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Uptake of liposomes by cultured cardiomyocytes

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Received July 2, 2004, accepted January 14, 2005

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Pharmazie 60: 844–848 (2005)

Small unilamellar liposomes (SUV) of different phospholipid/polymer composition were labeled with NBD-PC, which served as a bilayersituated fluorescence marker. Neonatal cardiomyocytes were incubated with liposomes and then the cell-associated fluorescence was measured. The factors influencing the liposome uptake by cardiomyocytes such as concentration of lipid, time of incubation, membrane fluidity of liposomes, charge lipid/polymer modification of liposomes and anoxia of cultured cardiomyocytes were investigated. The liposome uptake by cardiomyocytes increased dose-dependently and time-dependently. Liposome uptake was strongly influenced by the electrical charge and modified polymer. After 2 h incubation, the uptake of positively charged liposomes was 1.7-fold higher than that of negatively charged one and both higher than that of the neutral one. The presence of PE-PEG_{2000} distinctly reduced the liposome uptake and the difference between the uptake of charged and neutral liposome. Anoxia increased the uptake of liposome at the first hour (increased 20%), but after 2 h incubation the liposome uptake by hypoxia cells was less than that of normoxia cells (decreased 18%). Mechanisms involved are also discussed.

1. Introduction

Liposomes have been suggested as efficient carriers for delivery of drugs into ischemic myocardium (Phelan and Lange 1991; Smalling et al. 1995; Silva et al. 2001). Spontaneous accumulation of positively and neutrally charged liposomes in the regions of experimental myocardial infarction was initially described by Caride (Caride and Zaret 1977). Mueller also found that positive liposome concentration in the infarct myocardium was about 130% of normal values. Their studies demonstrated that liposomes injected into dogs with experimental myocardial infarction can release their contents into ischemic myocytes (Mueller et al. 1981). This targeting phenomenon can be explained by increased permeability in hypoxic areas of infarcted myocardium. Liposomes ranging from 10 to 500 nm in size can accumulate inside the interstitial space as a reservoir of drug (Torchilin 2000).

The interaction of liposomes with cells is a promising area of research (Osaka et al. 1994; Shimizu et al. 1997; Guo et al. 2000; Hong et al. 2000; Kawano et al. 2002). It is believed that a better understanding of the liposome-cell interactions *in vitro* could provide important clues to the behavior of liposomes *in vivo*. Mueller studied the interaction of liposomes containing fluorescent dyes and horseradish peroxidase with isolated contracting cardiomyocytes, fluorescence measurements by photometry showed that depolarization of the isolated cardiomyocytes membrane with 100 mM K^+ enhanced uptake of both neutral and positive liposomes by at least 5-fold (Müller et al. 1981). Chaoshu Tang used ^{14}C-labeled phospholipids as a liposome member marker and ^{3}H-inulin as a marker for liposome aqueous spaces, results of liposome uptake by freshly isolated cardiomyocytes demonstrated that physical characteristics of liposomes, such as solid or liquid phase and the electrical charge, were important factors influencing the interaction between adult cardiomyocytes and liposomes (Chaoshu et al. 1993).

In this study we investigated the interaction of liposomes with cultured neonatal cardiomyocytes. The liposome uptake was studied by incubating fluorescence labeled liposomes with cardiomyocytes *in vitro* and measuring the association of liposomes by a fluorescence spectrophotometer. The factors influencion the liposome uptake by cardiomyocytes such as concentration of lipid, time of incubation, membrane fluidity of liposomes, charge lipid/polymer modification of liposomes and anoxia of cultured cardiomyocytes were investigated.

2. Investigations, results and discussion

Liposomes of different phospholipid/polymer composition had approximate size and different zeta potential (Table).

It is evident that phospholipid composition and presence of PEG only minimally influence liposome size and mean diameter of liposomes prepared by saturated phospholipid (HSPC) was a little larger than that of unsaturated phospholipid liposomes (SPC) and PEG modified liposomes. When liposomes were forced through filters with 0.2 μm pore sizes, a liposomal population was obtained with a mean diameter that reflects the diameter of the filter pores and narrow size distribution. So the effect of liposome size on liposome uptake by cardiomyocytes could be neglected in our studies.

Table: Composition, size and zeta potential of liposomes

Composition (molar ratio)	Size (nm ± SD)	Zeta potential (mv ± SD)
SPC/Chol (2 : 1)	174 ± 22	−12.8 ± 0.8
SPC/Chol/SPG (2 : 1 : 0.2)	173 ± 16	−37.3 ± 1.2
SPC/Chol/SA (2 : 1 : 0.2)	177 ± 37	8.3 ± 0.7
SPC/Chol/PE-PEG_{2000} (2 : 1 : 0.2)	164 ± 31	−15.9 ± 1.4
SPC/Chol/SPG/PE-PEG_{2000} (2 : 1 : 0.2 : 0.2)	166 ± 26	−18.6 ± 1.1
SPC/Chol/SA/PE-PEG_{2000} (2 : 1 : 0.2 : 0.2)	169 ± 25	−5.4 ± 1.3
HSPC/Chol (2 : 1)	203 ± 34	−2.1 ± 1.0

The zeta potential of liposomes was strongly influenced by the presence of SA, PG and/or PE-PEG_{2000}. The addition of positively charged SA increased the zeta potential of SPC/Chol liposomes, whereas addition of negatively charged SPG decreased it. The attachment of PE-PEG_{2000} to charged liposomes brought their zeta potential back close to neutral. It was previously described that a larger PEG can effectively shield the charge of a phospholipid block as well as the charge of the whole liposome, and that the net zeta potential of such a PEG liposome is close to neutral under physiological conditions (150 mM NaCl) (Moribe et al. 1997; Levchenko et al. 2002).

The cultured cardiomyocytes were incubated with liposomes (SPC/Chol 2 : 1 molar ratio) of various concentration for 2 h at 37 °C. The uptake of liposomes increased dose-dependently and the increase was rather slow as the dose increased beyond 1000 nmol/dish (Fig. 1).

The cultured cardiomyocytes were incubated with liposomes (SPC/Chol 2 : 1 molar ratio) of 2000 nmol/dish at 37 °C or 4 °C for various lengths of time. The amount of liposome uptake by cells increased as the incubation time was prolonged (Fig. 2).

The interaction of liposomes with cultured cells *in vitro* is complex. It involves surface binding, internalization and possible release of liposomal contents (Lee et al. 1992). Our investigation focuses on the first two processes. It is known that at 4 °C, surface binding to a cell occurs but internalization dose not. So, the amount of liposome uptake by cells at 37 °C was total uptake including surface binding and endocytosis, while at 4 °C the liposome uptake measured was all surface binding without any contribution of endocytosis.

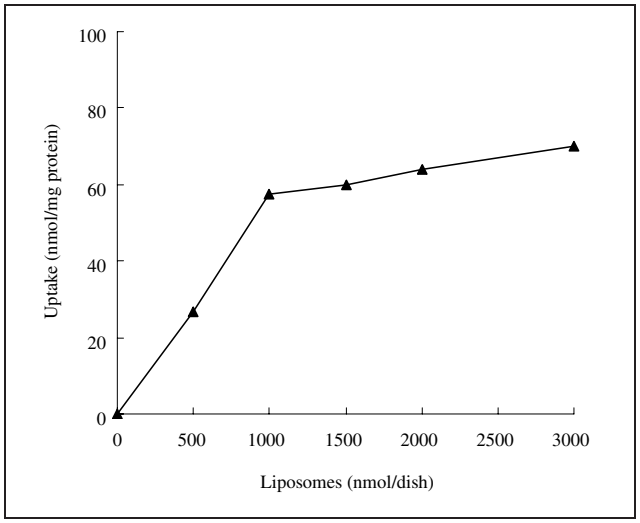

Fig. 1: Concentration dependence of liposome uptake by cardiomyocytes. Liposomes (SPC/Chol 2 : 1 molar ratio) were labeled with NBD-PC. The cells were incubated with liposomes at 37 °C for 2 h (▲). Each value represents the mean ± S.D. of three experiments

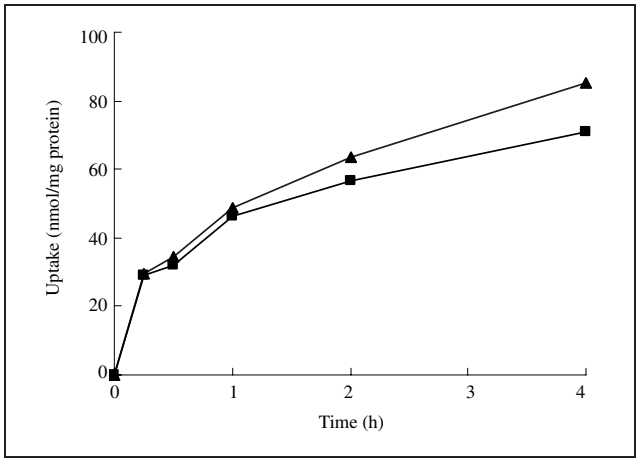

Fig. 2: Time dependence of liposome uptake by cardiomyocytes. Liposomes (SPC/Chol 2 : 1 molar ratio) were labeled with NBD-PC. One group cells were incubated at 37 °C with liposomes at a dose of 2000 nmol/dish (▲), while the cells were incubated at 4 °C with liposomes at a dose of 2000 nmol/dish in the other group (■). Each value represents the mean ± S.D. of three experiments

In our study, the increase of liposome uptake at 37 °C was rapid for the initial 15 min, and progressed slowly afterwards as the incubation time increased. The kinetics of liposome uptake at 4 °C was similar to that at 37 °C. It demonstrated that the majority of liposomes were not internalized by cardiomyocytes. We could draw the conclusion that the liposome uptake by cardiomyocytes was chiefly in the form of surface binding. The analogical conclusion had been proposed by Chaoshu Tang (Chaoshu et al. 1993).

To investigate the effect of membrane fluidity on liposome uptake, 2000 nmol/dish solid liposomes (HSPC/Chol 2 : 1 molar ratio) and liquid liposomes (SPC/Chol 2 : 1 molar ratio) were incubated with cultured cardiomyocytes at 37 °C for 2 h, the amount of solid liposome uptake was 4.4-fold higher than liquid ones (Fig. 3).

Since SPC has unsaturated fatty acid residues and HSPC has hydrogenated saturated ones, membrane fluidity of liposomes containing SPC is considered to be higher than that of liposomes containing HSPC. It was evident that the liposome uptake by cardiomyocytes was enhanced by the composition of saturated phospholipid. The result of our experiment was similar to earlier reports of Chaoshu Tang (Chaoshu et al. 1993). It was also reported that solid liposomes associated with different cultured cell types were more than fluid liposomes, irrespective of surface charge (Szoka 1980).

The cell uptake of liposomes modified with negative charge lipid (SPG) or positive charge lipid (SA) or/and PE-PEG_{2000} was investigated, in order to study the relative role of the liposome charge and the PEG chains on the liposome uptake by cardiomyocytes *in vitro*. The liposomes were incubated with cultured cardiomyocytes for 2 h, the amount of uptake was drastically different (Fig. 4). It was evident that liposome uptake was strongly influenced by the electrical charge and modified polymer.

After 2 h incubation, the uptake of positively charged liposomes (SPC/Chol/SA 2 : 1 : 0.2 molar ratio) by cardiomyocytes was 1.7-fold higher than that of negatively charged ones (SPC/Chol/SPG 2 : 1 : 0.2 molar ratio) and both higher than that of neutral ones (SPC/Chol 2 : 1 molar ratio). Though Szoka described previously that fluid

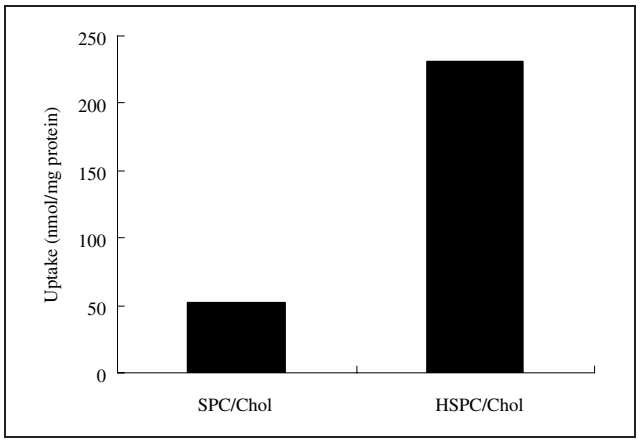

Fig. 3: Effect of membrane fluidity on liposome uptake by cardiomyocytes Liquid liposome (SPC/Chol 2 : 1 molar ratio) and solid liposome (HSPC/Chol 2 : 1 molar ratio) were labeled with NBD-PC. The cells were incubated at 37 °C with liposomes at a dose of 2000 nmol/dish for 2 h. Each value represents the mean ± S.D. of three experiments

liposomes, regardless of charge, were found to associate with cells to the same degree (Szoka 1980). Our study demonstrated that the electrical charge was an important factor influenced the interaction between cardiomyocytes and liposomes.

The surface modification of PEG created a steric barrier preventing liposome opsonization and subsequent rapid clearance by the reticulo-endothelial system (RES). Prolongation of liposome circulation in the blood has been achieved. But it was not clear how the modification of the liposome surface with PEG-lipid influenced liposome uptake by cultured cardiomyocytes. Our experiment showed that the presence of PE-PEG_{2000} distinctly reduced the liposome uptake and the difference between the uptake of charged and neutral liposomes. Two possible mechanisms of the reduction were electrostatic and stericrepulsion effect of PEG. As described above, the modification of charged liposome with PE-PEG_{2000} could provide efficient charge neutralization. On the other hand, PE-PEG_{2000} could reduce the binding constant between the specific headgroups of liposomes and the cell membrane binding site because of the bulky PEG group acting as a strong steric hindrance barrier.

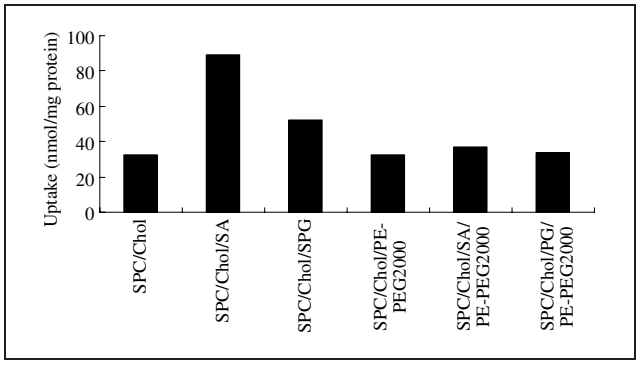

Fig. 4: Effect of charge lipid/polymer on liposome uptake by cardiomyocytes. Liposomes composed of SPC/Chol (2 : 1 molar ratio), SPC/Chol/PE-PEG_{2000} (2 : 1 : 0.2 molar ratio), SPC/Chol/SA (2 : 1 : 0.2 molar ratio), SPC/Chol/SA/PE-PEG_{2000} (2 : 1 : 0.2 : 0.2 molar ratio), SPC/Chol/SPG (2 : 1 : 0.2 molar ratio) and SPC/Chol/SPG/PE-PEG_{2000} (2 : 1 : 0.2 : 0.2 molar ratio) were labeled with NBD-PC. The cells were incubated at 37 °C with liposomes at a dose of 2000 nmol/dish for 2 h. Each value represents the mean ± S.D. of three experiments

To determine whether anoxia increases the uptake of liposomes, liposomes (SPC/Chol 2 : 1 molar ratio) were added into the cultured cardiomyocytes at a dose of 2000 nmol/dish, and incubated under the normoxia or hypoxia conditions respectively. When the incubation time was 1 h, the liposome uptake by hypoxia cells was more than that of normoxia cells (increased 20%). However, after 2 h incubation the liposome uptake by hypoxia cells was less than that of normoxia cells (decreased 18%) (Fig. 5).

Ischemia of the heart, which is usually caused by a reduction of coronary flow, induces hypoxia and then the death of cardiomyocytes. It has been reported that neonatal cardiomyocytes exposed to 20 min of anoxia shows the same amount of liposome uptake as cells in an oxygen-rich environment (Mueller et al. 1981). While the results of Chaoshu Tang et al. demonstrated that anoxia 30 min of adult cardiomyocytes not only increased the uptake of various kinds of liposomes (range of increase: 12–22%), but also lead to a change in uptake patterns, the endocytosis of various liposomes significantly increased (Chaoshu et al. 1993).

As the resistance of neonatal cardiomyocytes to anoxia was better than that of adult cardiomyocytes, the hypoxia incubation time we chose was different from the reports of Mueller and Chaoshu Tang. Because of the different physiological state after different hypoxia time, the results were different. At the first anoxia period, because hypoxia cells still had an intact structure and anoxia promoted the endocytosis of liposomes, the amount of liposome uptake by cells increased significantly. Continuing with anoxia, hypoxia began to induce the death of cardiomyocytes. Necrotic cells with disrupted membrane could no longer prevent the contents of cells from being released into the extracellular space, and contents released were washed away with PBS at the time of fluorescence measurement. So at the afterward period, anoxia decreased the liposome uptake by cardiomyocytes. It may be another important cause besides bloodstream that the quantity of liposomes distributed in the peripheral area of the infarct was higher than in infarcted areas *in vivo*.

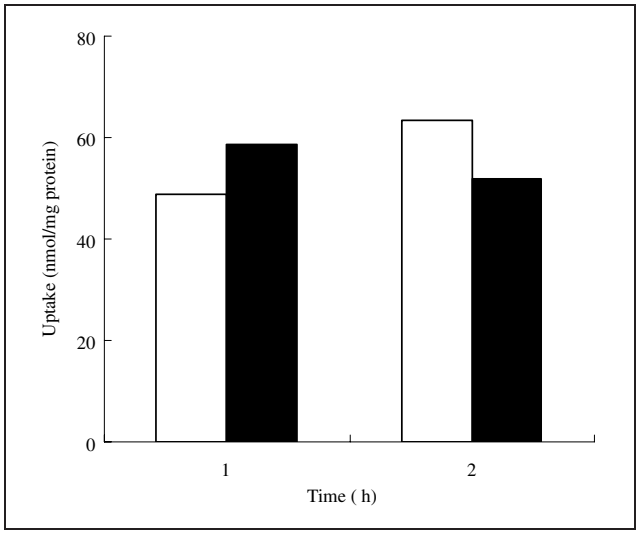

Fig. 5: Effect of anoxia on the liposome uptake by cardiomyocytes. Liposomes (SPC/Chol 2 : 1 molar ratio) were labeled with NBD-PC. In one group the cells were incubated under normoxia conditions with liposomes at a dose of 2000 nmol/dish for 1 h and 2 h (open columns), while the cells were incubated under hypoxia conditions with liposomes at a dose of 2000 nmol/dish for 1 h and 2 h in the other group (hatched columns). Each value represents the mean ± S.D. of three experiments

In conclusion, the above-mentioned factors were all important to the liposome uptake by cardiomyocytes *in vitro*. We believe that a better understanding of the liposome-cell interactions *in vitro* based on the information presented in this paper will provide important clues to the behavior of liposomes *in vivo*.

3. Experimental

3.1. Materials

Soybean phosphatidylcholine (SPC, trademark: Epikuron 200), hydrogenated soybean phosphatidylcholine (HSPC, trademark: Epikuron 200) and soybean phosphatidylglycerol (SPG, purity > 95%) were generous gifts from Degussa; cholesterol (Chol) and stearylamine (SA) were purchased from Sigma; 1-myristoyl-2-[6-[(7-nitro-2−1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine (NBD-PC) was purchased from Avanti Polar Lipids, Inc.; Iscove's Modified Dulbecco's Medium (IMDM) was purchased from Invitrogen Co.; Collagenas II and fetal bovine serum (FBS) were purchased from Gibco; Laminin was purchased from Sigma; BCA protein assay kit was purchased from Pierce Chemical Co.: Other materials used in cell culture were purchased from Coster; all other chemicals were reagent grade.

3.2. Preparation of liposomes

Liposomes were composed of SPC/Chol (2 : 1 molar ratio), SPC/Chol/PE-PEG_{2000} (2 : 1 : 0.2 molar ratio), SPC/Chol/SPG (2 : 1 : 0.2 molar ratio), SPC/Chol/SPG/PE-PEG_{2000} (2 : 1 : 0.2 : 0.2 molar ratio), SPC/Chol/SA (2 : 1 : 0.2 molar ratio) and SPC/Chol/SA/PE-PEG_{2000} (2 : 1 : 0.2 : 0.2 molar ratio) respectively. NBD-PC was incorporated into the lipids with 1 mol%, which served as a bilayersituated fluorescence marker. Small unilamellar liposomes (SUV) were prepared by the reverse-phase evaporation method (REV) as previously described (Szoka and Papahadjopoulos 1981). In brief, lipids and NBD-PC were dissolved in ether/chloroform (1 : 1 v/v), and then 150 mM NaCl solution was added into the lipid solution. A water in oil (w/o) emulsion was obtained after 5 min sonication using the bath-type sonicator. The organic solvent was dried in vacuum in round-bottomed flask, and then the emulsion changed into the gel form. Phase inversion occurred after the organic solvents were completely removed from the gel, and then the liposomes were obtained. To homogenize size distribution, the liposomes were extruded under high pressure nitrogen through sterile polycarbonate membrane (Nucleopore) with a final pore size of 0.2 μm subsequently. When saturated lipids were used, the extruder device was preheated to 60 °C. All of the NBD-PC added was entrapped in liposomes as determined by gel filtration.

3.3. Characterization of liposomes

Liposome size was determined by the NICOMP-380/ZLS submicron particle sizer (Nicomp Co., CA, U.S.A.). Zeta potential was measured using a Delsa 440SX Zeta Potential Analyzer (Beckman Co. CA, U.S.A.). All measurements were done at 25 °C.

3.4. Preparation of cardiomyocyte cultures

Cultured rat neonatal cardiomyocytes were prepared as described previously (Tanaka et al. 1994; Patten et al. 1996; Yonemochi et al. 1998). Briefly, ventricles were obtained from 1−2 day old Wister rats, and cardiomyocytes were isolated by digestion with 0.12% collagenase II. Cardiomyocytes were separated from nonmyocytes by 90 min differential plating. The estimated purity of cardiomyocytes after differential plating was 90% to 95%. The viability of the cardiomyocytes was tested using the Trypan Blue exclusion method, and more than 95% of the isolated cardiomyocytes were viable. IMDM medium (pH 7.2) containing 10% fetal bovine serum (FBS) was supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The purified cardiomyocytes suspension in the above medium was prepared at a concentration of 5×10^5 cells/ml and plated in laminin-coated 35-mm plastic culture dish at 2 ml per dish. The cardiomyocytes were incubated at 37 °C, and under humidified atmosphere of 95% air −5% CO_2. The 72−84 h cultured cardiomyocytes were used in the following experiments. At the time of use, the cells were single cells, small groups, or interconnected net works. Cells selected for study had spontaneous contraction frequencies of approximately 80−120 beats/min.

3.5. Liposome uptake by cardiomyocytes

For liposome uptake determination, liposomes were diluted with IMDM (without FBS) to a certain concentration and added into the cultured cardiomyocytes. The cardiomyocytes were incubated with liposomes under the conditions of 37 °C, humidified atmosphere of 95% air −5% CO_2. When hypoxia was needed, cardiomyocytes were incubated with liposomes under the conditions of 37 °C, humidified atmosphere of 95% N_2 −5% CO_2. And then the non-associated liposomes in solution were washed away at the end of incubation by rinsing three times with 2 ml ice-cold PBS. The washed cells in a dish were solubilized with 2 ml PBS containing 1% Triton X-100. The fluorescences intensity of the cellular lysate at 460 nm excitation and 534 nm emission was measured using a fluorescence spectrophotometer (Hitachi, 650−60). The protein concentration of the cells was determined by BCA protein assay. The association of liposomes by cells was expressed as nmol of liposomal lipid per mg of cell protein. Liposomal lipid was represented by SPC or HSPC concentration.

3.6. Effect of lipid concentration on uptake

The cultured cardiomyocytes were incubated with fluorescence labeled liposomes (SPC/Chol 2 : 1 molar ratio) at doses of 500, 1000, 1500, 2000, 3000 nmol/dish. After 2 h incubation, the amount of liposome uptake by cardiomyocytes was determined with the method described in 3.5.

3.7. Effect of incubation time on uptake

The cultured cardiomyocytes were incubated with fluorescence labeled liposomes (SPC/Chol 2 : 1 molar ratio) of 2000 nmol/dish at 37 °C or 4 °C for 0.25, 0.5, 1.0, 2.0, 4.0 h respectively. The amount of liposome uptake by cells was determined with the method described in 3.5.

3.8. Effect of membrane fluidity on uptake

Solid liposome (HSPC/Chol 2 : 1 molar ratio) and liquid liposome (SPC/Chol 2 : 1 molar ratio) 2000 nmol/dish labeled with fluorescence were incubated with cardiomyocytes at 37 °C for 2 h respectively, the amount liposome uptake by cells was determined with the method described in 3.5.

3.9. Effect of charge lipid/polymer on uptake

Liposomes composed of SPC/Chol (2 : 1 molar ratio), SPC/Chol/SPG (2 : 1 : 0.2 molar ratio), SPC/Chol/SPG/PE-PEG_{2000} (2 : 1 : 0.2 : 0.2 molar ratio), SPC/Chol/SA (2 : 1 : 0.2 molar ratio) and SPC/Chol/SA/PE-PEG_{2000} (2 : 1 : 0.2 : 0.2 molar ratio) of 2000 nmol/dish were incubated with cardiomyocytes at 37 °C for 2 h respectively. The amount liposome uptake by cells was determined with the method described in 3.5.

3.10. Effect of anoxia on uptake

Liposomes (SPC/Chol 2 : 1 molar ratio) of 2000 nmol/dish labeled with fluorescence were incubated with the cultured cardiomyocytes for 1 h and 2 h respectively. Normoxia groups were incubated under the conditions of 37 °C, humidified atmosphere of 95% air −5% CO_2. Hypoxia groups were incubated under the conditions of 37 °C, humidified atmosphere of 95% N_2 −5% CO_2. The amount liposome uptake by cells was determined with the method described in 3.5.

3.11. Statistics

Experiments were done in triplicate, and the maximum deviation from the average value was 15% within each experiment. Results were expressed as mean ± S.D., the significance of the difference obtained was evaluated by Student's-t test.

Acknowledgements: The authors are thankful to Dr. Jun Sheng (Changchun Institute of Biological Products, Jilin, China) for his friendly assistance. The study was supported by National Natural Science Foundation of China (30271548).

References

Caride VJ, Zaret BL (1977) Liposome accumulation in regions of experimental myocardial infarction. Science 198: 735−738.

Chaoshu T, Jingyi S, Zhaoping L (1993) Possibility of targeting treatment for ischemic heart disease with liposome (I). Sci Chin (Series B) 36: 590−598.

Guo W, Lee T, Sudimack J (2000) Receptor − specific delivery of liposomes via folate − PEG − Chol J Liposome Res 10: 179−195.

Hong L, Min L, Yongfeng J (2000) Delivery of folid acid conjugated liposomes into cultured hela cells. J Chin Pharma Sci 9(2): 96−99.

Kawano K, Nakamura K, Hayashi K (2002) Liver targeting liposomes containing β-sitosterol glucoside with regard to penetration-enhancing effect on HepG2 cells. Biol Pharm Bull 25: 766−770.

Lee KD, Hong K, Papahadjopoulos D (1992) Recognition of liposomes by cells: *in vitro* binding and endocytosis mediated by specific lipid headgroups and surface charge density. Biochim Biophys Acta 1103: 185−197.

Levchenko TS, Rammohan R, Lukyanov AN (2002) Liposome clearance in mice: the effect of a separate and combined presence of surface charge and polymer coating. Int J Pharma 240: 95−102.

Moribe K, Maruyama K, Iwatsuru M (1997) Estimation of surface state of poly(ethylene glycol)-coated liposomes using an aqueous two-phase partitioning technique. Chem Pharm Bull 45: 1683–1687.

Mueller TM, Marcus ML, Mayer HE (1981) Liposome concentration in canine ischemic myocardium and depolarized myocardial cells. Circ Res 49: 406–415.

Osaka S, Tsuji H, Kiwada H (1994) Uptake of liposomes surface-modified with glycyrrhizin by primary cultured rat hepatocytes. Biol Pharm Bull 17: 940–943.

Patten M, Hartogensis WE, Long CS (1996) Interleukin-1β is a negative transcriptional regulator of 1-adrenergic induced gene expression in cultured cardiac myocytes. J Biol Chem 271: 21134–21141.

Phelan AM, Lange DG (1991) Ischemia/reperfusion – induced changes in membrane fluidity characteristics of brain capillary endothelial cells and its prevention by liposomal – incorporated superoxide dismutase. Biochim Biophys Acta 1067: 97–102.

Shimizu K, Maitani Y, Takayama K (1997) Formulation of liposomes with a soybean-derived sterylglucoside mixture and cholesterol for liver targeting. Biol Pharm Bull 20: 881–886.

Silva-Barcellos NM, Frédéric F, Caligiorne S (2001) Long-lasting cardiovascular effects of liposome – entrapped angiotensin – (1–7) at the rostral ventrolateral medulla. Hypertension 38: 1266–1269.

Smalling RW, Feld S, Ramanna N (1995) Infarct salvage with liposomeal prostaglandin E_1 administered by intravenous bolus immediately before reperfusion in a canine infarction-reperfusion model. Circulation 92: 935–943.

Szoka F, Jacobson K, Derzko Z (1980) Fluorescence studies on the mechanism of liposome – cell interactions *in vitro*. Biochim Biophys Acta 600: 1–18.

Szoka F, Papahadjopoulos D (1981) Liposomes: preparation and characterization. In: Knight CG (Ed.) Liposomes: from physical structure to therapeutic applications, New York, p. 57–59.

Tanaka M, Ito H, Adachi S (1994) Hypoxia induces apoptosis with enhanced expression of fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. Circ Res 75: 426–433.

Torchilin VP, Klibannov AL, Huang L (1992) Targeted accumulation of polyethylene glycolcoated immunoliposomes in infracted rabbit myocardium. FASEB J 6: 2716–2719.

Torchilin VP, Narula J, Halpern E (1996) Poly (ethylene glycol) – coated anti – cardiac myosin immunoliposomes: factors influencing targeted accumulation in the infracted myocardium. Biochim Biophys Acta 1279: 75–83.

Torchilin VP (2000) Drug targeting. Eur J Pharma Sci 11: S81–S91.

Yonemochi H, Yasunaga S, Teshima Y (1998) Mechanism of β-adrenergic receptor upregulation induced by ACE inhibition in cultured neonatal rat cardiac myocytes. Circulation 97: 2268–2273.