 53.2 and 46.8% of the total content, respectively. If the subcellular distribution of glucocorticoid receptor is corrected for the artefactual redistribution induced by crosslinking, using the values obtained for lactate dehydrogenase, it can be concluded that glucocorticoid receptors in HTC cells are distributed between cytosol and nuclei in a ratio which is about 2:1. Our findings lend further support to the conclusion that only a portion of glucocorticoid receptor is cytosolic in intact cells.

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Steroid hormone receptors represent *trans*-acting transcription factors which can modulate the expression of specific genes after binding their cognate ligands [1]. While this role implies that nuclei must be the subcellular compartment of functioning receptors, a considerable debate has been developed on the subcellular location of steroid receptors before they become transformed to forms capable of tight binding to their hormone responsive elements on DNA.

The classical two-step model of steroid hormone action [2, 3], involving binding of hormone to receptors in cytosols of target cells, followed by nuclear translocation of steroid–receptor complexes, has been subjected to revision after it has been shown that hormone-free estrogen receptors are prevalently located inside nuclei in intact cells, and are released in the cytosolic fraction upon cell rupture [4, 5]. While it has been shown that progesterone and androgen receptors would also be located at the nuclear level in the absence of hormone [6, 8], the subcellular distribution of glucocorticoid receptor has remained controversial, as both cytosolic [9, 10] and nuclear [6, 10–12] location of receptor proteins has been documented by immunocytochemical and enucleation studies.

In our previous investigations employing chemical crosslinking of intact cells, we could show that covalent coupling of proteins to associated components *in vivo*, leads to nuclear immobilization of hormone-free glucocorticoid receptors and untransformed glucocorticoid–receptor complexes [13–15]. While those experiments provided biochemical evidence of a nuclear location of some untransformed glucocorticoid receptors, a precise estimate of their subcellular distribution could not be obtained.

In the present study we have reevaluated the issue by a more stringent analysis of experimental data, and we have found that in HTC cells glucocorticoid receptors are distributed between cytosol and nuclei in a ratio which is about 2:1.

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EXPERIMENTAL

Cell culture conditions and labeling of glucocorticoid receptor

HTC cells were grown in 5% carbon dioxide in air at 37°C in Petri dishes with a culture medium composed of RPMI 1640 containing 10% fetal calf serum. Cells were recovered, washed with PBS buffer (20 mM phosphate, pH 7.4, 0.15 M NaCl), and dispersed in the same buffer. Cell suspensions were then incubated for 2 h at 2°C with 50 nM tritiated dexamethasone (New England Nuclear, 43.9–44.7 Ci/mmol), and in the presence or in the absence of a 200-fold molar excess of unlabeled dexamethasone. At the end of the incubation, cells were washed once with PBS buffer, and were used for preparation of cell extracts after they were, or were not, subjected to crosslinking.

Preparation of cell fractions

Cells were disrupted by suspension in 1 ml of 20 mM Tris–HCl, pH 7.5 at 2°C, 1.5 mM $MgCl_2$, 10 mM NaCl (buffer A), containing 3% (w/v) digitonin, and incubation for 30 min at 2°C with occasional vortexing. The homogenate was centrifuged for 10 min at 1000 *g* to obtain a crude nuclear precipitate and a supernatant. The supernatant was centrifuged for 1 h at 105,000 *g*, to obtain the cytosolic extract. Nuclei were dispersed in 1 ml of buffer A to yield the nuclear fraction.

Determination of glucocorticoid receptor levels

Cytosolic binding was determined by the radioactivity detected after extracts were treated with a dextran-coated charcoal pellet, as described previously [14]. To determine nuclear binding, 0.4 ml portions of nuclear suspensions were centrifuged for 10 min at 1000 *g* and nuclei were washed once by resuspension with 0.3 ml of buffer A and centrifugation for 10 min at 1000 *g*. The supernatants obtained in these two centrifugations were combined, treated with a dextran-coated charcoal pellet, and used to resuspend their own nuclear pellets. Aliquots of these samples were then taken for determination of radioactivity. Specific glucocorticoid binding in cytosol and nuclei was calculated by subtraction of unspecific binding (+competitor) from total binding (−competitor).

Crosslinking of intact cells

Cell suspensions in PBS buffer were subjected to crosslinking by incubation for 90 min at 2°C with the indicated glutaraldehyde concentrations, as described previously [14]. Crosslinked cells were washed once with PBS buffer before being used for preparation of cell extracts.

Other methods

Radioactivity was measured in a Packard liquid scintillation spectrometer, using Scintillator 299 (Packard) as the scintillation cocktail. Lactate dehydrogenase (LDH) activity was determined by the procedure of Wróblewski and LaDue [16]. DNA was measured by the diphenylamine test with calf thymus DNA as the standard [17].

RESULTS

The distribution of glucocorticoid–receptor complexes between cytosolic and nuclear fractions from HeLa cels has been found to depend on the concentration of reagents used in crosslinking of intact cells [13, 14]. In our preliminary experiments, HTC cells were exposed to tritiated dexamethasone, before being subjected to treatments with different glutaraldehyde concentrations, and the levels of glucocorticoid–receptor complexes were next measured in cytosolic and nuclear fractions prepared from our samples. As it is shown in Fig. 1, HTC cell treatment with increasing glutaraldehyde concentrations induced a progressive loss of cytosolic receptor complexes which was accompanied by their detection in the nuclear fraction.

This observation was confirmed by a second independent experiment, but we noticed some variability in the redistribution of glucocorticoid–receptor complexes between the two fractions induced by crosslinking of HTC cells at any glutaraldehyde concentration tested. We then checked whether the cell density of our samples might be responsible for this phenomenon. The results we obtained in a typical experiment are reported in Fig. 2, and show that the extent of the redistribution of glucocorticoid binding

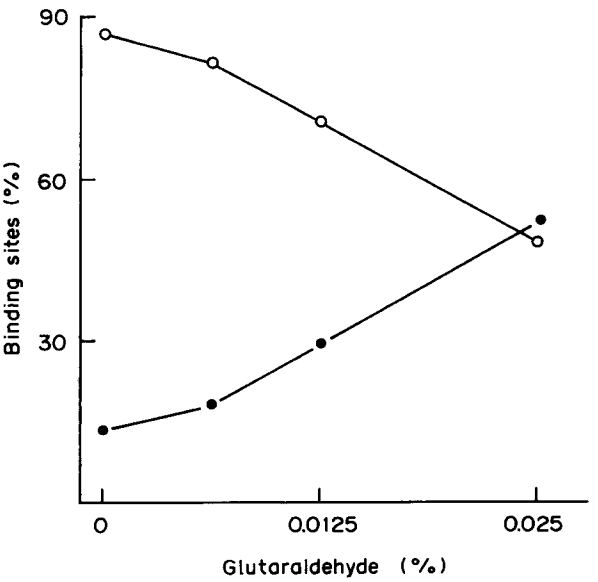

Fig. 1. Effect of HTC cell treatment with increasing glutaraldehyde concentrations on the distribution of glucocorticoid binding sites between cytosolic and nuclear fractions. HTC cells were incubated for 2 h at 2°C with 50 nM tritiated dexamethasone and in the presence or absence of a 200-fold molar excess of unlabeled competitor. At the end of the incubation, cells were treated for 90 min at 2°C with the indicated glutaraldehyde concentrations, and were then processed to determine specific glucocorticoid binding in cytosolic (○) and nuclear (●) fractions, as described in the Experimental.

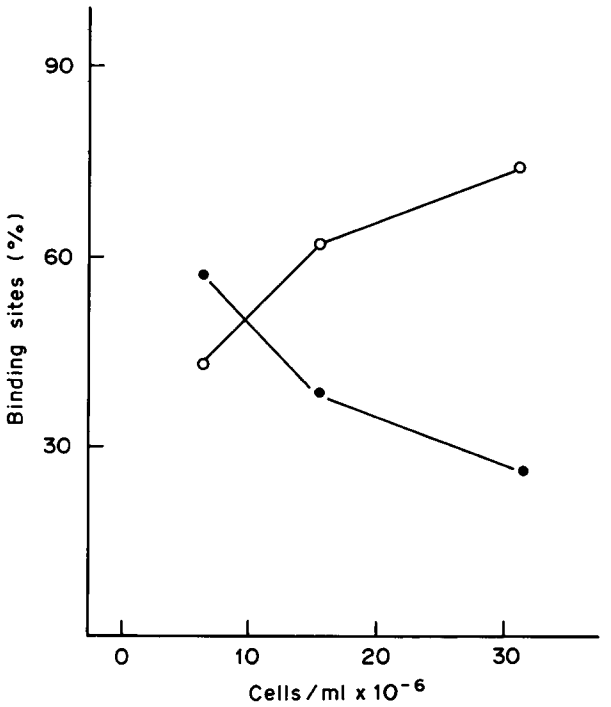

Fig. 2. Effect of cell density on the distribution of glucocorticoid binding sites between cytosolic and nuclear fractions from HTC cells crosslinked with glutaraldehyde. HTC cells were incubated for 2 h at 2°C with 50 nM tritiated dexamethasone and in the presence or in the absence of a 200-fold molar excess of unlabeled competitor. At the end of the incubation, cells were recovered, were dispersed in PBS buffer, and three identical aliquots of cell suspensions were diluted with PBS buffer in order to obtain the indicated cell densities. Cells were then treated for 90 min at 2°C with 0.0125% (w/v) glutaraldehyde, and were then processed to determine specific glucocorticoid binding in cytosolic (○) and nuclear (●) fractions, as described in the Experimental.

sites between cytosol and nuclei was decreased upon increasing the cell density of samples during treatment with glutaraldehyde.

This finding was reproduced in the four independent experiments we have performed, and indicated that the efficiency of crosslinking depends on the concentrations of both glutaraldehyde and the components reacted upon by the crosslinker. We then sought to define optimal conditions to perform crosslinking, by a series of experiments in which the concentrations of the reactants varied. To this end, we incubated samples containing HTC cells at different densities with fixed concentrations of glutaraldehyde. When the levels of glucocorticoid–receptor complexes in cytosolic and nuclear fractions were plotted vs the DNA content of samples (Fig. 3), we obtained two sets of lines whose slopes depended on the glutaraldehyde concentration used in HTC cell treatments. The extrapolation of these lines would intercept at the maximal dilution of samples, yielding a value of cytosolic and nuclear receptor complexes corresponding to 41.1 ± 3.3 and $58.9 \pm 3.3\%$ ($n = 5$) of total cellular receptors, respectively.

These data indicated that the extent of the subcellular redistribution of glucocorticoid–receptor complexes in cytosolic and nuclear fractions depended on the ratio between the concentrations of the chemical groups of cellular components involved in crosslinking (expressed by the DNA content of samples) and that of glutaraldehyde. The data obtained in eleven separate experiments were then combined, and analyzed by linear regression of the receptor content in cytosolic and nuclear fractions vs the ratio between the DNA and glutaraldehyde concentrations of samples. As it is shown in Fig. 4, two lines were obtained ($r = 0.890$, $P < 0.001$), whose intercepts on the ordinate yielded values for cytosolic and nuclear contents of glucocorticoid–receptor complexes corresponding to 37.5 and 62.5% of the total cellular pool, respectively.

On the basis of these findings, we thought it was important to compare the subcellular redistribution of glucocorticoid receptor to that of a cytosolic component, such as the enzyme lactate dehydrogenase (LDH), upon glutaraldehyde treatment of HTC cells. We then repeated those experiments, and measured the LDH activity in cytosolic and nuclear fractions. The results we obtained (Fig. 5) showed that even in the case of LDH activity the levels detectable in cytosolic and nuclear fractions from crosslinked HTC cells could be analyzed by linear regression vs the ratio between the DNA and glutaraldehyde concentrations

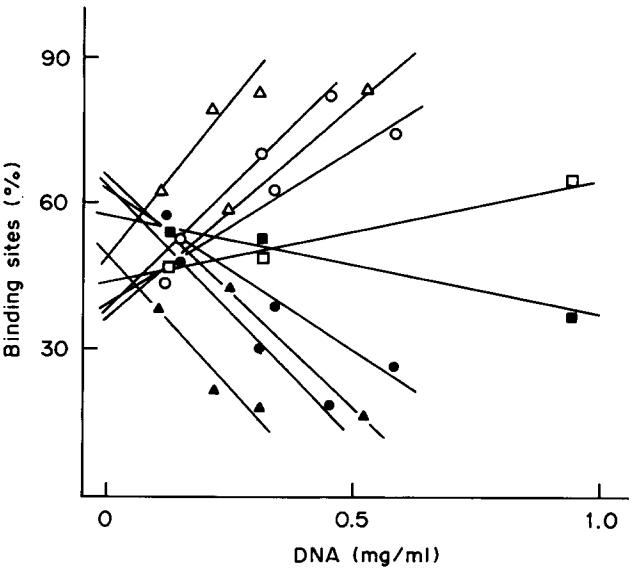

Fig. 3. Effect of changes in both cell density and glutaraldehyde concentrations on the distribution of glucocorticoid binding sites between cytosolic and nuclear fractions from HTC cells crosslinked with different concentrations of glutaraldehyde. HTC cells were incubated for 2 h at 2°C with 50 nM tritiated dexamethasone and in the presence or in the absence of a 200-fold molar excess of unlabeled competitor. At the end of the incubation, cells were recovered, were dispersed in PBS buffer, and aliquots were diluted with different volumes of PBS buffer. Cells were then treated for 90 min at 2°C with 0.00625 (△,▲), 0.0125 (○,●), and 0.025 (□,■) % (w/v) glutaraldehyde, and were then processed to determine specific glucocorticoid binding in cytosolic (open symbols) and nuclear (closed symbols) fractions, as described in the Experimental. Cell density has been expressed as the DNA content in 1 ml of sample during incubation with glutaraldehyde.

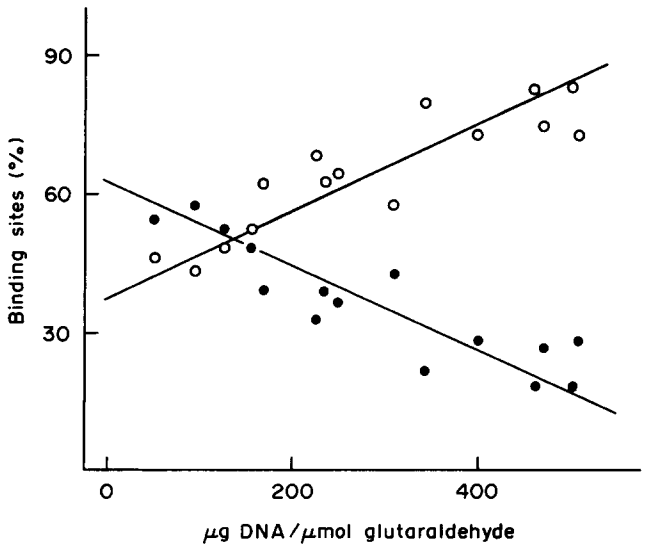

Fig. 4. Linear regression of glucocorticoid binding in cytosolic and nuclear fractions from HTC cells crosslinked with glutaraldehyde. The experimental conditions are as described in the legend to Fig. 3. The levels of specific binding sites in cytosolic (○) and nuclear (●) fractions have been plotted as a function of the ratio between the DNA and the glutaraldehyde contained in 1 ml of sample during crosslinking.

of samples, whose intercepts on the ordinate yielded values corresponding to 53.2 and 46.8% ($r = 0.931$, $P < 0.001$) of the total cellular content, respectively.

DISCUSSION

Chemical crosslinking of intact cells has been shown to be an appropriate tool to immobilize glucocorticoid receptors at the nuclear level, thereby preventing leakage into cytosol upon cell rupture [13, 14].

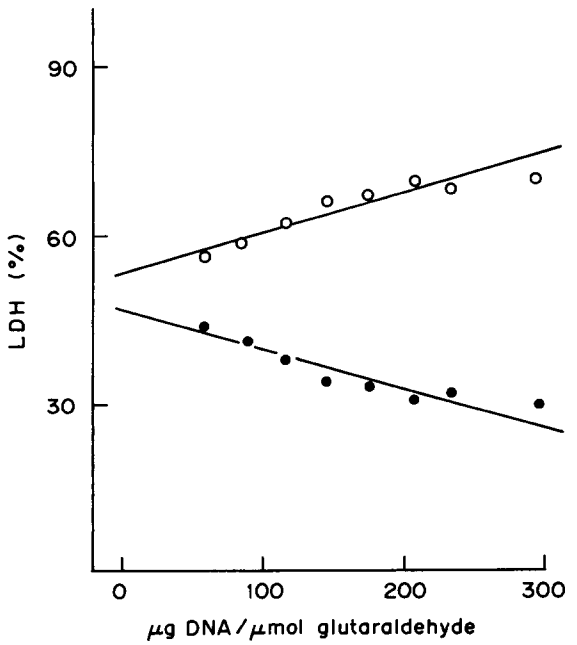

Fig. 5. Linear regression of LDH activity in cytosolic and nuclear fractions from HTC cells crosslinked with glutaraldehyde. The experimental conditions are as described in the legend to Fig. 4. LDH activity in cytosolic (○) and nuclear (●) fractions was determined as described in the Experimental.

Using the HTC cell line we have confirmed the data obtained with HeLa cells [15], regarding the increase in the extent of covalent coupling of glucocorticoid receptors to nuclear components upon increasing the glutaraldehyde concentrations used to crosslink intact cells (Fig. 1), and have shown that it is also inversely related to the concentration of chemical groups of cellular components (expressed by either the cell density or the DNA content of samples) reacted upon by the crosslinker (Figs 2 and 3).

Although it can be assumed that the immobilization of the entire pool of glucocorticoid receptors at the nuclear level can be obtained by increasing the concentration of crosslinker used to treat intact cells, the attempts to precisely estimate the subcellular distribution of receptors by this approach are subjected to a major constraint. Chemical crosslinking, in fact, represents a fixation procedure and, in the extreme, treated cells would become solid blocks. The occurrence of ongoing "fixation" upon glutaraldehyde treatment of intact cells, is documented by the finding that a cytosolic component, such as the enzyme lactate dehydrogenase, accumulates at the nuclear level as a function of the concentration of crosslinker [13].

In our previous investigations this constraint to a careful evaluation of the subcellular distribution of glucocorticoid receptors in crosslinked cells was dealt with by measurements of cytosolic and nuclear levels of receptors under experimental conditions which do not induce extensive redistribution of components, including LDH activity, between the two fractions [13]. While the data we obtained could provide evidence that part of the cellular glucocorticoid receptor pool is located at the nuclear level [13–15], they could not allow a precise estimate of the amounts of receptors in the nuclear and cytosolic fractions of intact cells.

In the present study we approached the issue in an opposite way, by the attempt to evaluate the subcellular distribution of glucocorticoid receptor complexes under extreme conditions, and correct our data by measurements of a cytosolic marker, such as LDH.

When experimental data have been expressed as a function of the ratio between the DNA and crosslinker concentrations of samples, we could obtain a linear regression, whose intercepts with the ordinate represented the percentage of the cellular content of components in cytosolic and nuclear fractions under extreme crosslinking conditions. As expected, when the crosslinker is in a vast excess over the concentration of reactive groups in the cell, the formation of a macromolecular network stabilized by covalent bonds inside the cells, leads to recovery of cytosolic components in nuclear fractions. Our data, however, show that the extent of crosslinking differently affects the redistribution of LDH and glucocorticoid–receptor complexes between cytosolic and nuclear fractions of HTC cells, so that under extreme conditions the cytosolic content of LDH activity is about 50% of the cellular content,

whereas cytosolic receptors account for <40% of the total cellular pool (Figs 4 and 5).

These findings then show that part of the cellular pool of glucocorticoid receptor does not co-distribute with LDH between cytosolic and nuclear fractions of crosslinked HTC cells, being located at the nuclear level, where proteins become immobilized as a consequence of crosslinking.

If the cytosolic content of glucocorticoid receptor is corrected for the artefactual redistribution induced by crosslinking, using the value obtained for cytosolic LDH activity, it can be calculated that in intact HTC cells about 70% of total receptor is cytosolic and the remaining portion is located at the nuclear level.

As we have already shown that our experimental conditions do not induce transformation of glucocorticoid–receptor complexes to states displaying high affinity for DNA [13, 14], it can be concluded that in HTC cells most untransformed glucocorticoid receptor is cytosolic. The nuclear pool, however, is a significant fraction, as it represents about 30% of the total cellular content.

The subcellular distribution of glucocorticoid receptor in HTC cells quantitatively defined in the present study cannot be directly compared to the more qualitative evaluations obtained by immunocytochemical studies [9–12]. The simplicity of our experimental design and the high reproducibility of results (the data contained in Fig. 4 have been obtained over a 15 month period), however, can be easily extended to other systems for direct comparisons of the subcellular distribution of different receptors in the same cell line or among different cells. This information will be particularly important to gain a better understanding of subcellular distribution of steroid receptors. In any case, our finding that glucocorticoid receptors in HTC cells are distributed between cytosol and nuclei in a ratio which is about 2:1, supports the contention that these proteins are in equilibrium between the two compartments [13, 14, 18].

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