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Intracellular Transport of Benzo(a)pyrene by Chemically Modified Low Density Lipoproteins into Hep 2 Cells.

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Facilitated transport of lipophilic benzo(a)pyrene into human fibroblast cells by low density lipoproteins (LDL) was examined. Amounts of [^{3}H]-labeled B(a)P taken up by the Hep 2 fibroblast was increased 3 folds by the addition of LDL(100μg of protein/10^5 cells) in the media. However, we have found that the facilitated B(a)P transport into cells were diminished by the addition of LDL of which the apoproteins were modified by copper(II) ion-catalyzed oxidation in 10μM copper sulfate. The results of the present study suggest that lipophilic compounds are taken up via adsorptive endocytosis which is mediated by interactions between apoprotein-specific receptors on the cell membrane and the specific apoproteins on LDL.

INTRODUCTION

Benzo(a)pyrene (B(a)P) is a potent chemical carcinogen to which humans are routinely exposed (Miller and Miller, 1981; Hutcheon *et al.*, 1983; Willingford and Que Hee, 1985). The biological activities of B(a)P occurs as a result of metabolic derivatives, the most important of which appears to be 7r, 8t, dihydroxy-9, 10t-epoxy-7, 8, 9, 10-tetrahydrobenzo(a)pyrene (BPDE) (Melikian *et al.*, 1987). Numerous studies have shown that B(a)P administrations to animals result in different levels of BPDE bound to nuclear DNA that are similar in the tissues with capacities to activate B(a)P (Lu *et al.*, 1986, Stowers and Anderson, 1984). An hypothesis has been proposed that B(a)P is transported into cells by serum components especially by low density lipoproteins (LDL) (Remsen *et al.*, 1981). This hypothesis is supported by the data showing that lipoproteins can sequester and release B(a)P to lymphocytes, which results in DNA adducts formation (Busbee *et al.*, 1982). Further,

the studies using experimental animals have demonstrated that electrophilic metabolites of B(a)P are actually present in the mouse serum and that the levels of BPDE in serum is sufficient to account for a substantial portion of the tissue containing BPDE-DNA adducts (Ginsberg and Atherholt, 1989).

The mechanism for the transport of lipophilic compounds including B(a)P into nucleus of cells via lipoprotein carriers has been suggested by the works of Goldstein and Brown (1974) who showed that LDL and very low density lipoproteins (VLDL), but typically not high density lipoproteins (HDL), are bound to receptors specific for apoprotein B and E. Upon apoprotein specific binding of LDL to the receptors at coated-pits regions of cell membrane, LDL are internalized into cells and are subsequently degraded.

In this study we examined B(a)P transport into cells using chemically modified LDL as a carrier of the carcinogen, and found that copper-modified LDL are inefficient carriers of B(a)P into cells.

MATERIALS AND METHODS

LDL Preparation

LDL was isolated from human plasma by differential density preparative ultracentrifugation between $\rho > 1.006$ and $\rho < 1.06$ (Fisher *et al.*, 1972). Homogeneity was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Kobylka *et al.*, 1972) and LDL concentration was determined as described by Lowry *et al.* (1951).

LDL Modification

LDL (100μg protein /ml) was incubated with 10μM copper sulfate in 2ml of Dulbeco's Modified Eagles Medium (DMEM) at 37°C for 24 hrs. (Parthasarathy, 1987).

Fluorescence Measurements

Fluorescence measurements were carried out on Perkin Elmer Model LS 3B spectrofluorometer. Emission spectra were taken between 450 nm and 530 nm with the excitation wavelength set at 375 nm. LDL and B(a)P (1.0μg/ml) dissolved in a buffer containing 5mM sodium phosphate, 0.15M NaCl and 0.3mM EDTA were examined for the complex formation by fluorescence quenching.

[^{3}H]-B(a)P Association with LDL

Partitioning of [^{3}H]-benzo(a)pyrene ([^{3}H]-B(a)P, G-[^{3}H] benzo(a)pyrene, 5.3 x 10^{16} dpm/mol, 0.375 mol/1; New England Nuclear) into components of LDL were examined using the method of Busbee *et al.* (1982). To measure total associated [^{3}H]-B(a)P, 500μl LDL (100μg of protein) was added to 690μl of phosphate buffered saline (PBS), pH 7.4, containing bovine serum albumin, 435 mg/l (BSA/PBS). To this was added 10μl of an ethanol solution containing [^{3}H]-B(a)P. The preparation was vigorously vortex-mixed and was incubated for 30 min at 37°C in the dark. After incubation, the tubes were placed on ice and 200μl of the reaction mixture was removed to measure total [^{3}H]-B(a)P. To the remaining solution was added 200μl of a charcoal/dextran suspension (6% activated, washed NORIT A and 0.06% dextran, Mr=70,000, in PBS). The preparation was vortex-mixed, placed on ice for 30 min, centrifuged at 3000 x g for 10 min, and 200μl of the supernatant (equivalent to 167μl of the original incubation mixture) was carefully removed

and counted. The 200μl aliquots were added to 5 ml of Beckman Ready Proteins$^+$ and radioactivity was determined in a liquid scintillation counter. Quenching was corrected by automatic external standardization (H-number). Counting efficiency was routinely 38-42%. The amount of [^{3}H]-B(a)P associated with a sample is expressed as the percentage of the [^{3}H]-B(a)P remaining in the supernatant after treatment with charcoal (i.e., the percentage of [^{3}H]-B(a)P bound to LDL components and thus unavailable for adsorption to charcoal).

[^{3}H]-B(a)P Uptake into Hep 2 Cells

Transport of B(a)P into cells was examined by measuring cellular uptake of [^{3}H]-B(a)P into Hep 2 cells. Hep 2 cells originated from the epidermoid carcinoma tissue, were maintained in 100 mm petri dishes in DMEM supplemented with 10% heat inactivated fetal calf serum. For experiments, cells were depleted of serum for 4 hrs, and media were removed by aspiration, [^{3}H]-B(a)P (1ng/10^5 cells) was then added to the fresh media with LDL (100μg of protein/10^5 cells) or oxidized LDL (100μg of protein/10^5 cells) for the specified incubation time periods. Cells were harvested using trypsin EDTA solution. Cell samples were centrifuged at 2,000 x g for 5 mins, supernatants were removed, and cell pellets were resuspended in 1 ml aliquots in PBS. Samples were poured onto glass fiber filter held in Millipore 1225 sampling manifold. Each sample was washed twice with 5ml of cold ethanol, an once with 10ml of 10% trichloroacetic acid. After drying the samples were counted in a liquid scintillation counter using Beckman Ready Proteins$^+$ as a counting cocktail.

RESULTS

The association between LDL and B(a)P were examined by measuring the quenching of fluorescence emission of B(a)P by LDL. The emission spectra of B(a)P excited at 375 nm before and after the addition of LDL were shown in Fig 1. Upon addition of 500μg/ml of LDL protein concentration of 1.0μg/ml of free B(a)P, about 49% of the fluorescence is quenched and suggest the accessibility of B(a)P in the LDL : B(a)P complex.

In an attempt to prove the LDL : B(a)P complex transport by LDL receptor mediated internalization into cells, we firstly compared the carcinogen transport using copper ion (II) oxidized LDL with the transport by normal LDL as the carrier. To the chemically modified LDL (100μg of protein/ml), we add [^{3}H]-B(a)P at 1μg/ml and reacted the mixture at 24°C for 30 mins in the dark. Excess B(a)P which was not associated with LDL was removed by adsorption to activated charcoal as given in materials and methods. Data in Fig 2. indicate that the binding efficiency of [^{3}H]-B(a)P to human LDL was approximately 15% under this experimental conditions and that the efficiency of chemically modified lipoproteins association to [^{3}H]-B(a)P was almost the same as that of normal lipoproteins.

Comparative measurements of B(a)P uptake into Hep 2 cells either in the presence of normal human LDL or modified LDL were seen in Fig 3. The binding of [^{3}H]-B(a)P to acid precipitable cellular macromolecules was found to increase over a period of 8 hrs in preparations containing [^{3}H]-B(a)P bound to LDL. Identical cell preparations containing [^{3}H]-B(a)P bound to oxidized LDL showed reduced binding of carcinogen to cellular

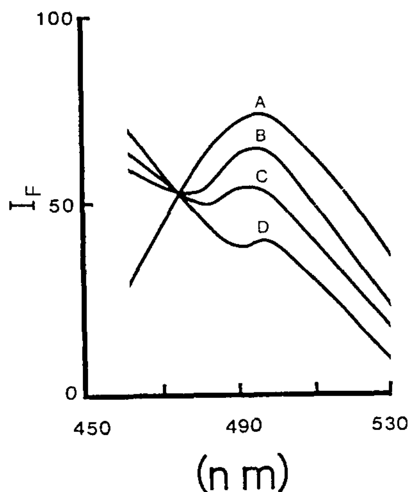

Fig. 1. Association of B(a)P with LDL. B(a)P (1μg/ml) was excited at 375nm, and fluorescence emission spectra were examined. LDL was added to the cuvet as separate aliquots, with the final concentration of B(a)P being maintained at 1μg/ml.

A) free B(a)P
B) B(a)P + LDL (10μg of protein/ml)
C) B(a)P + LDL (100μg of protein/ml)
D) B(a)P + LDL (500μg of protein/ml)

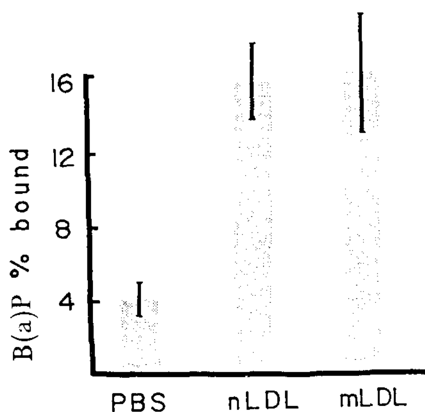

Fig. 2. An analysis of the efficiency of binding of normal and chemically modified LDL to [^{3}H]-B(a)P. [^{3}H]-B(a)P binding is expressed as percent bound as described in Materials and Methods. n LDL : normal LDL, m LDL : copper oxidized LDL, PBS : Background level of [^{3}H]-B(a)P in the supernatant obtained by performing the assay only in the presence of bovine serum albumin and PBS.

macromolecules, while B(a)P binding to acid precipitable cellular component in PBS without LDL shows very low level of binding.

DISCUSSION

Lipoproteins in human plasma are known to bind and transport a variety of lipophilic compounds including α-carotin, lycopene, dolichol, β-tochopherol, and polynuclear aromatic hydrocarbon chemicals (Keenan *et al.*, 1977; Shu and Nichols, 1979). Human cells are also known to internalize LDL by receptor mediated adsorptive endocytosis, a process which could potentiate the entry of lipoproteins sequestered chemicals into cells. To examine this phenomenon we reacted human LDL with [^{3}H]-B(a)P. Fluorescence quenching studies with free and LDL associated B(a)P indicate the formation of LDL : B(a)P complex. This complex formation is most likely to depend upon the presence of hydrophobic components in LDL, which consist mainly of a cholesterol ester / triglyceride core and a cholesterol / phospholipid domain located between the core and the surface of the LDL particle. These lipid phases might assure soluble regions for the ring structures of polynuclear aromatic hydrocarbon compounds including B(a)P.

It is reported that the modification of lysine residues of the apoprotein B of LDL by acetylation or acetoacetylation inhibit the binding of modified LDL to the LDL receptors on the cell membrane (Pitas *et al.*, 1985). Chemical

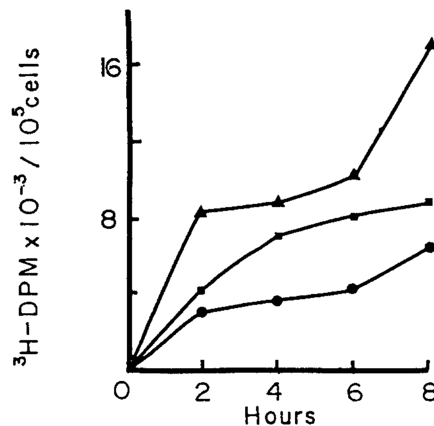

Fig. 3. An analysis of binding of [^{3}H]-B(a)P to acid precipitable cellular macromolecules. Hep 2 cells were held in fresh DMEM for 4 hrs to deplete cells of membrane bound LDL before the addition of [^{3}H]-B(a)P to the media. Serum depleted cells received [^{3}H]-B(a)P (1 ng/10^5 cells) either with normal human LDL (▲) or with copper oxidized LDL (■). Control cells received same concentration [^{3}H]-B(a)P under the same condition (●).

modification of LDL that block ε-amino group of lysine residues are known to generate modified forms of LDL that are degraded avidly by macrophages. These negatively charged forms of LDL are degraded by special receptors, of which the acetyl receptors or scavenger receptor are commonly refered (Pathasarathy *et al.,* 1987). In Fig. 2, we compared the binding of B(a)P with normal and copper ion oxidized LDL. The copper induced modification of LDL is reported to induce the peroxidation of the lipid and breakdown of apoprotein B-100 into smaller peptides (Parthasarathy *et al.,* 1987). We reacted [^{3}H]-B(a)P either with normal human LDL or copper ion oxidized LDL, removing excess [^{3}H]-B(a)P from the LDL preparation by adsorbing unbound carcinogen to activate charcoal. The levels [^{3}H]-B(a)P bound to both LDL preparation were almost the same at 15% efficiency and the data show that the accessibility of B(a)P in LDL is not affected by oxidative modification of apoprotein in LDL.

When LDL plus bound [^{3}H]-B(a)P were added to media containing Hep 2 cells, there was an increase in [^{3}H]-B(a)P bound to cellular macromolecules over an 8 hr period. However, oxidized LDL apparently reduced the transfer of the [^{3}H]-B(a)P into cells in a form capable of binding to acid precipitable cellular molecules. The control cell preparations, containing no LDL, were exposed to the same concentration of [^{3}H]-B(a)P at 1μg/ml and show low level of labeled [^{3}H]-B(a)P transfer into Hep 2 cells. These data are supportive of the transfer of polynuclear aromatic hydrocarbon compounds into cells as a function of cellular internalization of LDL.

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화학적으로 변형된 저밀도 지방단백질에 의한 벤조피렌 화합물의 Hep 2 세포내 이동에 관한 연구

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수용체를 통한 저밀도 지방단백질(LDL)의 세포내 이동과 함께 일어나는 지용성 돌연변이원인 벤조피렌의 세포내 이동에 관하여 조사하였다. [^{3}H]로 표지된 벤조피렌의 Hep 2 세포내 이동을 조사한 결과, 배지에 LDL을 첨가한 경우, LDL이 첨가되지 않은 경우보다 3배나 많은 양의 벤조피렌이 세포내로 이동하여 세포내 물질과 결합하였음을 관찰하였다. 그러나 10 μM의 황산구리로 LDL의 아포단백질을 변형시킨 후 변형된 LDL을 배지에 첨가하고 벤조피렌의 세포내 이동을 조사한 결과, 정상 LDL이 첨가된 경우보다 벤조피렌의 세포내 이동이 현저히 감소하였다. 이 결과는 LDL을 이동체로 이용한 벤조피렌의 세포내 이동이 세포의 수용체와 LDL의 아포 단백질의 인식과정을 거친다는 사실을 뒷받침 한다고 여겨진다.