Cytosol-nucleus traffic and colocalization with FXR of conjugated bile acids in rat hepatocytes

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Bile acids (BAs) are the main metabolic products accounting for cholesterol elimination. BA homeostasis is largely controlled by nuclear receptors, mainly the farnesoid X receptor (FXR). Whether, in addition to protein-mediated cytosolic-nuclear BA translocation, other mechanisms are involved in the access of BAs to nuclear FXR was investigated. When rat hepatocytes were incubated with radiolabeled taurocholic acid, taurodeoxycholic acid, taurocholate, and tauroursodeoxycholic acid, their nuclear accumulation was proportional to their intracellular levels. With the use of flow cytometry analysis, the accumulation by nuclei isolated from rat liver cells was found to differ for several fluorescent compounds of similar molecular weight and different charge, including fluorescein-tagged BAs (cholylglycyl amidofluorescein (CGamF), ursodeoxychlorylglycyl amidofluorescein, or chenodeoxychlorylglycyl amidofluorescein). When we varied nuclear volume by incubation with different sucrose concentrations, a similar relationship between nuclear clearance was proportional to their intracellular levels. With the use of flow cytometry analysis, the accumulation by nuclei isolated from rat liver cells was found to differ for several fluorescent compounds of similar molecular weight and different charge, including fluorescein-tagged BAs (cholylglycyl amidofluorescein (CGamF), ursodeoxychlorylglycyl amidofluorescein, or chenodeoxychlorylglycyl amidofluorescein). When we varied nuclear volume by incubation with different sucrose concentrations, a similar relationship between nuclear clearance was proportional to their intracellular levels. With the use of flow cytometry analysis, the accumulation by nuclei isolated from rat liver cells was found to differ for several fluorescent compounds of similar molecular weight and different charge, including fluorescein-tagged BAs (cholylglycyl amidofluorescein (CGamF), ursodeoxychlorylglycyl amidofluorescein, or chenodeoxychlorylglycyl amidofluorescein). When we varied nuclear volume by incubation with different sucrose concentrations, a similar relationship between nuclear clearance was proportional to their intracellular levels. With the use of flow cytometry analysis, the accumulation by nuclei isolated from rat liver cells was found to differ for several fluorescent compounds of similar molecular weight and different charge, including fluorescein-tagged BAs (cholylglycyl amidofluorescein (CGamF), ursodeoxychlorylglycyl amidofluorescein, or chenodeoxychlorylglycyl amidofluorescein). When we varied nuclear volume by incubation with different sucrose concentrations, a similar relationship between nuclear clearance was proportional to their intracellular levels.

Bile acids (BAs) are the main metabolic products accounting for cholesterol elimination. BA homeostasis is largely controlled by nuclear receptors, mainly the farnesoid X receptor (FXR) (12, 17, 28), which specifically activates the transcription of genes involved in BA synthesis, hepatobiliary transport, and intestinal absorption. In general, FXR activation results in the protection against BA accumulation and toxicity in tissues that express this receptor (for review see Refs. 3 and 5). Different BA species have different abilities to bind FXR. Thus the major primary BA, cholic acid (CA) and its glycine (GCA) and taurine (TCA) conjugates, as well as ursodeoxycholic acid (UDCA), a BA used in the treatment of cholestatic liver diseases, bind very weakly to FXR (9, 12), whereas the CA-derived secondary BA deoxycholic acid (DCA) and the primary BA chenodeoxycholic acid (CDCA) bind with higher affinity and are stronger activators of FXR (9, 12). Other nuclear receptors sensitive to BAs include the pregnane X receptor and the vitamin D receptor (VDR), of which both can be activated by lithocholic acid (11, 26, 31).

To bind to these nuclear receptors, BAs must reach the nucleus. Since the majority of BAs traversing the hepatocyte from blood to bile are in amimidated form, it can be assumed that most BAs reaching the nuclei of this cell type under physiological circumstances should also be conjugated BAs. The presence of BAs in the nuclei of rat hepatocytes has been reported previously (14, 16, 21). The amount of BAs in the nucleus increases after common bile duct ligation (21). Moreover, nuclear BA pool composition was different from that of the cytoplasmic pool, and this difference changes during liver regeneration (16) and carcinogenesis (14). Whether cytosolic-nucleus exchange of BAs is due to simple diffusion through nuclear pore complexes, translocation after binding to cytosolic-soluble proteins, or other mechanisms is not known.

Despite the important advances made in recent decades in our understanding of the mechanisms responsible for the nuclear-cytoplasmic traffic of proteins and nucleic acids (2, 4, 6), very little is known about the nuclear trafficking of small organic molecules (molecular mass <1,000 Da), including ligands of nuclear receptors, such as BAs, retinoic acids, oxysterols, and several xenobiotics.

The access of BAs to the hepatocyte nucleus has been associated to soluble protein-dependent translocation to the nucleus after binding to these carriers in the cytosol. These include glucocorticoid receptors (23, 27, 30) and probably histone deacetylases (15).

The aim of the present study was to investigate the access of conjugated BAs to the nuclei of rat hepatocytes, as well as to define their nuclear localization and to gain insight into the mechanisms accounting for their exchange between the nucleus and the cytosol.

MATERIALS AND METHODS

Chemicals and animals. Glycoursodeoxycholic acid (GUDCA) was from Calbiochem (Darmstadt, Germany), and sodium salts of cholesterol, taurocholate, and tauroursodeoxycholic acid (UDCA), a BA used in the treatment of cholestatic liver diseases, bind very weakly to FXR (9, 12), whereas the CA-derived secondary BA deoxycholic acid (DCA) and the primary BA chenodeoxycholic acid (CDCA) bind with higher affinity and are stronger activators of FXR (9, 12). Other nuclear receptors sensitive to BAs include the pregnane X receptor and the vitamin D receptor (VDR), of which both can be activated by lithocholic acid (11, 26, 31).

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compounds were carried out after 30-min incubation when nuclear sucrose diffusion, measurements of nuclear content of fluorescent dextran at 37°C. To minimize initial solvent drag effects linked to (0 –250 mM), together with 10 mM HEPES/Tris, pH 7.4, and varying concentrations of sucrose (CGamF), ursodeoxycholylglycyl amidofluorescein (UDCGamF), and chenodeoxycholylglycyl amidofluorescein (CDCGamF) were synthesized by coupling the amido group of FITC to the carboxyl group of the glycine moiety (19) of GCA, GUDCA, or GCDCA, respectively (22).

Male Wistar rats (Animal House, University of Salamanca, Spain) received humane care as established and approved by the ethical committee of the University of Salamanca in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experiments on isolated hepatocytes. Rat hepatocytes were obtained as described elsewhere (13). Nuclear content of radiolabeled BAs was determined as previously described (16). When rat hepatocytes (50 × 10⁶ cells suspended in 20 ml of Earle’s Balanced Salts Solution) were maintained in suspension at 37°C with continuous gentle shaking and oxygenation (5% CO₂-95% O₂), cell viability was >90% for at least 60 min, on the basis of Trypan blue dye exclusion (data not shown). These cells were incubated with [³H]TCA, [³H]TCDCA, [³H]TDCA, or [³H]TUDCA to measure cellular accumulation, extracellular binding, and nuclear content in isolated nuclei. To calculate cellular accumulation at 37°C, two samples were collected: 1) sample A from the whole cell suspension and 2) sample B from the supernatant obtained by centrifuging the suspension at 43 g at 4°C for 2 min. Total BA cell retention was calculated from the difference of radioactivity between A and B. Extracellular binding was calculated in a similar way in separate cell suspension samples maintained at 4°C in which the radiolabeled BA was added immediately before cell separation by centrifugation. To isolate the nuclei, pelleted hepatocytes were washed twice with ice-cold PBS, resuspended in swelling solution (1 mM Trizma base, 25 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.15 mM spermidine, and 1% protease inhibitor cocktail from Sigma), and placed on ice for 60 min. The cells were then broken in a motor-driven tight-fitting Teflon-glass homogenizer. Correction of the degree of BA crosscontamination between the nuclei and the cytosol occurring during the isolation procedure was carried out as previously reported (16) by adding [¹⁴C]TCA to broken cells at approximately the same amount of [³H]BA as that retained by the cells. Nuclei were recovered by centrifugation at 500 g for 4 min at 4°C, washed three times with ice-cold PBS, and resuspended in ice-cold PBS before the DNA content and the amount of radioactivity were measured.

Nuclear uptake of fluorescent BA derivatives. Nuclei were obtained from rat liver homogenates as previously described (16), purified by serial centrifugation (7), suspended in sucrose-Tris magnesium solution (250 mM sucrose, 50 mM Tris ·HCl, and 5 mM MgSO₄, pH 7.4), and counted in a Neubauer chamber. The mean yield of the isolation procedure was 173 ± 14 × 10⁶ nuclei/g liver (n = 20 preparations, from 2 rat livers each). These preparations consisted of suspensions of cell debris-free nuclei, which were kept at 4°C until use within the following 24 h.

Flow cytometry using a FACSort flow cytometer (BD Biosciences, Madrid, Spain) was used to determine nuclear uptake of fluorescent compounds.

To investigate the effect of nuclear size, in some experiments this was modified by incubating isolated nuclei in media containing 10 mM HEPES/Tris, pH 7.4, and varying concentrations of sucrose (0–250 mM), together with 10 μM CGamF, FITC, or 4-kDa FITCdextran at 37°C. To minimize initial solvent drag effects linked to sucrose diffusion, measurements of nuclear content of fluorescent compounds were carried out after 30-min incubation when nuclear size and presumably sucrose concentrations were already in steady-state conditions (data not shown).

Confocal microscopy studies. After 3 h of culturing rat hepatocytes (10⁶ hepatocytes/well) in collagen-coated eight-well Lab-Tek chamber slides (BD Biosciences), the medium was removed and replaced by a fresh one containing 20 μM of FITC, CGamF, CDCGamF, or UDCGamF. After 30 min of incubation at 37°C in the dark, cells were washed with PBS and fixed with ice-cold paraformaldehyde (4% in PBS).

Purified isolated hepatocyte nuclei preparations were incubated for 30 min with the desired fluorescent compound. Nuclei were then precipitated by centrifugation (4,000 g for 2 min at 4°C), washed with
PBS, and fixed with ice-cold paraformaldehyde. After a wash with PBS, nuclei were concentrated on slides by centrifugation.

Immunofluorescence studies were carried out using H-130 antibody for FXR, C23 (MS-3) antibody for nucleolin (both from Santa Cruz Biotechnology, Santa Cruz, CA), 3B6/PPAR-activated receptor (PPAR) antibody for PPAR-α (Affinity BioReagents, Golden, Colorado), and Alexa Fluor 488 or Alexa Fluor 594-conjugated antirabbit or antimouse secondary antibody (Molecular Probes, Leiden, The Netherlands) as appropriate. Negative controls were obtained by omitting the primary antibody.

**Analytical and statistical methods.** Radioactivity was measured by using the Ready Safe Scintillation Cocktail (Beckman Instruments, Madrid, Spain) as scintillant. DNA measurements were carried out with Hoechst-33258 as fluorescent probe (8) and calf thymus DNA as standard.

To calculate the statistical significance of differences among groups, the paired Student's t-test or the Bonferroni method of multiple-range testing were used, as appropriate. Linear regression analyses were carried out using the least-squares method.

**RESULTS**

Incubation of rat hepatocytes with [3H]TCA resulted in radioactivity accumulation by these cells and their nuclei (Fig. 1A).

TCA extracellular binding, as well as the total amount accumulated by the cells (Fig. 1B) and their nuclei (Fig. 1C), was progressively higher in the presence of increasing TCA concentrations in the incubation medium. As the preparations contained binucleate and tetraploid hepatocytes, the results were normalized per amount of DNA. The mean value was 2.86 ± 0.34 μg DNA/million cells, and this was not significantly changed when the cells were incubated with TCA. Plotting net intracellular TCA accumulation vs. nuclear content resulted in a linear correlation (Fig. 1C, inset). There was a positive value of x-axis intercept, which was probably due to intracellular binding. Similar results were obtained when other conjugated BAs (TDCA, TCDCA, and TUDCA) were used instead of TCA (data not shown).

Since in experiments using radiolabeled BAs a certain degree of efflux probably occurred during nuclei isolation, additional experiments on nuclear accumulation of conjugated BAs were carried out. In these studies, we used isolated nuclei and flow cytometry analysis together with three fluorescent BA derivatives, CGamF (molecular mass = 794 Da), CDCGamF (molecular mass = 778 Da), and UDCGamF (molecular mass = 778 Da).

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**Fig. 2.** Flow cytometry analysis of isolated rat hepatocyte nuclei. A and B: representative dot plots showing the 2 nuclear populations obtained when nuclei were isolated from liver tissue and stained with propidium iodide to label their DNA content. C: representative histogram of DNA contents in both populations. LN, large nuclei; SN, small nuclei. D–H: representative histograms corresponding to isolated hepatocyte nuclei after 30-min incubation at 37°C with 20 μM FITC (G), FITC-labeled dextrans of decreasing molecular weight: FD-70S (70 kDa, D), FD-40S (40 kDa, E), and FD-10S (10 kDa, F) or the fluorescent bile acid derivative cholyglycyl amidofluorescein (CGamF, H). The nuclei were suspended in loading medium (LM; 250 mM sucrose, 100 mM KNO₃, 10 mM MgCl₂, 0.2 mM CaCl₂, and 10 mM HEPES/Tris pH 7.4) containing the fluorescent compound to be assayed and were incubated at 37°C in the dark with mild shaking before a sample was collected and fluorescence was measured. Data from 10,000 events (nuclei) were collected in each analysis.
The preparations of isolated nuclei contained two populations of nuclei of different sizes (Fig. 2A). DNA content was approximately twofold higher in the large than in the small nuclei (Fig. 2, B and C), indicating that these populations were nuclei obtained from tetraploid and diploid hepatocytes, respectively. Similar qualitative (although quantitatively different) effects were observed regarding the nuclear uptake of fluorescent compounds by large and small nuclei (data not shown).

When isolated nuclei were incubated at 37°C for 30 min with FITC-labeled dextrans of different molecular masses, the fluorescence detected in the nuclei was low but increased as the molecular mass of the FITC-dextrans decreased (Fig. 2). In contrast, incubation with FITC (molecular mass = 389 Da) resulted in a marked increase in fluorescence. Using fluorescein-BA conjugates, lower values (although still 5–8 times higher than those found for 10-kDa FITC-dextran) were found (Fig. 2H).

Time course of FITC and fluorescent BA derivatives uptake by isolated nuclei at 37°C was initially rapid, with no significant increase in fluorescence values after the first 10 min of incubation (data not shown). The nuclear uptake of these compounds was temperature dependent; i.e., at 4°C the fluorescence levels reached in the nuclei were lower (data not shown). Measurement of the nuclear uptake of other small fluorescent compounds revealed that, after the initial retention (presumably due mainly to binding), almost no further accumulation of calcein (molecular mass = 666 Da) or the smaller cationic compound rhodamine (molecular mass = 380 Da) occurred. In contrast, the fluorescence due to FITC, CGamF, UDCGamF, and doxorubicin (molecular mass = 580 Da) increased over 5 min (Fig. 3).

When isolated nuclei were incubated with different sucrose concentrations (0–250 mM) two populations, i.e., large and small nuclei, were no longer distinguishable. The nuclei were distributed in a range of different sizes (Fig. 4A), whose mean value was dependent on sucrose concentration (Fig. 4B). When combining data obtained from the same isolated nuclei preparation (incubated with different sucrose concentrations), it was possible to analyze a large number of nuclei of different sizes. These were divided into five segments of sizes containing similar numbers of nuclei (>1,000). When mean values for each segment (corrected by the number of events in it) were plotted, significant correlations between nuclear volume and accumulation of 4-kDa FITC-dextran, FITC, or CGamF were found (Fig. 4C). These curves had similar values of y-axis intercepts (presumably due to retention in a volume-independent compartment) but different slopes (FITC ≈ 4-kDa FITC-dextran > CGamF) (presumably due to accumulation in a volume-dependent compartment) (Fig. 4D). The amount of sucrose in the medium had no effect on the intensity of the fluorescence due to these compounds and did not affect the values of y-axis intercepts or slopes of the correlation curves for each compound (data not shown). To elucidate whether the process of BA incorporation into isolated nuclei has saturation characteristics, self-inhibition of UDCGamF and crossinhibition by natural BAs was investigated. UDCGamF nuclear load was lineal up to 120 μM UDCGamF in the extranuclear medium (data not shown). Moreover, GCA, TCA, GUDCA, TUDCA, TDCA, and TCDCA were not able to inhibit the UDCGamF load when isolated nuclei were incubated with 5 μM of this fluorescent BA derivative and 100 μM of one of the major BAs mentioned above for 2 min (data not shown).

To visualize BA derivatives by confocal laser microscopy, rat hepatocytes were incubated with CGamF at 37°C for 30 min. The fluorescence was observed in the cytosol, together with a less prominent signal in the nuclei (Fig. 5A), where CGamF was in different compartments (Fig. 5B). In the intranuclear space, a dispersed fluorescence, together with some spots of more intense fluorescence, was observed. When isolated nuclei were incubated directly with CGamF, the fluoresc-
cence was more clearly observed both at the nuclear envelope and inside the nuclei; there it was also both dispersed and concentrated in spots (Fig. 5C). In these regions, DNA seemed to be less densely packed as suggested by the lower intensity of fluorescence due to propidium iodide staining (Fig. 5, D and E). The same pattern of nuclear distribution of BA derivatives was found when either cells (data not shown) or isolated nuclei (Fig. 6) were incubated with FITC (Fig. 6B), 10-kDa FITC-dextran (Fig. 6C), CGamF (Fig. 6D), CDCGamF (Fig. 6E), or UDCGamF (Fig. 6F). In contrast, only a small degree of nuclear accumulation of either calcine (Fig. 6G) or rhodamine (Fig. 6H) occurred, and neither of these two compounds was found to be concentrated in intranuclear spots. Moreover, incubation with doxorubicin, which entered the nucleus readily, resulted in an intense homogeneously distributed fluorescence (Fig. 6I).

Fig. 5. Representative confocal microscopy image of a binucleated rat hepatocyte in primary culture after incubation for 30 min with 20 μM CGamF (A) and schematic representation of the nuclear localization of fluorescence indicated with an asterisk (B). Similar studies were carried out in isolated nuclei, which, after incubation with CGamF (green fluorescence, C), were fixed and DNA stained with 50 μg/ml propidium iodide (red fluorescence, D). Colocalization of CGamF and DNA (E).
When FXR was localized by immunolabeling, the fluorescence was observed mainly inside the nuclei although some extranuclear staining was also detected (Figs. 7 and 8). Intranuclear FXR was localized in the same regions of lower DNA density where CGamF was concentrated (Fig. 7). Nucleolin was used here as a marker of transcriptional active chromatin regions, whereas PPAR-α, due to its cytosolic localization, was used as a negative control nuclear receptor. Immunolabeling of nucleolin and PPAR-α resulted in the expected subcellular distribution (Fig. 8). When immunodetection of FXR and nucleolin was carried out on the same cells, colocalization of both proteins in intranuclear spots was observed (Fig. 8). In contrast, when immunodetection of PPAR-α and FXR was performed, different subcellular localization was observed (mainly cytosolic and mainly nuclear, respectively) (Fig. 8).

DISCUSSION

The results of the present study show that conjugated BAs are able to enter the nucleus of rat hepatocytes even though cytosolic proteins are not present, which is not compatible with the existence in vivo of BA translocation to the nucleus after binding in the cytosol to carrier proteins (15, 23, 27, 30).

In addition to a certain degree of rapid accumulation in a nuclear volume-independent compartment, which probably is accounted for extranuclear binding and/or retention in the intermembrane space of the nuclear envelope, there is a protein-independent mechanism of exchange of BAs and other small organic molecules between the nucleus and the extranuclear space. The simplest interpretation of our results is that this is determined by simple diffusion. However, this pathway seems to have a certain degree of molecular selectivity because molecules of similar weight and positive or negative charge are more (e.g., FITC) or less (e.g., calcein and rhodamine) able than BAs to enter the hepatocyte nucleus.

This is not consistent with the concept that nuclear pore complexes permit the simple diffusion of all compounds below a molecular mass of 20–40 kDa (18). The present study confirms that a diffusional component that permits limited passage of small dextrans does exist. However, our results suggest that, in addition to simple diffusion, there are factors other than molecular size that determine nuclear content of small organic compounds, including BAs. The findings that intranuclear accumulation of FITC and 4-kDa FITC-dextran was similar, whereas that of CGamF, which has an intermediate molecular weight, was markedly lower support this hypothesis.

It should be considered that, compared with in vivo physiological circumstances, the diffusional component of BA exchange between the cytosol and the nucleus across the nuclear pore complexes has been probably overestimated in the experimental conditions of the present study. 1) In isolated nuclei preparations used here, nuclear pore complexes are not in-

Fig. 6. Representative images obtained by laser confocal microscopy of isolated rat hepatocyte nuclei after incubation with different fluorescent compounds. Autofluorescence of nonincubated nuclei was used as a negative control (A). Hepatocyte nuclei were incubated for 30 min with 20 μM FITC (B), 10-kDa FITC-dextran (FD-10S) (C), CGamF (D), chenodeoxycholylglycyl amidofluorescein (CDCGamF) (E), or UDCGamF (F) or with 5 μg/ml of calcein (G), rhodamine 123 (H), or doxorubicin (I).
involved in the trafficking of the macromolecules, which normally occurs in living cells. It could be speculated that proteins and RNA crossing these structures probably behave as plugs to the diffusion of small molecules across them. Although there is little information on the concentrations of monomeric BAs in hepatocytes, this is believed to be about 1 μM (29). Thus the magnitude of the BA concentration gradients between the cytosol and the nucleus are probably smaller than those employed in the present study.

Owing to their hydrophobic face, conjugated BAs are likely to be bound to intracellular proteins and lipids, which would diminish their efficient concentration gradients for simple diffusion across nuclear pore complexes.

When rat hepatocytes were incubated with fluorescent BA derivatives, the fluorescence in the nucleus was less marked than in the rest of the cell. This was probably due to the fact that there is little free water space in the nucleus unoccupied by structural elements such as chromatin and nuclear bodies. However, other possibilities cannot be ruled out. Thus the quantum yield of fluorescein-tagged BAs is dependent on the polarity of the medium, and this may differ between the nucleus and the cytosol (10).

An important observation of the present study was that fluorescein-BAs accumulated in nuclear regions with less densely packed DNA and that FXR was also present in these regions. This is consistent with the interaction of BAs and FXR in transcriptionally active zones of the nucleus. The localization of nucleolin in the same nuclear regions supports this idea. Colocalization inside the nucleus of nucleolin and other nuclear receptors, such as those for glucocorticoids (20) and...
estrogens (24), has been already reported. This supports the concept that, in addition to the presence in the nucleoli contributed to polymerase I-mediated synthesis of rRNA, nucleolins may facilitate chromatin transcription (behave as FACT), facilitating mRNA synthesis by polymerase II in transcriptionally active areas (1).

Regarding the specificity of this location for BAs, the accumulation in these less densely packed DNA regions of FITC and 10-kDa FITC-dextran suggests that the interaction of small organic compounds, such as BAs, with nuclear receptors present in these regions, such as FXR, is the consequence rather than the cause of the observed pattern of subnuclear colocalization of BAs and FXR. This means that ligand and receptor coincide in the same spot, facilitating the mutual interaction rather than the opposite; i.e., the specific binding to FXR determines the subnuclear localization of BAs.

In conclusion, these results indicate that conjugated BAs can enter the hepatocyte nucleus by a cytosolic protein-independent mechanism. This process depends on BA concentrations in the extranuclear space, which is physiologically relevant because it may constitute a rapid mechanism for the cell-to-sense variations in BA levels and hence to respond by modulating the expression of transporters and enzymes involved in BA and cholesterol homeostasis. However, although intranuclear levels of small organic molecules depend on their concentrations in the extranuclear space, the existence of certain molecular selectivity (not strictly dependent on molecular weight or charge) suggests that, in addition to simple diffusion, other mechanisms may also be involved in determining their overall nuclear content.

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