FEBS Letters 580 (2006) 5974–5978

Inhibition of MMP-2 activation and release as a novel mechanism for HDL-induced cardioprotection

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Received 18 September 2006; accepted 3 October 2006

Available online 12 October 2006

Edited by Miguel De la Rosa

Abstract **High density lipoproteins (HDL) protect the heart against ischemia/reperfusion (I/R) injury, and matrix metalloproteinase-2 (MMP-2) directly contributes to cardiac contractile dysfunction after I/R. To investigate the possible involvement of MMP-2 inhibition in HDL-mediated cardioprotection, isolated rat hearts underwent 20 min of low-flow ischemia and 30 min of reperfusion. Plasma-derived and synthetic HDL attenuated the I/R-induced cardiac MMP-2 activation and release in a dose-dependent way. The attenuation of I/R-induced MMP-2 activation by HDL correlated with the reduction of post-ischemic contractile dysfunction and cardiomyocyte necrosis. These results indicate prevention of MMP-2 activation as a novel mechanism for HDL-mediated cardioprotection.**

Keywords: High density lipoproteins; Gelatinase A; Matrix metalloproteinases; Myocardial ischemia; Ischemia/reperfusion injury

1. Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases involved in the remodeling of extracellular matrix (ECM). They are synthesized and secreted as pro-enzymes (zymogens), and then activated by proteinases or by non-proteolytic agents that can modify the "cysteine switch" [1]. MMP-2, or gelatinase A, is expressed in the myocardium, where it is synthesized by fibroblasts and cardiomyocytes [2]. Within the heart, MMP-2 degrades almost all ECM components and participates in the long-term cardiac remodeling which follows a myocardial infarction [2]. Indeed, targeted deletion or pharmacological inhibition of MMP-2 improves cardiac activity and survival rate after acute myocardial infarction [3]. Recently, a series of non-ECM substrates for MMP-2 has been described; among these, troponin I (TnI) [4], thus implying MMP-2 as a primary mediator of cardiac dysfunction following I/R [5].

We have recently demonstrated that plasma-derived or synthetic HDL cause a rapid improvement of post-ischemic cardiac function in isolated rat hearts undergoing ischemia/reperfusion (I/R) injury; this cardioprotective effect is dose-dependent and occurs at HDL concentrations that are normally found in the plasma of healthy individuals [6,7].

HDL have been shown to inhibit the expression and activation of MMP-2 and MMP-9 induced by oxidized LDL in smooth muscle cells and macrophages [8,9]. Moreover, the infusion of plasma-derived and synthetic HDL prevented MMP-9 expression in rabbit aortic lesions induced by a combination of cholesterol rich diet and balloon denudation [10]. The aim of the present study was thus to investigate whether a modulation of MMP-2 expression and/or activation could contribute to the cardioprotective effect of plasma-derived and synthetic HDL in isolated hearts undergoing I/R injury.

2. Materials and methods

2.1. Experimental protocol

I/R was carried out in isolated rat hearts as previously described [6,7]. Briefly, hearts were equilibrated with Krebs–Henseleit (K–H) buffer at a flow rate of 15 ml/min for 30 min and underwent a 20 min low-flow ischemia (1 ml/min) followed by 30 min of reperfusion at the starting flow. Human plasma HDL, low density lipoproteins (LDL) and synthetic HDL (sHDL) were prepared as previously described [6,7], and administered during the 10 min immediately before ischemia ($n = 3$ for each treatment group); a separate group of hearts was similarly treated with an equal volume of saline. Control hearts ($n = 3$) were mounted and perfused with K–H buffer at the constant flow rate of 15 ml/min for 80 min. At the end of the experiment, hearts were snap-frozen in liquid nitrogen and stored at −80 °C. Cardiac homogenates were prepared as described [6] and suspended in PBS containing AEBSF 2 mM, EDTA 1 mM, Bestatin 130 μM, E-64 14 μM, Leupeptin 1 μM, Aprotinin 0.3 μM (Sigma–Aldrich). After 1 h incubation at 4 °C, the homogenates were centrifuged for 20 min at 20 000 × *g* at 4 °C and protein content measured by the BCA method (Pierce).

2.2. Zymography and Western blotting for MMP-2

The gelatinolytic activity of cardiac homogenates (40 μg of total protein) was evaluated by zymography, as previously described [11]. Band densities were evaluated with a GS-690 Imaging Densitometer and a Multi-Analyst software (Bio-Rad Laboratories). Aliquots of coronary effluent (10 ml) collected in the first 5 min of reperfusion were

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Abbreviations: ApoA-I, apolipoprotein A-I; CK, creatine kinase; ECM, extracellular matrix; FN, fibronectin; HDL, high density lipoproteins; I/R, ischemia/reperfusion; K–H, Krebs–Henseleit; LDL, low density lipoproteins; LOOH, lipid hydroperoxide; LVDP, left ventricular developed pressure; MMP-2, matrix metalloproteinase 2; ROS, radical oxygen species; sHDL, synthetic HDL; TnI, troponin I

0014-5793/$32.00
doi:10.1016/j.febslet.2006.10.006

lyophilized and subjected to SDS–PAGE; the separated proteins were transferred on nitrocellulose membranes. Membranes were saturated with 5% non-fat dried milk at 4 °C, and incubated with a mouse monoclonal antibody against MMP-2 (Calbiochem) and with an HRP-conjugated rabbit anti-mouse antibody (Dakocytomation). Bands were visualized by enhanced chemiluminescence (Amersham Biosciences). Each experiment was performed at least three times with different heart preparations.

2.3. Western blotting for fibronectin

Cardiac homogenates (20 μg of total protein) were subjected to SDS-PAGE (8% acrylamide) under reducing conditions and the separated proteins were transferred on nitrocellulose membranes. Membranes were saturated overnight with 5% non-fat dried milk at 4 °C, incubated with a rabbit polyclonal antibody against rat fibronectin (Sigma–Aldrich) and with an HRP-conjugated goat anti-rabbit antibody (Dakocytomation). Bands were visualized by enhanced chemiluminescence (Amersham Biosciences). Membranes were then stripped and reprobed with an antibody against α-actin (Sigma–Aldrich).

2.4. RT-PCR

Total RNA was extracted from cardiac homogenates with Trizol Reagent (Life Technologies); cDNA was prepared by reverse transcription of 1 μg total RNA using the iScript cDNA Synthesis kit (Bio-Rad Laboratories), and amplified for 30 cycles with *iTaq* DNA polymerase (Bio-Rad laboratories) in a MyCycler (Bio-Rad Laboratories). The following primers were used: MMP-2 sense 5′-CCCCTATCTACACCTACACCAAGAAC-3′, antisense 5′-CATTCCAGGAGTCTGCGATGAGC-3′, producing a 576 bp fragment; GAPDH sense 5′-ACGACCCCTTCATTGACC-3′, antisense 5′-TGCTTCACCACCTTCTTG-3′, producing a 691 bp fragment. PCR products were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide, visualized by ultraviolet irradiation and photographed with Polaroid film. Band densities were evaluated with a GS-690 Imaging Densitometer and a Multi-Analyst software (Bio-Rad Laboratories). MMP-2 band intensities were normalized by their GADPH values.

2.5. Determination of cardiac lipid hydroperoxides

Aliquots of cardiac homogenates were suspended in PBS containing 5 mM BHT and centrifuged at 4 ° C for 10 min at 20000 × *g*. Protein concentration was measured by the method of Lowry [12]. Lipid hydroperoxide (LOOH) concentration was estimated by a iodometric procedure: after incubation with phosphate-buffered potassium iodide (0.12 M) in the dark for 30 min, the conversion of iodide to iodine by LOOH was measured at 365 nm. Concentrations of LOOH in cardiac homogenates were calculated by the molar absorption of I_3 at 365 nm (24,600 M^{-1} cm^{-1}) and corrected for protein concentration.

2.6. Statistical analysis

Results are reported as mean ± SEM. Differences among the groups were evaluated by 1-way ANOVA, with post hoc evaluation by the Neuman–Keuls test. Statistical significance was defined as $P < 0.05$. Simple regression analyses were performed and significance of the correlation was determined by the Pearson method.

3. Results

3.1. HDL attenuate cardiac MMP-2 activation and release

Gelatinolytic activities in homogenates of non-ischemic (Control) hearts were detected by zymography at 75, 72 and 62 kDa (Fig. 1). The 72 kDa and the 62 kDa bands were identified as proMMP-2 and MMP-2, respectively. It is important to note that in the SDS-containing gels, and differently from the in vivo condition, both latent and active MMP-2 display gelatinolytic activity. As expected, proMMP-2 represented the major gelatinolytic activity in control hearts. I/R caused an almost complete loss of both MMP-2 forms, as shown by the clear-cut reduction of gelatinolytic activity in saline-treated hearts (Fig. 1). In parallel, both pro- and active MMP-2 were

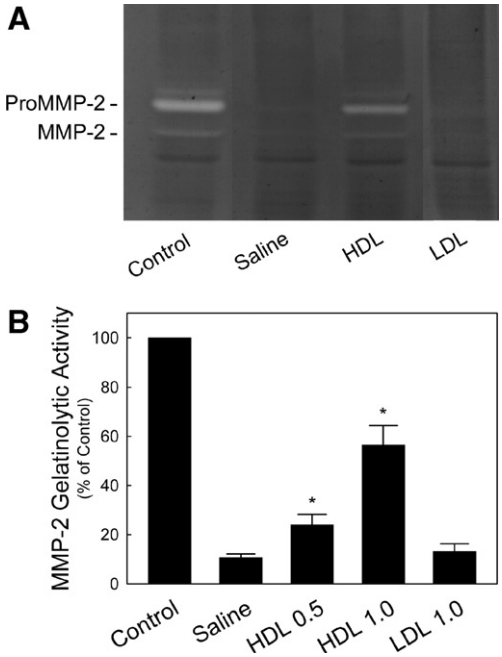

Fig. 1. Effect of HDL on cardiac MMP-2 content. Panel A: Representative zymography of homogenates from non-ischemic hearts (Control) and I/R hearts treated with saline, HDL or LDL (both at 1 mg of protein/ml) during the 10 min immediately before ischemia. Panel B: Densitometric analysis of total MMP-2 gelatinolytic activity in cardiac tissue samples. Data are expressed as mean ± SEM, $n = 3$ for each group. $^{*}P < 0.05$ versus saline.

detected in the coronary effluent collected during the first 5 min of reperfusion (Fig. 2). Active MMP-2 was the predominant form in the coronary effluent, indicating that I/R actually caused the activation and release of cardiac MMP-2. The degradation of fibronectin, one of the non-collagenous components of ECM and substrate of MMP-2 [3], confirmed that MMP-2 is activated in ischemic hearts (Fig. 3).

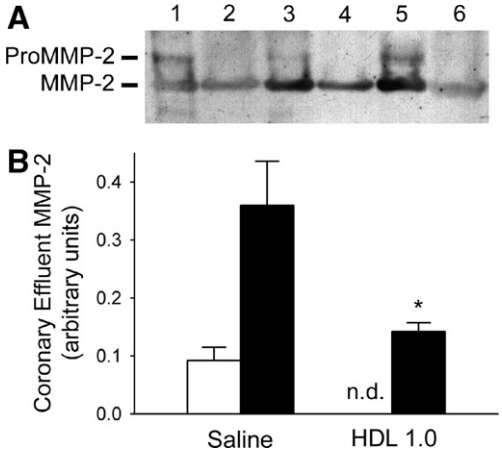

Fig. 2. MMP-2 content in the coronary effluent. Panel A: Immunoblotting analysis for MMP-2 of coronary effluents from I/R hearts treated with saline (lane 1, 3 and 5) or HDL at 1 mg/ml (lane 2, 4 and 6). Panel B: Densitometric analysis of proMMP-2 (white bars) and active MMP-2 (black bars) bands. ProMMP-2 was undetectable (n.d.) in the coronary effluent of HDL-treated hearts. Data are mean ± SEM, $n = 3$ for each group. $^{*}P < 0.05$ versus saline.