Targeting of Drug to the Hepatocytes by Fatty Acids. Influence of the Carrier (Albumin or Galactosylated Albumin) on the Fate of the Fatty Acids and Their Analogs

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Purpose. The aim of this study was to evaluate the potential of fatty acids as shuttles to deliver xenobiotic inside the hepatocytes as well as to study the mechanism of incorporation into isolated hepatocytes when bound to native albumin or galactosylated albumin. Theoretically, they can enter into the hepatocytes after recognition of the Fatty Acid Binding Protein ($FABP_{PM}$), or remain bound to galactosylated proteins and enter into these cells by a process known as receptor mediated endocytosis after selective recognition of the asialoglycoprotein receptor (ASGPR).

Methods. We synthesized a ^{3}H-benzoyl adduct of lauric acid (BLA) (benzoyl adduct choosen to mimick any low molecular weight drug or contrast agent), and compared the behavior of BLA with oleic acid for their binding properties to carrier-proteins and the uptake mechanism by isolated hepatocytes.

Results. No significant difference was found in the binding properties of BLA for albumin and galactosylated albumin. The incorporation into the hepatocytes was found essentially depending on the $FABP_{PM}$ transport system whenever BLA was bound to albumin or to galactosylated albumin in the incubation medium: indeed, the transport was inhibited by phloretin (inhibitor of sodium dependent transport), increased when the free part of BLA was higher, and BLA was recovered in the cytosolic fraction of the hepatocytes.

Conclusions. This study showed the convenience in using fatty acids as drug carriers possessing tropism for the hepatocytes.

KEY WORDS: liver; fatty acid; asialoglycoprotein; albumin; selective; drug delivery.

INTRODUCTION

Selective drug delivery at the convenient site is of major interest in drug design in order to avoid non specific pharmacological activity or toxicity to the whole organism. Sometimes, it also is interesting to provide this drug delivery into a specific compartment of the cell. In order to deliver drugs or contrast agents inside the hepatocytes, we recently focalized our attention on two major pathways: the transport system of the fatty acids on the one hand [1], the receptor mediated endocytosis after selective recognition of the asialoglycoprotein receptor (ASGPR) on the other hand [2]. Fatty acids present a high affinity for the liver: their efficient cellular uptake is the result of the dissociation of the complex albumin/fatty acid, and of a specific interaction with the Fatty Acid Binding Protein (h-$FABP_{PM}$) present at the sinusoidal pole of the hepatocyte [3], then they become bound in the cytoplasm to the cytosolic Fatty Acid Binding Protein ($FABP_c$). The ASGPR recognizes endogenous neoglycoproteins and macromolecules bearing polygalactose residues (e.g. galactosylated albumin [4]). The receptor-ligand complexes are internalized, and the ligands are finally transported to lysosomes.

Because fatty acids theoretically can bind to albumin or to galactosylated albumin, we can potentially expect two different mechanisms of uptake and intracellular fate when the fatty acid is bound to albumin or galactosylated albumin. In the first condition, the fatty acid is bound to albumin or galactosylated albumin. In the first condition, the fatty acid is known to recognize the $FABP_{PM}$ and to have a cytosolic fate, in the latter, we can expect a recognition of the ASGPR by the complex galactosylated albumin/fatty acid, and an accumulation inside the lysosomes. The purpose of this study was (1) to synthesize a radiolabelled adduct of fatty acid, with a benzoyl adduct choosen to mimick any low molecular weight drug or contrast agent; (2) to check the binding of this compound to both albumin and galactosylated albumin; (3) to verify the potential use of fatty acids as delivery systems of drug in the hepatocytes, (4) to study the mechanism of the uptake when the fatty acid analog is bound to albumin or galactosylated albumin; (5) to appreciate the intracellular fate of the compound; (6) to compare the behavior of this adduct with a natural fatty acid.

MATERIALS AND METHODS

Chemistry

Synthesis of 12-(benzoylamino)dodecanoic Acid (benzoyl lauric acid, BLA). The synthesis scheme is shown in Fig. 1. 1.9 g of aminododecanoic acid (1 on Fig.1, EGA-Chemie 8.8 mmol) were added to 2g of benzoic anhydride (2 on fig 1, 8.8 mmol, Aldrich) in 100 ml of acetic acid. The mixture was stirred for 2.5 hour at room temperature. The mixture was evaporated under reduced pressure. The powder was washed with 50 ml of CCl_4 (Merck), filtrated, and recristallized 2 times in ethanol.

Yield: 86 %

The compound was found pure in TLC (Silicagel, Merck) using a mixture of hexane / ethylacetate 1:9 as eluent (Rf = 0.43).

F : 99.5-101.5°

Infra-red spectra, mass spectra, ^{13}C-NMR spectra, and elemental analysis were consistent with the structure.

Tritiation of BLA. BLA was tritiated using the method adapted from Hesk *et al* [5]. Briefly, 120 mg of BLA and 120 mg of $RhCl_3$ were dissolved in 2 ml of anhydrous dimethyl-

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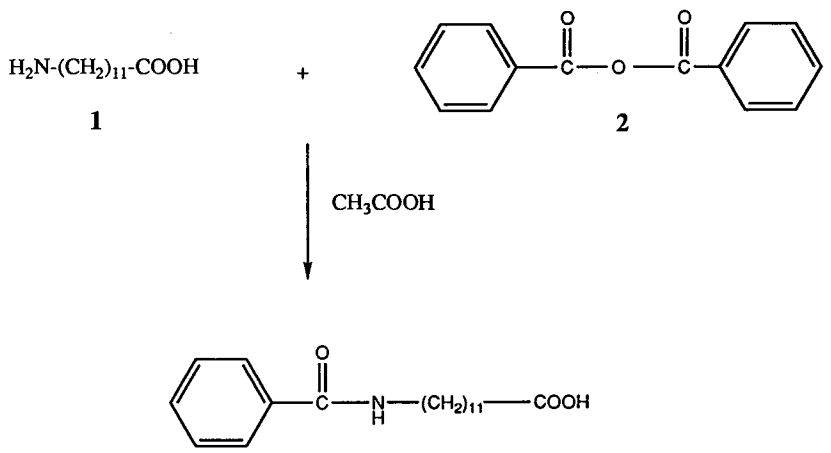


BLA

Fig. 1. Chemical synthesis of BLA.

formamide (Aldrich). 90 µl of ^3H_2O (Amersham, 185 GBq/ml) were added and the mixture was stirred during 24 hours at 108°C. The reaction flask was cooled until room temperature, 8 ml of hydrogen chloride 4 M were added. The media was extracted 3 times by 20 ml of ethylacetate. The organic phase was dried on $MgSO_4$, filtrated, and evaporated under reduced pressure. The residue is then dissolved in ethylacetate, and purified by preparative thin layer chromatography using Silicagel plates (Merck) and a mixture hexane-ethylacetate (1:9, v:v) as eluent. The radiochemical purity was determined using HPTLC (RP-18F 254S, Merck) and an eluent containing a mixture of an aqueous solution of NaH_2PO_4 6mM 30%, acetonitrile 50%, and methanol 20%. The radioactivity on the plate was analysed with a Bioscan System 200 Imaging Scanner, and was found >99 %. The typical specific activity obtained was ~ 2.10^9 dpm/mmol.

Galactosylated Albumin. The galactosylated albumin was prepared following the method described by Schwartz [6] and characterized as previously described [2]. We found that 13 galactose residues were linked per one albumin molecule. We previously demonstrated that the galactosylated albumin synthesized by this procedure was able to recognize the ASGPR [2].

Binding to Albumin and Galactosylated Albumin

A solution of BLA in absolute ethanol was diluted in a PBS buffer (pH = 7.2) to reach a final concentration of 150 µM (final concentration in ethanol : 5%). To 250 µl of this solution were added different amounts of a solution of albumin or galactosylated albumin (10 %), and PBS buffer was added to reach a final volume of 400 µl. The mixture was vortexed. After 10 minutes incubation at room temperature, the solution is deposed on a ultrafiltration membrane (Ultrafree-MC Filters Millipore), centrifugated for 1h at 4°C (5000 g). The amount of free BLA was determined by adding 10 ml of Hionic-Fluor (Camberra Packard) and counting the radioactivity using a Wallac 1410 liquid scintillation counter. These values were corrected for the aspecific adsorption on the cellulose membranes.

Uptake of ^3H-Oleic Acid and ^3H-BLA by Isolated Hepatocytes

Isolated hepatocytes from rat were obtained using the collagenase perfusion method [7]. The hepatocytes were suspended in a Krebs-Ringer solution supplemented with $NaHCO_3$ 25 mM, $CaCl_2$ 2.5 mM, glucose 25 mM. This suspension was gently stirred in a bath at 37°C. Isolated hepatocytes solutions (5.10^5 cells/ml) were incubated in the presence of complexes of BLA-albumin or BLA-galactosylated albumin (molar ratio : 1/1 and 1/2), oleic acid-albumin or oleic acid-galactosylated albumin (molar ratio 1/1), without (=control) or with phloretin or arabinogalactan as potential competitor of the uptake. The concentration of BLA or oleic acid was 0.11 nmol per ml of suspension of hepatocytes. The uptake of the fatty acid was stopped at different times of the incubation by adding 1 ml of the hepatocytes suspension to 500 µl of a Krebs-Ringer solution of phloretin 0.2 mM cooled in an ice bath [8]. The solutions were centrifugated (700 g, 2 minutes, 4°C), the pellet was washed twice with 500 µl of a cooled Krebs-Ringer solution containing phloretin 0.2 mM, and the radioactivity present in the final pellet and the supernatant was quantified by addition of Aquasol® (NEN) and using a Wallac 1410 liquid scintillation counter. The viability of the hepatocytes was followed by measuring the LDH (cytosolic enyzme) activity in the supernatant and the hepatocytes [8]. Each uptake assay was performed in triplicate on three independent batches of hepatocytes. In order to follow the intracellular fate of the compounds, a subcellular fractionation was performed, and the differents fractions were analyzed for their content. After an incubation of 10 minutes (in the conditions described earlier), the subcellular fractionation was performed using the method adapted from Krack et al. [8]. Briefly : the entire procedure was carried out at 4°C. The cell suspension (30×10^6 cells/ml) was centrifuged at 45 g for 1 min. The supernatant was discarded and the cells were resuspended in 0.25 M sucrose buffered with 3 mM imidazole (pH 7.4) so as to reach a concentration of 10^7 cells/ml. The homogeneization was performed operating several times with the tight pestle of a Dounce tissue grinder, and checking the integrity of the cells using a microscope.

Fractionation of the cytoplasmic extract was carried out by differential centrifugation according to the method of De Duve [9] to obtain three different fractions: nuclei, mitocondria + lysosomes, cytosol + microsomes. The radioactivity was quantified in each fraction.

RESULTS

Binding to Albumin

Fig. 2 presents the binding curve of BLA to albumin and galactosylated albumin. Interestingly enough, both curves are roughly superposable indicating a similar affinity of BLA for the native and the modified protein. Moreover, we find that more than 90 % of BLA is bound when the protein/BLA molar ratio is greater than 1. This observation is consistent with previous results obtained with other analogs of fatty acids [3, 10].

Uptake by the Hepatocytes

Fig. 3a shows the initial uptake of BLA by the hepatocytes when the molar ratio BLA/Albumin is 1:1 and 1:2. Interestingly enough, we can notice the increased incorporation inside the cells for the highest molar ratio (in other words, for the highest concentration of free BLA). The same result was obtained when BLA was bound in the incubation medium to the galactosylated albumin (Fig. 3b). We obtained the same amount and kinetics of incorporation whenever BLA was bound to native albumin or galactosylated albumin in the molar ratio 1:1, and very slightly different when the molar ratio was 1:2.

The influence of phloretin, an inhibitor of the transport of the fatty acids [8], on the uptake of BLA was checked : from one minute to five minutes of incubation, the inhibition was between 40 and 60%, with typical values of 64 and 53 % of the control (incubation without phloretin) incorporated into the hepatocytes after five minutes, respectively for BLA bound to albumin or galactosylated albumin (molar ratio 1:1). In the same experimental conditions (molar ratio 1:1, 5 min incubation), we observed 28% of inhibition of oleic acid incorporation by phloretin. The results obtained with ^{3}H-oleic acid (incubated in the presence of albumin, molar ratio 1:1) are summarized in Fig. 4.

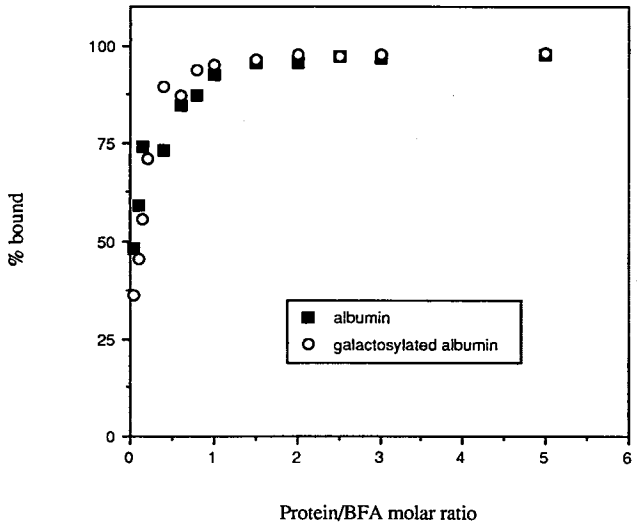
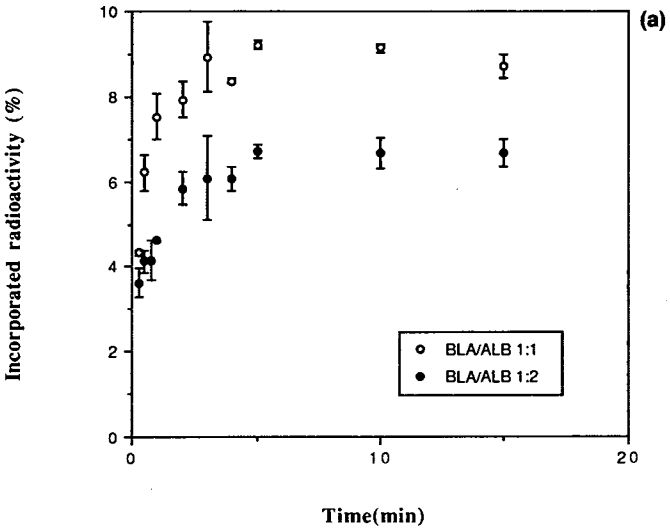


Fig. 2. Binding curve of BLA to albumin and galactosylated albumin. Note that the amount of BLA bound to the protein is roughly superposable for both proteins.



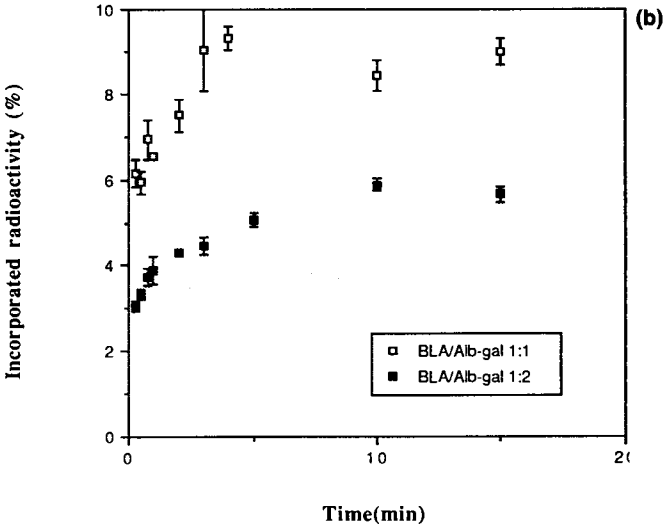


Fig. 3. (a) Time course of BLA uptake by isolated hepatocytes suspensions. In the curves illustrated, incubations of BLA were in the presence of a BLA/albumin molar ratio of 1:1 and 1:2. (b) Time course of BLA uptake by isolated hepatocytes suspensions. In the curves illustrated, incubations of BLA were in the presence of a BLA/galactosylated albumin molar ratio of 1:1 and 1:2.

The results of the fractionnation of the hepatocytes after incubation with BLA bound to albumin or galactosylated albumin are shown on Table 1. The carrier protein does not influence the intracellular fate of BLA since the results are very similar in both cases. The radioactivity was essentially found in the fraction cytosol-microsomes. A redistribution of BLA can be excluded as the radioactivity found inside the different fractions do not correlate with the partition that

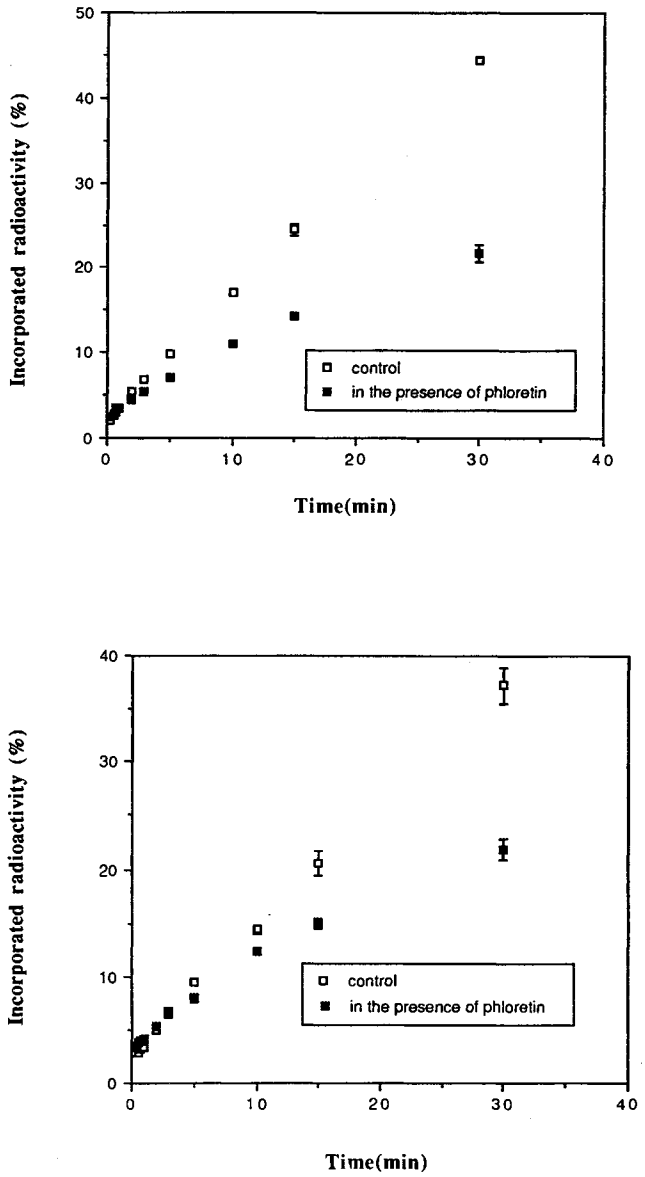


Fig. 4. Time course of oleic acid uptake by isolated hepatocytes suspensions, without (=control), or in the presence of phloretin. (Top) incubation of oleic bound to albumin (molar ratio 1:1). (Bottom) incubation of oleic acid bound to galactosylated albumin (molar ratio 1:1).

should possess a product which could distribute or diffuse freely in the different cell compartments (such a compound should possess a much higher concentration in the nuclei fraction).

During prolonged incubations, we observed that the amount of BLA increases to reach a plateau after 5 minutes, and starts to decrease, while oleic acid continues to enter inside the hepatocytes. In typical experiments the amount of BLA incorporated inside the hepatocytes was 5.85% after 5 min, 3.79 % after 20 min, and 2.04 % after 30 min.

In order to determine if this decrease of radioactivity in the hepatocytes was correlated with a release of BLA or another product, we analysed the content of the cells by radiochromatography HPTLC : RP-18F 254S, (Merck) and

Table I. BLA Found in the Different Fractions of the Hepatocytes 10 Minutes After the Incubation of the Complex Albumin/BLA or Galactosylated Albumin/BLA[a]

Fraction	BLA/albumin	BLA/galactosylated albumin
Nuclei	4.2%	3.4%
Mitochondria + lysosomes	18.9%	15.2%
Cytosol + microsomes	76.9%	81.4%

[a] The results are expressed as % of the radioactivity incorporated inside the hepatocytes.

an eluent containing a mixture of an aqueous solution of NaH_2PO_4 6mM 30%, acetonitrile 50%, and methanol 20%. This analysis showed that BLA (R_f = 0.26) did not remain completely intact, and that the radioactivity was partially linked to a product possessing the same R_f than benzoic acid (R_f = 0.66). This observation demonstrated that BLA is metabolized, likely by the breaking of the amide bond. It is likely that the decrease of the radioactivity with the time is related to a release of this metabolite by the hepatocytes.

DISCUSSION

The derivatization of fatty acids with some hindered groups seems to preserve the fatty acid character of these analogs, such as the binding to $FABP_c$ [11], the binding to albumin at the site of the fatty acids [1], the accumulation in liver and heart [1,12,13], the β-oxidation metabolism [14]. However, to our knowledge, the uptake mechanism of these fatty acids analogs by hepatocytes was never studied until now. This consideration is a fundamental aspect in the concept of receptor imaging where the image is the mapping of the receptors expressed at the surface of the cells of a tissue. Besides the simple tissue accumulation, it is also primordial in drug design to know the uptake mechanisms of the targeted drug : because there is a potential change in the expression of receptors at the surface of pathological cells, that knowledge can potentially predict the succes of this targeting. The radiolabelled analog of fatty acid BLA allow us to check the potential modulation in the uptake mechanisms by modifying the carrier-protein.

The binding of this compound to albumin and galactosylated albumin was similar (Fig. 2) : this observation shows that the modifications of the albumin does not substantially affect the binding to the analogs of fatty acids. BLA was found to penetrate inside the hepatocytes whenever this compound was bound to albumin or galactosylated albumin. The mechanism of uptake still seems to be the same : our results indeed are not significantly different between BLA bound to albumin and BLA bound to galactosylated albumin as demonstrated by a similar extent of the uptake by the hepatocytes (Fig.3), a similar kinetics of this uptake, a like inhibition by some agents introduced in the media, and a similar intracellular fate of the compound (Table 1). The same observations were obtained when BLA was replaced by oleic acid, choosen to be representative of the natural fatty acids (Fig.4). The change in the amount of BLA incorporated into the hepatocytes, when the protein/BLA molar ratio was modified (Fig.3a and b), also is well representative

of the situation. The fatty acids are known to be incorporated inside the hepatocytes after interaction of the free fatty acid with the $FABP_{PM}$. For a larger amount of free fatty acids in the medium (obtained for instance by decreasing the protein/fatty acid molar ratio), we can expect a higher amount of fatty acid recovered inside the cells. This situation, extensively studied with oleic acid by Stremmel [3], is found here again with the modified fatty acid BLA. The situation should be different if the mechanism of uptake was a receptor mediated endocytosis after selective recognition of the ASGPR : in this particular case, that is the complex "galactosylated albumin-BLA" which should penetrate inside the cells, and the amount of BLA recovered inside the cells should increase when the protein/BLA molar ratio is increasing. We found the opposite result (Fig.3b), demonstrating that increase of free BLA was responsible for a higher uptake : he dissociation of the complex BLA/ galactosylated albumin most probably takes place before the entry inside the cells. That means that the simple adsorption of BLA on a galactosylated protein does not affect the mechanism of uptake, and that the change of the carrier cannot give any advantage for the targeting of the fatty acid analogs. In a previous study [1], we showed that the *in vivo* uptake of oleic acid by the liver can be inhibited by an analogous of fatty acid, and consequently that this compound is likely to recognize the $FABP_{PM}$. In the present study, we also demonstrated that the uptake is dependent on the presence of inhibitors such as phloretin, known to inhibit the mechanism of $FABP_{PM}$, and, consequently, still corresponds to an active process. As already described for oleic acid, the residual part of the entry inside the cells, non inhibited by phloretin, is due to the diffusion through the membrane.

Another interesting finding is related to the metabolism of the adduct "fatty acid-drug", with a cleavage of the amide bond and the release of the drug. This is an essential point if we consider the adduct "fatty acid-drug" as a prodrug. The release of the drug will permit the expression of the therapeutic activity of molecules where the carboxylate groups are necessary for this activity.

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