HDL protects against ischemia reperfusion injury by preserving mitochondrial integrity

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ABSTRACT

Objective: High density lipoproteins (HDL) protect against ischemia reperfusion injury (IRI). However, the precise mechanisms are not clearly understood. The novel intrinsic prosurvival signaling pathway named survivor activating factor enhancement (SAFE) path involves the activation of tumor necrosis factor (TNF)-alpha and signal transducer and activator of transcription 3 (STAT3). SAFE plays a crucial role in cardioprotection against IRI. We propose that HDL protect against IRI via activation of the SAFE pathway and modulation of the mitochondrial permeability transition pore (mPTP) opening.

Methods and results: Isolated mouse hearts were subjected to global ischemia (35 min) followed by reperfusion (45 min). HDL were given during the first 7 min of reperfusion. In control hearts, the post-reperfusion infarct size was 41.3 ± 2.3%. Addition of HDL during reperfusion reduced the infarct size in a dose-dependent manner (HDL 200 μg protein/ml: 25.5 ± 1.6%, p < 0.001 vs. control). This protective effect was absent in TNF deficient mice (TNF-KO) or cardiomyocyte-STAT3 deficient mice (STAT3-KO). Similarly, HDL, given as a preconditioning stimulus, improved cell survival and inhibited mPTP opening in isolated cardiomyocytes subjected to simulated ischemia. These protective responses were inhibited in cardiomyocytes from TNF-KO and STAT3-KO mice.

Conclusion: Our data demonstrate that HDL protect against IRI by inhibition of mPTP opening, an effect mediated via activation of the SAFE pathway.

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1. Introduction

For the past decades, high density lipoproteins (HDL) have been identified as a strong negative predictor of cardiovascular events. This beneficial effect on the cardiovascular system was attributed, in the first instance, to their ability to facilitate cholesterol excretion [1]. Recent evidence attributes more widespread, beneficial actions to the HDL particle, such as antioxidant, anti-inflammatory and anti-apoptotic influences on vascular cells [2]. The direct actions of HDL on the heart have not been extensively investigated although experimental data show that HDL protect from ischemia reperfusion injury (IRI) [3,4], hypoxia reoxygenation [5], and apoptosis induced by doxorubicin, a potent anti-cancer drug with cardiotoxic side effects [6]. However, the mechanisms of action remain poorly elucidated. It is essential to define precisely the mechanisms involved in HDL-induced cardioprotection especially in the perspective of potential, therapeutic applications of synthetic HDL.

The Survivor Activating Factor Enhancement (SAFE) pathway is a novel, powerful prosurvival signaling pathway involved in cardioprotection against IRI. Two major components of the SAFE pathway, tumor necrosis factor alpha (TNF) and the transcription factor signal transducer and activator of transcription 3 (STAT3), are both required for protection by ischemic pre- and post-conditioning [7,8]. In cultured cardiomyocytes, HDL activate STAT3 and protect against the deleterious effect of doxorubicin Refs. [6,9]. Whether HDL protect against IRI via the SAFE pathway remains unknown.

The mitochondrion plays a key role in protection against IRI. Inhibition of the mitochondrial permeability transition pore
tein/ml), as indicated in the figure legend. At the end of the reperfusion period, the hearts were sliced and stained with triphenyltetrazolium chloride (TTC). Infarct size (IS) was evaluated using computerized planimetry (Planimetry, Boreal Software, Norway). A minimum of 6 hearts was used in all groups.

2.4. Isolation of adult mouse cardiomyocytes

Mice were heparinised (250IU) and anesthetized via an intraperitoneal injection of sodium pentobarbital (60 mg/kg). The heart was quickly excised and the aorta was cannulated for retrograde perfusion in a Langendorff apparatus at a constant flow rate of 3 ml/min at 37 °C. The heart was perfused for 1 min with oxygenated perfusion buffer (PB) (NaCl 113 mM, KCl 4.7 mM, KH2PO4 0.6 mM, Na2HPO4 0.6 mM, MgSO4 7H2O 1.2 mM, NaHCO3 12 mM, KHCO3 10 mM, taurine 30 mM, HEPES 10 mM, glucose 11 mM, 2,3-butanediol monoxide 10 mM), followed by digestion for 13 min with collagenase II (0.9 mg/ml, Worthington) in PB (collagenase buffer (CB)). After digestion, the heart was removed and cardiomyocytes were suspended in CB. This suspension was oxygenated and shaken for 20 min at 37 °C. Cardiomyocytes were centrifugated at 600 rpm for 3 min and the pellet was resuspended in PB2 (CaCl2 12.5 µM and bovine serum albumin (BSA) 1% in PB). Calcium was reintroduced via a stepwise manner over a period of 20 min and cardiomyocytes were left to settle via gravity sedimentation for 10 min. The pellet was resuspended in culture media (BSA 0.2%, creatine 5.8 mM, taurine 5 mM, carmine 2 mM and penicillin/streptomycin 1% in medium 199). Cardiomyocytes were plated onto 6 well plates precoated with laminin and incubated for 1 h at 37 °C.

2.5. Experimental protocols for isolated cardiomyocytes

Normoxic controls were cardiomyocytes without treatment and kept under normoxic conditions. Hypoxic controls were cardiomyocytes without treatment and submitted to 2 h of hypoxia in simulated ischemia (SI) buffer (MgCl2·6H2O 1.2 mM, KCl 16 mM, KH2PO4 1 mM, NaCl 74 mM, CaCl2 1.2 mM, NaHCO3 10 mM, sodium lactate 20 mM, HEPES 25 mM, pH 6.7) using a Gentronics CO2 and O2 Gas Controller. O2 levels were maintained at 1% and CO2 levels at 5%. Treated cardiomyocytes were exposed to HDL (400 µg protein/ml) for 30 min, followed by a 30 min washout period in culture medium and 2 h of hypoxia in SI buffer.

2.6. Measurement of cell viability

Following hypoxia, cells were loaded with 0.04% trypan blue and cell viability was immediately analyzed using a light microscope at 40× magnification. The number of viable (unstained) and nonviable (blue stained) cardiomyocytes in 4 random microscopic fields was recorded, with at least 100 cells counted in each well as previously described [17]. Cell viability was normalized to the normoxic control.

2.7. Analysis of mPTP opening

Following hypoxia, cardiomyocytes were incubated with the dye TetramethylRhodamine Methylester (TMRM, Life Technologies) for 10 min followed by a 10 min washout period. Cardiomyocytes were lifted from the wells and analyzed by flow cytometry (FACS Calibur, BD). 10,000 events were counted. Fluorescence intensity was measured, analyzed using the software FlowJo and normalized to the normoxic control.

2.8. Western blot analysis

Control or HDL treated mouse hearts were subjected to a 30 min stabilization period followed by 35 min of global ischemia and 7 min of reperfusion. The hearts were then snap-frozen in liquid nitrogen and stored at −80 °C until protein extraction was performed. Mitochondrial and cytosolic proteins were extracted by homogenization of the heart tissue, as previously described [18]. Phosphorylated STAT3 (Phospho-STAT3, serine 727), was analyzed by sodium dodecyl sulfate–polyacrylamide gel immunoelectrophoresis (SDS–PAGE) with antibodies from Cell Signaling Technologies. Levels of phosphorylated proteins were normalized to voltage-dependent anion selective channel (VDAC) for...
mitochondria or β-actin for the cytosol. Relative band density was determined with the use of computerized software package (UVI Soft, UVI Band, UVI Tech, Cambridge, UK). A minimum of 4 independent hearts was used per group.

2.9. Chemicals and pharmacological agents

Unless otherwise stated, all chemicals were obtained from Sigma–Aldrich Chemicals, Germany.

2.10. Statistical analysis

All statistical analysis was performed using the software Instat. Data are expressed as mean ± the standard error of the mean (SEM). Comparisons were performed by Student t-test (two tailed) or one- or two-way ANOVA (multiple groups) followed by Turkey or Bonferroni post hoc test, where requested. P < 0.05 was accepted as significantly different.

3. Results

3.1. HDL induce cardioprotection

In order to evaluate the direct, beneficial impact of HDL on the heart, we investigated the protective actions of HDL against IRI using the ex vivo heart Langendorff technique. Following global ischemia, HDL (isolated from human serum) were perfused at the ex vivo level to assess the protective actions of HDL against IRI. We found that HDL treatment reduced the infarct size by approximately 40% of dead cells. The protective effect of native HDL was absent in TNF-KO littermates (41.6 ± 2.2%) (Fig. 2A). Similarly, the protective effect of native HDL observed in STAT3-WT (23.3 ± 2%) was absent in the STAT3-KO littermates (37.2 ± 2.2%) (Fig. 2B).

Similar data were obtained in isolated adult mouse cardiomyocytes subjected to hypoxia. Normoxic cardiomyocytes from wild type littermates (TNF-WT and STAT3-WT) and knockout mice (TNF-KO and STAT3-KO) had mean survival rates of 77.1 ± 1.6%, 84.3 ± 1.5%, 77.5 ± 1.2% and 78.7 ± 3.3% respectively, indicating that approximately 20% of cardiomyocytes did not survive the isolation and plating process. Exposure to 2 h of hypoxia reduced cardiomyocyte survival to 48.5 ± 2.3% (TNF-WT) and 59.6 ± 2.5% (STAT3-WT) (p < 0.001 vs. normoxia), with comparable effects in TNF-KO (55.6 ± 1.3%) and STAT3-KO (60.2 ± 4.8%) (p < 0.001 vs. normoxia). Pretreatment with HDL restored cell survival to 71.2 ± 2.8% and 76.7 ± 1.8% in TNF-WT and STAT3-WT, respectively (p < 0.0001 vs. hypoxia). However, HDL pretreatment did not confer protection against hypoxia in cardiomyocytes isolated from TNF-KO (54.2 ± 1.2%) or STAT3-KO (60.7 ± 2.4%) (Fig. 3A+B).

3.2. HDL induce cardioprotection via the SAFE pathway

To evaluate the role of the SAFE pathway in HDL-induced cardioprotection, we used TNF-KO and STAT3-KO mice. The infarct size of all control hearts (IR) subjected to IRI (from TNF-WT, TNF-KO, STAT3-WT or STAT3-KO mice) were very similar with approximately 40% of dead cells. The protective effect of native HDL (200 μg protein/ml) in TNF-WT littermate mice was comparable to that previously recorded in Fig. 1 (TNF-WT, infarct size of 25.8 ± 2.9%). However, the protective effect of HDL was absent in TNF-KO littermates (41.6 ± 2.2%) (Fig. 2A). Similarly, the protective effect of

Fig. 1. HDL protect against ischemic reperfusion injury. Isolated hearts from (C57black6) mice were subjected to global ischemia (35 min) followed by reperfusion (45 min). At the onset of reperfusion, hearts were treated or not (control mice IR) with HDL (100, 200 or 400 μg protein/ml) during the first 7 min of reperfusion. Quantification of infarct size is expressed in percentage of total heart area. *p < 0.05, ***p < 0.001 vs. IR, n = 6–16.

Fig. 2. HDL protect against ischemic reperfusion injury via the SAFE pathway. Isolated hearts from TNF-KO (A) and STAT3-KO (B) mice and their corresponding wild type littermates (WT) were submitted to global ischemia (35 min) followed by reperfusion (45 min). At the onset of reperfusion, hearts were treated or not (control mice IR) with HDL (200 μg protein/ml) during the first 7 min of reperfusion. Quantification of infarct size is expressed as percentage of total heart area. **p < 0.01, ***p < 0.001 vs. IR. ###p < 0.01 vs. same treatment in WT mice, n = 6.

Fig. 3. HDL protect against ischemic reperfusion injury by activating the STAT3 pathway. Isolated myocytes from TNF-WT and STAT3-WT mice were subjected to hypoxia in order to evaluate the direct, beneficial impact of HDL on the heart, we investigated the protective actions of HDL against IRI using the ex vivo heart Langendorff technique. Following global ischemia, HDL (isolated from human serum) were perfused at the ex vivo level to assess the protective actions of HDL against IRI. We found that HDL treatment reduced the infarct size by approximately 40% of dead cells. The protective effect of native HDL observed in STAT3-WT (23.3 ± 2%) was absent in the STAT3-KO littermates (37.2 ± 2.2%) (Fig. 2B).

Similar data were obtained in isolated adult mouse cardiomyocytes subjected to hypoxia. Normoxic cardiomyocytes from wild type littermates (TNF-WT and STAT3-WT) and knockout mice (TNF-KO and STAT3-KO) had mean survival rates of 77.1 ± 1.6%, 84.3 ± 1.5%, 77.5 ± 1.2% and 78.7 ± 3.3% respectively, indicating that approximately 20% of cardiomyocytes did not survive the isolation and plating process. Exposure to 2 h of hypoxia reduced cardiomyocyte survival to 48.5 ± 2.3% (TNF-WT) and 59.6 ± 2.5% (STAT3-WT) (p < 0.001 vs. normoxia), with comparable effects in TNF-KO (55.6 ± 1.3%) and STAT3-KO (60.2 ± 4.8%) (p < 0.001 vs. normoxia). Pretreatment with HDL restored cell survival to 71.2 ± 2.8% and 76.7 ± 1.8% in TNF-WT and STAT3-WT, respectively (p < 0.0001 vs. hypoxia). However, HDL pretreatment did not confer protection against hypoxia in cardiomyocytes isolated from TNF-KO (54.2 ± 1.2%) or STAT3-KO (60.7 ± 2.4%) (Fig. 3A+B).

3.3. HDL induce mitochondrial and cytosolic STAT3 phosphorylation

New evidence suggests a protective role of STAT3 in mitochondria. We therefore evaluated the effect of HDL treatment on mitochondrial STAT3 phosphorylation. In isolated heart, we observed that treatment with HDL (given during the first 7 min of reperfusion) induced the phosphorylation of STAT3 (serine 727) in

Fig. 4. HDL induce mitochondrial and cytosolic STAT3 phosphorylation. Isolated hearts from TNF-KO (A) and STAT3-KO (B) mice and their corresponding wild type littermates (WT) were subjected to global ischemia (35 min) followed by reperfusion (45 min). At the onset of reperfusion, hearts were treated or not (control mice IR) with HDL (200 μg protein/ml) during the first 7 min of reperfusion. Quantification of infarct size is expressed as percentage of total heart area. **p < 0.01, ***p < 0.001 vs. IR. ###p < 0.01 vs. same treatment in WT mice, n = 6.
both mitochondrial and cytosolic extracts (to 153% and 238% respectively vs. control heart, Fig. 4AþB).

3.4. HDL prevent mPTP opening via the SAFE pathway

Mitochondria are crucial in protection against IRI and are currently considered to be the common end point of different cardioprotective signaling pathways. We therefore evaluated whether HDL improve cardiomyocyte survival via protection of the mitochondria by analyzing their impact on mPTP opening. Following 2 h of hypoxia, TMRM fluorescence intensity was decreased to 67.0±C6±3.1% and 61.0±C6±11.9% in TNF-WT and STAT3-WT respectively (p<0.01 vs. normoxia), indicating mPTP opening. The same effect was observed in TNF-KO and STAT3-KO cardiomyocytes (72.2±C6±2.2% and 68.1±C6±3.0% respectively, p<0.01 vs. normoxia). HDL pretreatment restored the fluorescence intensity to 82.8±C6±2.6% and 87.1±C6±8.1% in TNF-WT and STAT3-WT, respectively (p<0.05 vs. hypoxia), indicating prevention of mPTP opening. In contrast, HDL pretreatment did not restore the fluorescence intensity after hypoxia in TNF-KO (70.2±C6±2.2%) and STAT3-KO mice (63.6±C6±6.9%) (Fig. 5A þB þC).

4. Discussion

Our present data demonstrate that perfusion of physiological concentrations of native HDL, given prior to ischemia or at the onset of reperfusion, confer cardioprotection against IRI and/or reperfusion injury. This effect is mediated via the activation of the SAFE pathway as HDL fail to reduce infarct size in hearts isolated from TNF-KO or STAT3-KO mice. Furthermore, our studies conducted in isolated cardiomyocytes suggest that the prosurvival effect of the SAFE pathway is mediated by preservation of mitochondrial integrity with inhibition of mPTP opening.

The cardioprotective effect of HDL against IRI has previously been reported for both in vitro and in vivo settings. In a model of isolated perfused rat heart, pretreatment with HDL (0.5 or 1 mg/ml) for 10 min prior to ischemia induces a rapid and dose-dependent, postischemic improvement of functional recovery of the left ventricle [3]. Indeed, HDL preserve left ventricular-developed pressure, inhibit the increase of coronary perfusion pressure and limit the release of creatine kinase. These beneficial effects are conserved when HDL (1 mg/ml) are administrated at the onset of reperfusion for 10 min, but to a lesser extent [3]. In a similar model, treatment during the first 5 min of reperfusion with HDL (0.8 mg/ml) significantly reduces infarct size following an ischemia reperfusion insult [19]. The preconditioning action of HDL is confirmed in vivo in mice where HDL (100 µg/g), injected 30 min before ischemia, reduce infarct size by approximately 30% [4]. In our experiments, we show that administration of HDL, given during the first minutes of reperfusion at a lower concentration than previously reported in the literature (0.2–0.4 mg/ml), is sufficient to reduce the infarct size after an ischemia reperfusion insult. This concentration is lower than the physiological circulating concentration of HDL (1 mg/ml) and underlines its potential protective capacity. Moreover, our results obtained in an isolated heart model allow us to exclude the participation of circulating cells, cytokines and circulating endogenous HDL that may confound the effect of exogenous HDL on the heart.

Fig. 3. HDL improve cell survival via the SAFE pathway. Freshly isolated cardiomyocytes from A) TNF-KO and B) STAT3-KO mice and wild type littermates (WT) were treated with HDL (400 µg protein/ml) for 30 min before hypoxia (2 h). Dead cells (trypan blue positive cells) were measured by microscopy counting and cell survival was normalized to the normoxic treatment. **p < 0.001 vs. normoxic controls, ###p < 0.0001 vs. hypoxic controls. n = 6–11.
The SAFE pathway was first described in 2009 and involves the activation of the cytokine TNF and transcription factor STAT3. These two major components of the SAFE pathway have been shown to be involved in cardioprotection. They play a role in ischemic pre- and post-conditioning [7,8] as do other, pharmacological cardioprotective compounds such as melatonin, resveratrol [20] and insulin Ref. [21].

Both ischemic pre- and post-conditioning attenuate the increase of circulating TNF concentrations following ischemia. Paradoxically, ischemic post-conditioning also requires activation of TNF to confer cardioprotection [8,17]. In the isolated heart, HDL has been postulated to play a role in protection against IRI [3], where it decreases myocardial tissue levels of TNF but increases release of TNF from the tissue. In this context, they have suggested that HDL bind and neutralize TNF, affecting its bioavailability [3]. Yet our present data clearly demonstrate that activation of TNF at the onset of reperfusion is necessary for HDL-induced cardioprotection as HDL failed to protect in TNF-KO mice hearts. One possible explanation is that moderate activation of TNF at the onset of reperfusion subsequently leads to cardioprotection whilst continued, excessive production of TNF would be deleterious. It underlines the complex role of TNF is cardiac pathologies. TNF is a multifaceted molecule, being deleterious or beneficial depending of its dose, time and type of receptor activated [8,22]. In the model of IRI, both effects have been shown. Indeed pharmaceutical treatment with a low dose of TNF (0.5 ng/ml) before (preconditioning) or at the reperfusion (post-conditioning) reduces the infarct size induced by IRI, while a high dose of exogenous TNF (10–20 ng/ml) leads to irreversible damage [8,22]. Here, we also show that TNF is needed for inhibition of mPTP opening induced by HDL. Whether TNF has a direct action on the mitochondria is still controversial. However, very recent data show that treatment of isolated mitochondria with TNF (0.5 ng/ml) improves recovery following anoxia reoxygenation, therefore suggesting that TNF can modulate mitochondrial function independently of its cell surface TNF receptor subtypes [23]. Further work is required to delineate the precise mechanisms, including the cellular origin of the cardioprotective TNF but the fact that isolated cardiomyocytes from TNF-KO mice failed to protect against simulated ischemia suggests that TNF may originate from the cardiomyocytes.

STAT3 is the other major component of the SAFE pathway. Our present data implicate mitochondrial STAT3 in cardioprotection.
induced by HDL. STAT3 was originally identified as a transcription factor. However, as its cardioprotective actions occur in a very short time frame, it seems unlikely that it protects against IRI via its transcriptional role. STAT3 plays a major role in the cardioprotective effect of ischemic pre- and post-conditioning [8,17], as well as the protective effects of other pharmacological compounds such as melatonin, resveratrol [20] and insulin Ref. [21]. Recent data demonstrate that the activation of STAT3 in mitochondria, (serine 727 phosphorylation) plays an important role in the regulation of cell respiration, cell metabolism [11] and mPTP opening [12]. Interestingly, Heusch and colleagues show that STAT3 is implicated in ischemic post-conditioning, but identified tyrosine 705 phosphorylation as key to this response [24]. Our previous study showed that a short treatment (5 min) with HDL induces STAT3 serine phosphorylation, but a longer treatment (90 min) is required for STAT3 tyrosine phosphorylation in cultured cardiomyocytes [9]. These data suggest a role for phosphorylation of STAT3 serine rather tyrosine in the rapid effects mediated by HDL. Moreover, targeted-overexpression of STAT3 protects mitochondrial function from ischemia by reducing formation of reactive oxygen species and decreasing cytochrome C release from mitochondria [25]. Our results suggest a pathway whereby HDL may induce cardioprotection via phosphorylation of mitochondrial STAT3 serine 727 and subsequent inhibition of mPTP opening. However, further investigations are necessary to determine the exact role of serine and tyrosine phosphorylation in this response.

Other important mediators involved in cardioprotection, such as extracellular signal-regulated kinase 1/2 (ERK1/2), Akt or connexin 43 (Cx43) are activated by HDL in cardiac cells. These proteins are known to play key roles in protective, signal transduction cascades induced by ischemic conditioning. Short-duration treatment with HDL can induce phosphorylation of ERK1/2 and Akt thus playing a role in protection against hypoxia [5,6]. Cx43 is the major myocardial gap junction protein and is responsible for rapid and synchronous transmission of the cardiac action potential. In neonatal rat cardiomyocytes, short-term treatment with HDL induces phosphorylation of Cx43 which requires activation of protein kinase C [19]. Interestingly, Cx43 can be expressed in mitochondria and might be a downstream mitochondrial target protein of the SAFE pathway.

Fig. 5. HDL inhibit mPTP opening via the SAFE pathway. Freshly isolated murine cardiomyocytes from A) TNF-KO and B) STAT3-KO mice and their wild type littermates (WT) were treated with HDL (400 μg protein/ml) for 30 min before hypoxia (2 h). mPTP opening was measured by TMRM fluorescence intensity, as represented in C). Results were normalized to normoxic treatment. *p < 0.01 vs. normoxic controls, #p < 0.05 vs. hypoxic controls. n = 5–6.
With respect to the various signaling pathways that can be activated to confer cardioprotection, the composition of the HDL particle must be of importance. The HDL particle is highly heterogeneous, although different studies on HDL-induced protection against IRI have attributed principal roles to two components of the complex: apolipoprotein AI (apoAI, the major structural protein) and sphingosine-1-phosphate (S1P). Whilst the cardioprotective effect of HDL in IRI was first attributed to apoAI, several recent studies propose a key role for the S1P content of the HDL particle. Interestingly, our laboratory has demonstrated that protection with S1P against IRI also requires activation of the SAFE pathway [26]. Although platelets are the major source of S1P in circulation, HDL is the main lipidprotein carrier of S1P in plasma [27]. Because S1P is bound to HDL particle, laboratory has demonstrated that protection with S1P against IRI also in IRI was gosine-1-phosphate (S1P). Whilst the cardioprotective effect of HDL apolipoprotein AI (apoAI, the major structural protein) and sphin-

must be of importance. The HDL particle is highly heterogenous, 

References

None declared.

Acknowledgment

The contributions of X Moren, MC Brulhart and S Bioletto are gratefully acknowledged. We also want to acknowledge COST Action BM0904 for its support.


