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Pravastatin reverses the membrane cholesterol reorganization induced by myocardial infarction within lipid rafts in CD14$^+$/CD16$^-$ circulating monocytes

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ABSTRACT

Large numbers of monocytes are recruited in the infarcted myocardium. Their cell membranes contain cholesterol-rich microdomains called lipids rafts, which participate in numerous signaling cascades. In addition to its cholesterol-lowering effect, pravastatin has several pleiotropic effects and is widely used as secondary prevention treatment after myocardial infarction (MI). The aim of this study was to investigate the effects of pravastatin on the organization of cholesterol within monocyte membrane rafts from patients who had suffered myocardial infarction. Monocytes from healthy donors and acute MI patients were cultured with or without 4 μM pravastatin. Lipid rafts were extracted by Lubrol WX, caveolae and flat rafts were separated using a modified sucrose gradient. Cholesterol level and caveolin-1 expression in lipid rafts were determined. In healthy donors, cholesterol was concentrated in flat rafts (63 ± 3 vs 13 ± 1%, $p<0.001$). While monocytes from MI patients presented similar cholesterol distribution in both caveolae and flat rafts. Cholesterol distribution was higher in flat rafts in healthy donors, compared to MI patients (63 ± 3 vs 41 ± 2%, $p<0.001$), with less distribution in caveolae (13 ± 1 vs 34 ± 2%, $p<0.001$). Pravastatin reversed the cholesterol distribution in MI patients cells between flat rafts (41 ± 2 vs 66 ± 3%, $p<0.001$) and caveolae (34 ± 2 vs 18 ± 1%, $p<0.001$). In conclusion, MI redistributes cholesterol from flat rafts to caveolae indicating monocyte membrane reorganization. In vitro pravastatin treatment restored basal conditions in MI monocytes, suggesting another effect of statins.

1. Introduction

Acute myocardial infarction (MI) is associated with activation of CD14$^+$/CD16$^-$ monocytes, which participate in the healing of ischemic tissue after MI [1,2]. This activation leads to phenotype modification and release of proinflammatory factors, involving membrane reorganization [1,3]. The cell membrane contains lipid raft microdomains, or detergent-resistant membranes (DRM), enriched in cholesterol and sphingolipids and packed together to create a liquid-ordered phase [4] that is insoluble in detergents [5]. Lipids rafts float in the membrane and constitute distinct signaling platforms, depending on the lipid raft subtype and composition [6–8]. There are two major subtypes of lipid rafts, namely caveolae and flat rafts [6,9,10]. Caveolae are small invaginations, approximately 50–100 nm in size, that are present in many mammalian cell types. Caveolae appear to be stabilized by the protein caveolin-1, the main isoform found in non-muscle cells [11,12]. The second major subtype of lipid rafts, namely flat rafts, are coplanar and rich in ganglioside GM3 [13].

Lipid rafts are involved in several cell mechanisms, such as numerous signaling cascades [6,14,15], notably in immune effector cells [16,17], and in endothelial dysfunction, leading to the initiation of atherosclerotic plaque [18].

Furthermore, monocyte adhesion, migration through the endothelium and differentiation in foam cells are the first steps of plaque formation [19] which are prevented and delayed by HMG-CoA reductase inhibitors (statins). Besides the well-known effect of statins on the reduction of total serum and LDL-cholesterol [20], some of the pleiotropic effects of statins may be due to the disruption and/or depletion of cholesterol-rich lipid rafts [17,20].

We determined the most suitable method for extraction of lipid rafts, with the primary objective of analyzing the lipid distribution

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of flat rafts and caveolae in monocytes from acute MI patients and investigating the *in vitro* impact of pravastatin on cholesterol reorganization within lipids rafts.

2. Materials and methods

2.1. Study population

Heparinized peripheral blood was obtained from 14 acute MI patients and 16 age-matched healthy donors. Patients aged <75 years referred to the cardiology department for a first acute MI and admitted within 12 h of onset of symptoms were considered eligible for inclusion. MI was defined by the guidelines of the joint Task Force of the European Society of Cardiology (ESC), the American College of Cardiology (ACC), the American Heart Association (AHA), and the World Heart Federation (WHF) [21]. This includes both ST and non-ST elevation MI, confirmed by detection of elevated levels of creatine kinase (CK) and troponin I (TnI) (at least one value above the 99th percentile of the upper reference limit (URL)), together with evidence of myocardial ischemia (i.e., new ST-T changes or new left bundle branch block, or development of pathological Q waves in the ECG). Exclusion criteria were: previous cardiovascular or pulmonary diseases, prior treatment with statins, history of renal or hepatic diseases, hematological or coagulative disorders. All patients were referred to the catheterization laboratory within the first 24 h for coronary angiogram, and received medication according to current guidelines [22,23].

The study protocol was approved by the local ethics committee and informed consent was obtained from all enrolled subjects in accordance with the Declaration of Helsinki.

2.2. Monocyte isolation and cell culture

Peripheral blood mononuclear cells (PBMC) were obtained from healthy and acute MI patients and isolated by Ficoll density gradient centrifugation. Circulating $CD14^+/CD16^-$ monocytes were isolated by Cell Isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany) by negative selection [24]. Non-monocytes are indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies (CD3, CD7, CD16, CD19, CD56, CD123 and glycophorin A), as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labeling reagent. Between the two labeling steps, no washing steps are required. The magnetically labeled non-monocytes are depleted by retaining them on a light-scattering MACS Column in the magnetic field of a MACS Separator, while the unlabeled monocytes pass through the column (Miltenyi Biotec, Bergisch-Gladbach, Germany). The purity was determined by FACS analysis using PE-conjugated anti-CD14. Ten million monocytes from healthy and acute MI patients were cultured in Dulbecco's Modified Eagle Medium (DMEM) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) (Invitrogen, CA) supplemented with 10% heat-inactivated fetal calf serum (Lonza, Verviers, Belgium). Pravastatin 4 μM (Sigma Aldrich, MO) was introduced into the culture medium for12 h at 37 °C in 5% CO_2. These culture conditions were chosen after a preliminary study with 2, 4, 6, 8 and 10 μM pravastatin and 6, 12 and 24 h of incubation.

2.3. Extraction and composition analysis of monocytes lipid rafts

Lipid rafts are membrane microdomains possessing a resistance to solubilisation in detergents. Two detergents (non-ionic Triton X-100 and Lubrol WX) and a detergent-free sodium carbonate (SC) solution at pH 11 associated with the conventional three-step sucrose density gradient (5–35–45% of sucrose) were used to extract lipid rafts in circulating human monocyte [4].

Briefly, 10,000,000 cells were washed with PBS, then lysed on ice for 30 min in 1 ml MBS buffer (Sigma Aldrich, MO) containing 1% (w/v) of either Triton X-100 or 0.5 M sodium carbonate (SC) pH 11 (Sigma Aldrich, MO) or Lubrol WX (Serva, Paris, France). Cellular plasma membrane extracted with 1 ml of 0.5 M SC was sonicated. Lysates were homogenized by a Dounce homogenizer and mixed with 2 ml of 90% sucrose solution to obtain a final concentration of 45% sucrose.

2.3.1. Conventional sucrose density gradient

The sucrose density gradient of 4 ml of sucrose at 45%, 5 ml of sucrose at 35% and 3 ml of sucrose at 5% (5–35–45% of sucrose) was prepared in MBS buffer. The gradient was centrifuged as reported previously [4]. Twelve fractions of 1 ml were collected from the top to the bottom, vortexed and stored at −80 °C before lipid and protein analysis.

2.3.2. Modified four-step sucrose density gradient

As it is not possible to separate the two subtypes of lipid rafts using the conventional sucrose density gradient, a modified gradient was developed to separate caveolae from flat rafts. In this modified gradient, 4 ml of sucrose at 45%, 3 ml of sucrose at 35%, 4 ml of sucrose at 20% and 1 ml of sucrose at 5% (5–20–35–45% of sucrose) were overlaid on 4 ml of 1% Lubrol WX extraction mixture.

The cholesterol, sphingomyelin (SM) and phosphatidyl-choline (PC) composition of the fractions were used as markers of lipid rafts. Cholesterol, SM and PC were extracted from these fractions by the Folch method [25] and analyzed respectively by gas chromatography/mass spectrometry (GC/MS) [26] and liquid chromatography/mass spectrometry (LC/MS) methods [27].

2.4. Determination of raft protein composition by Western blot

In order to distinguish flat rafts and caveolae obtained using the modified sucrose density gradient, flotillin-1 and caveolin-1 were used as markers of lipid rafts. Sixty microliters of each fraction were mixed with a 4× Laemmli sample buffer, boiled for 5 min, separated on a 12% polyacrylamide SDS-containing gel and transferred onto a nitrocellulose membrane (Whatman, NJ). After blocking non-specific binding sites for 1 h with 5% non-fat milk in PBS-Tween (PBS with 0.1% Tween 20), the membrane was incubated overnight at 4 °C with anti-flotillin-1 (Santa Cruz, CA) and anti-caveolin-1 (Transduction Laboratories, CA) primary antibodies (1:600), washed twice with PBS-Tween and incubated for 1 h at 4 °C with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (1:5000) (Santa Cruz, CA). The membrane was washed twice with PBS-Tween and revealed using an ECL detection kit (Amersham, NJ) and autoradiography.

2.5. Statistical analysis

Continuous variables were tested for normal distribution with the use of the Kolmogorov–Smirnov test. Data are expressed as mean values ± standard error of the mean (SEM) or as median with interquartile range [IQR], as appropriate. Between-group comparisons were performed using the unpaired *t*–test or the Mann–Whitney *U*-test and Kruskal–Wallis test for continuous data, as appropriate. A p value of <0.05 was considered statistically significant. All analyses were performed using SAS statistical software, version 9.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Baseline characteristics

The baseline characteristics of the study population are listed in Table 1. Acute MI patients were age-matched with healthy donors (48 ± 7 vs 50 ± 1 years, p = NS).