

Native and reconstituted HDL protect cardiomyocytes from doxorubicin-induced apoptosis

Miguel A. Frias, Ursula Lang, Christine Gerber-Wicht, and Richard W. James*

Faculty of Medicine, University of Geneva, Service of Endocrinology, Diabetology, and Nutrition, University Hospital, 4, rue Gabrielle-Perret-Gentil, CH-1211 Geneva 14, Switzerland

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Aims

We analysed the impact of native and reconstituted HDL on doxorubicin-induced cardiomyocyte apoptosis. While it is an effective anti-cancer agent, doxorubicin has serious cardiotoxic side effects. HDL has been shown to protect cardiomyocytes, notably against oxidative stress.

Methods and results

Cultured neonatal rat ventricular cardiomyocytes were subjected to doxorubicin-induced stress, monitored as caspase3 activation, apoptotic DNA fragmentation and cell viability. The protective effects of HDL and sphingosine-1-phosphate (S1P) were investigated using native HDL, reconstituted HDL of varied composition and agonists and antagonists of S1P receptors. Anti-apoptotic signalling pathways were identified with specific inhibitors. Native and reconstituted HDL significantly decreased doxorubicin-induced cardiomyocyte apoptosis, essentially due to the S1P component of HDL. The latter was mediated by the S1P2 receptor, but not the S1P1 or S1P3 receptors. The extracellular signal-regulated kinases 1 and 2 (ERK1/2) signalling pathway was required for the anti-apoptotic effects of HDL and S1P. The transcription factor Stat3 also played an important role, as inhibition of its activity compromised the protective effects of HDL and S1P on doxorubicin-induced apoptosis.

Conclusion

HDL and its sphingosine-1-phosphate component can protect cardiomyocytes against doxorubicin toxicity and may offer one means of reducing cardiotoxic side effects during doxorubicin therapy. The study identified anti-apoptotic pathways that could be exploited to improve cardiomyocyte survival.

Keywords

HDL • Oxidative stress • Signalling pathways • Cardiomyopathies • Apolipoprotein AI

1. Introduction

Doxorubicin (DOX) is an extremely effective antineoplastic agent used for the treatment of solid tumours and haematologic malignancies. However, its use is hampered by serious cardiotoxic side effects leading to cardiomyopathy and heart failure.^{1,2} The mechanism of DOX-induced cardiac injury is not entirely clear. The free radical hypothesis of DOX-induced cardiotoxicity has garnered most support as DOX has been shown to generate reactive oxygen species.^{3–5} The increase in oxidative stress could cause membrane and macromolecular damage, including the activation of intrinsic apoptotic pathways in cardiomyocytes,^{6–8} leading to heart injury. Large areas of myocardial apoptotic cells

have been observed in DOX-treated patients, and could contribute to dilated cardiomyopathy and heart failure.^{9,10}

High-density lipoprotein (HDL) promotes reverse cholesterol transport to the liver thereby reducing the risk of atherosclerosis. However, there is increasing evidence that HDL exerts other biological effects corresponding to anti-inflammatory, anti-thrombotic, and anti-oxidative functions.^{11–13} In the heart, HDL and its sphingosine-1-phosphate (S1P) component have been shown to counteract ischaemia/reperfusion (I/R) injury.^{14,15} As with DOX, the deleterious effects of I/R injury involve the generation of reactive oxygen species.¹⁶ However, there is no information concerning direct protective effects of HDL or S1P on DOX-induced myocardial injury.

* Corresponding author. Tel: +41 22 3729304, Fax: +41 22 3729329, Email: richard.james@hcuge.ch

The signal transducer and activator of transcription 3 (Stat3) appears to play a cardioprotective role in the heart.¹⁷ Mice with cardiomyocyte-specific deletion of Stat3 showed increased susceptibility to cardiac injury.¹⁸ The cardioprotective role of Stat3 may also extend to DOX.¹⁹

At present, there is little information concerning the effect of HDL on Stat3 activity. We have recently demonstrated in ventricular cardiomyocytes that HDL activates Stat3 mainly via its constituent S1P. However, whether Stat3 acts as a signalling molecule in HDL-induced cell survival is unknown.

There are few therapeutic options currently available to increase HDL. One novel strategy, synthetic or reconstituted HDL (rHDL), is under development. HDL is a heterogeneous group of small lipoproteins whose basic elements are apolipoprotein (apo) AI and phospholipids. A recent innovation has been to complement rHDL with other bioactive molecules that can extend its physiological impact. Thus, rHDL containing in addition S1P was shown to induce endothelial cell tube formation.²⁰

In the present study, we examined whether native and rHDL could counteract DOX-induced apoptosis in ventricular cardiomyocytes, determined the importance of S1P and investigated potential roles for the Stat3 and ERK1/2 (extracellular signal-regulated kinases 1 and 2) pathways.

2. Methods

2.1 Materials

S1P, DOX, and phosphatidylcholine were purchased from Sigma-Aldrich (Buchs, Switzerland). The S1P agonists and antagonists FTY720, VPC23019 were from Calbiochem (La Jolla, CA, USA) and Avanti Polar Lipids Inc. (Alabaster, AL, USA), respectively. SEW2871 and JTE013 were obtained from Tocris Bioscience (Ellisville, MO, USA). SB203580, AG490, and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were purchased from Calbiochem, while U0126 was from Biomol Research Laboratories (Plymouth Meeting, PA, USA).

2.2 Cell culture

The study was approved by the federal (Swiss) and cantonal (Geneva) veterinary authorities and conforms to the US guidelines on animal experimentation (NIH Publication No. 85-23, revised 1966). Neonatal cardiomyocytes were isolated from 1- to 2-day-old Wistar rat ventricles as previously described.²¹

2.3 Western blotting

After treatment as indicated in the figure legends, cardiomyocytes were washed with phosphate buffer saline (PBS) and lysed (50 μ L of Tris-HCl (50 mM, pH 7.4), NaCl (150 mM), glycerol (10%v/v), EDTA (2 mM), EGTA (2 mM), Triton X-100 (1%v/v), β -glycerophosphate (40 mM), NaF (50 mM) containing a mixture of protease inhibitors (Roche, Mannheim, Germany), and phenyl-methyl-sulfonyl fluoride (1 mM)). Total cell proteins (20 μ g) were separated by SDS-PAGE (12% acrylamide gel) and blotted onto nitrocellulose membrane. Membranes were probed for cleaved caspase3 (catalytically active 17 kDa fragment; Cell Signaling Technology, Denver, MA, USA) to monitor apoptosis, and reprobbed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon International Inc., Hampshire, UK). Specific bands were visualized with a chemiluminescence kit (Amersham, Zürich, Switzerland).

2.4 Preparation of HDL and rHDL

HDL ($d = 1.063-1.21$ g/mL) was isolated by cumulative flotation ultracentrifugation²² from a plasma pool provided by healthy volunteers, dialysed against PBS containing EDTA (1 mM) and stored at 4°C.

ApoAI was isolated from delipidated human HDL.²³ Three different forms of rHDL were prepared by the cholate dialysis procedure:²⁴ rHDL A contained phospholipids and S1P (for convenience labelled rHDL despite the absence of apoAI); rHDL B contained phospholipids, S1P and apoAI; rHDL C contained phospholipids and apoAI. The phospholipid:apoAI molar ratio was 100:1. For preparations containing S1P, aliquots from a stock solution (1.0 mg/mL in chloroform:methanol, 2:1 v/v) giving the required concentration (indicated in figure legends) were dried in glass tubes and the phospholipid/cholate mixture then apoAI added, as prescribed by the cholate dialysis procedure.²⁴ All preparations were dialysed against Tris 10 mM, pH 8.0, NaCl, 0.15 M and stored in sodium azide (0.05%w/v).

2.5 DNA fragmentation

DNA fragmentation was quantified by measuring the content of intracellular nucleosomes. Cardiomyocytes (10^6 cells per well) were deprived of serum for 24 h and subjected to treatment as indicated in the figure legends. After stimulation in serum-free DMEM, cells were washed with ice-cold PBS and lysed (60 μ L of the buffer used for western blotting). Cellular lysates were used for protein determination and quantitative evaluation of histone-associated DNA fragments (mono- and oligonucleosomes) by photometric enzyme immunoassay (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics, Germany). Results are reported as arbitrary absorbance units normalized to milligrams of proteins.

2.6 Determination of cardiomyocyte viability

Cell viability was evaluated by the colorimetric MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), as described previously.¹⁹

2.7 Statistical analysis

All values are expressed as mean (SEM). Differences between groups were determined using either two-tailed unpaired Student's *t*-tests. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1 HDL and S1P counteract DOX-induced cell death

The anti-apoptotic effect of HDL and its constituent S1P was studied by investigating whether they could counteract DOX-induced caspase3 activation and DNA fragmentation. As illustrated in *Figure 1A*, HDL (400 μ g protein/mL, corresponding to physiological extravascular concentrations²⁵), caused a significant decrease in DOX-induced activation of caspase3 at 8 h ($72 \pm 7\%$) and 16 h ($62 \pm 7\%$). Likewise, S1P inhibited DOX-induced caspase3 activation by $54 \pm 4\%$ (8 h) and $35 \pm 8\%$ (16 h) (*Figure 1B*). The HDL- and S1P-induced inhibitory effects on DOX-induced caspase3 activation were concentration-dependent, with significant responses already observed at 200 μ g/mL HDL (*Figure 1C*) and 100 nM S1P (*Figure 1D*). DOX also increased apoptotic DNA fragmentation ($319 \pm 54\%$ at 24 h) (*Figure 2A*) and reduced cardiomyocyte viability by 60% ($40 \pm 5\%$; *Figure 2B*). The presence of native HDL or S1P inhibited DOX-induced

DNA fragmentation (64 ± 5 and $58 \pm 6\%$, respectively) and increased cell viability of DOX-treated cells to 76 ± 3 and $67 \pm 6\%$, respectively (Figure 2B). Interestingly, HDL and S1P also reduced basal DNA fragmentation (33 and 21%, respectively).

Our results indicate that in ventricular cardiomyocytes, both HDL and S1P counteract DOX-induced apoptotic effects.

3.2 Effect of HDL components on DOX-induced apoptosis in ventricular cardiomyocytes

To investigate the specific role of the HDL constituents, S1P and apoA1, in the inhibitory action on DOX-induced apoptosis, we

pre-incubated cells with three different formulations of rHDL, containing S1P alone (rHDL A), S1P+apoA1 (rHDL B), and apoA1 (rHDL C). Cells were also pretreated with native, human HDL and S1P as positive controls. As illustrated in Figure 3A, the rHDLs containing S1P decreased DOX-induced caspase3 activation to an extent comparable to control treatments. In contrast, rHDL C did not significantly affect the DOX-induced increase in caspase3 activation (Figure 3A). Interestingly, rHDL B containing S1P and apoA1 appeared to induce a stronger (albeit non-significantly different) response than rHDL A containing only S1P.

A comparable result was obtained for DOX-induced apoptotic DNA fragmentation (Figure 3B). Native HDL and its constituent S1P as well as the rHDLs containing S1P markedly decreased

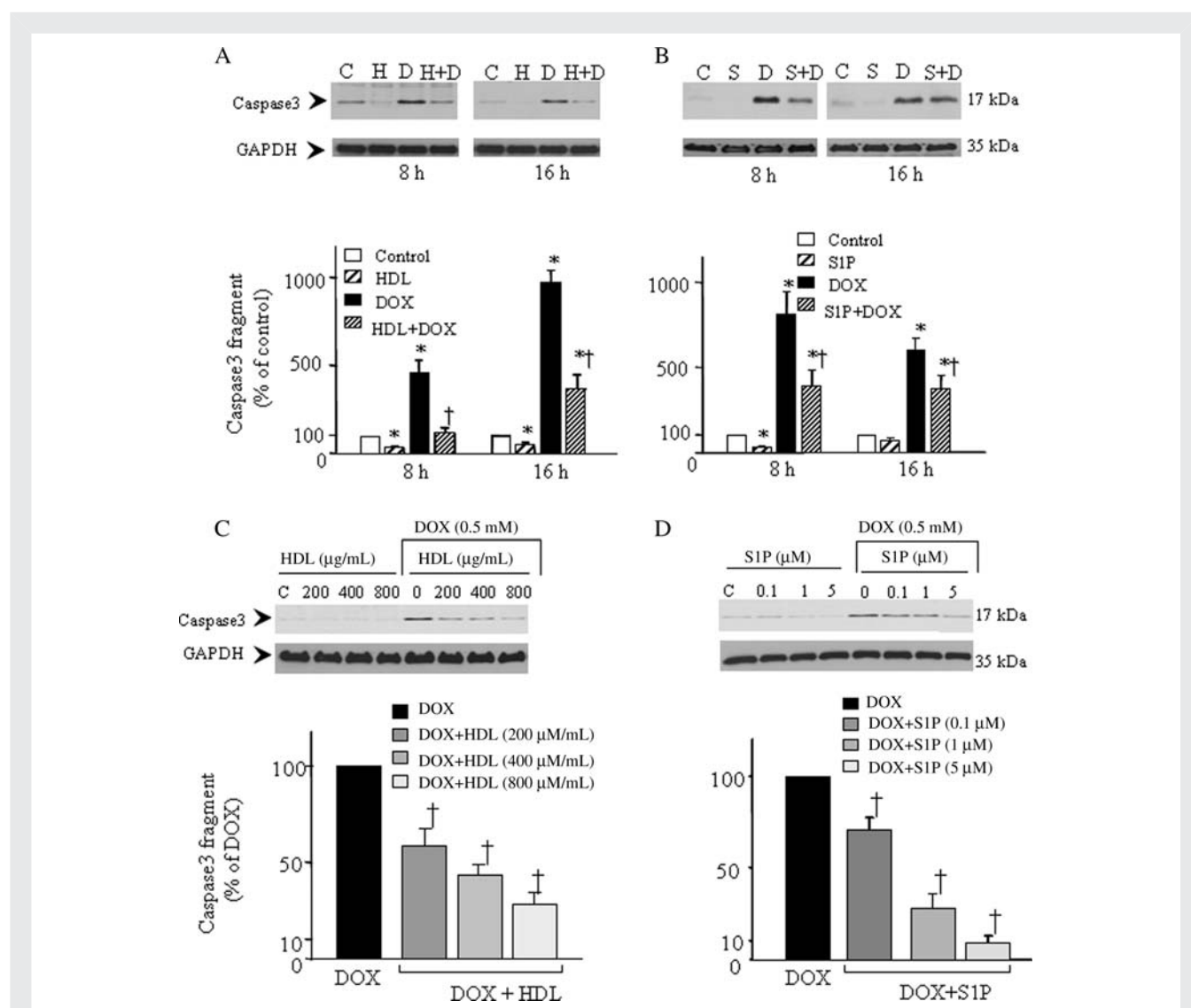


Figure 1 HDL and S1P inhibit DOX-induced caspase3 activation in ventricular cardiomyocytes. (A and B) Cardiomyocytes were pretreated or not (control, C) with HDL (H, 400 $\mu\text{g}/\text{mL}$) or S1P (S, 1 μM), for 30 min prior to the incubation with DOX (D, 0.5 μM ; 8 h and 16 h). (C and D) Cells were pretreated or not (control, C) with increasing concentrations of HDL (200–800 $\mu\text{g}/\text{mL}$) or S1P (S, 0.1–5 μM) for 30 min prior to the incubation with DOX (0.5 μM , 8 h). Caspase3 activation was analysed in cellular extracts by determining the level of the catalytically active 17 kDa fragment by western blot. Representative blots are shown at the top. Equal gel loading was assessed by immunoblotting GAPDH. Specific bands corresponding to activated caspase3 were quantified by densitometry and expressed as percentage of DOX-induced formation of the 17 kDa fragment. * $P < 0.05$ compared with control values, † $P < 0.05$ compared with values from DOX-treated cells ($n = 3-5$).

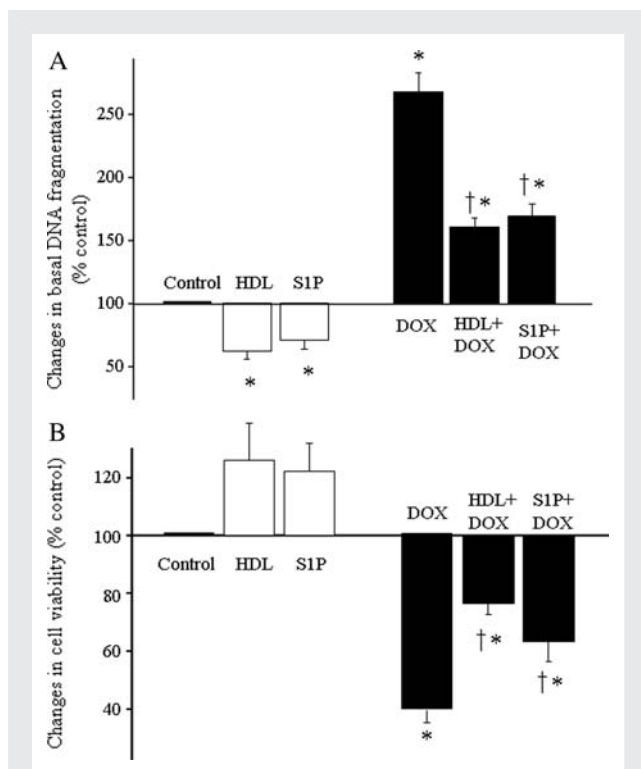


Figure 2 HDL and S1P inhibit DOX-induced apoptotic DNA fragmentation and increase cell viability in ventricular cardiomyocytes. Cells were pretreated or not (control) with HDL (400 $\mu\text{g}/\text{mL}$) or S1P (1 μM), for 30 min prior to incubation with DOX (0.5 μM , 24 h). (A) DNA fragmentation was analysed by measuring histone-associated DNA fragments. Changes in basal DNA fragmentation were calculated as arbitrary absorbance units normalized to protein and are expressed as percentage of the control (attributed 100%). (B) Cell viability determined using the MTT assay. Results are expressed as percentage of the control (attributed 100%). * $P < 0.05$ compared with control values, † $P < 0.05$ compared with values from DOX-treated cells ($n = 3-5$).

DOX-induced DNA fragmentation, whereas rHDL C containing only apoAI had no significant effect.

Our results indicate that HDL exerts its anti-apoptotic action principally via its sphingolipid constituent S1P.

3.3 HDL and S1P counteract the apoptotic effects of DOX via the S1P2 receptor

To determine which S1P receptors are involved in the anti-apoptotic action of HDL and S1P, we used available agonists and antagonists of the S1P receptors.²⁶⁻²⁸

The highly selective S1P2 antagonist, JTE013, abolished the ability of HDL and S1P to inhibit DOX-induced caspase3 activation (Figure 4A and B). In contrast, incubation of cardiomyocytes with VPC23019, a competitive antagonist of S1P1/S1P3 receptors, did not significantly affect the inhibitory action of HDL and S1P on DOX-induced caspase3 activation (Figure 4A and B). Similarly, SEW2871 (S1P1 receptor-specific agonist) and FTY720 (high-

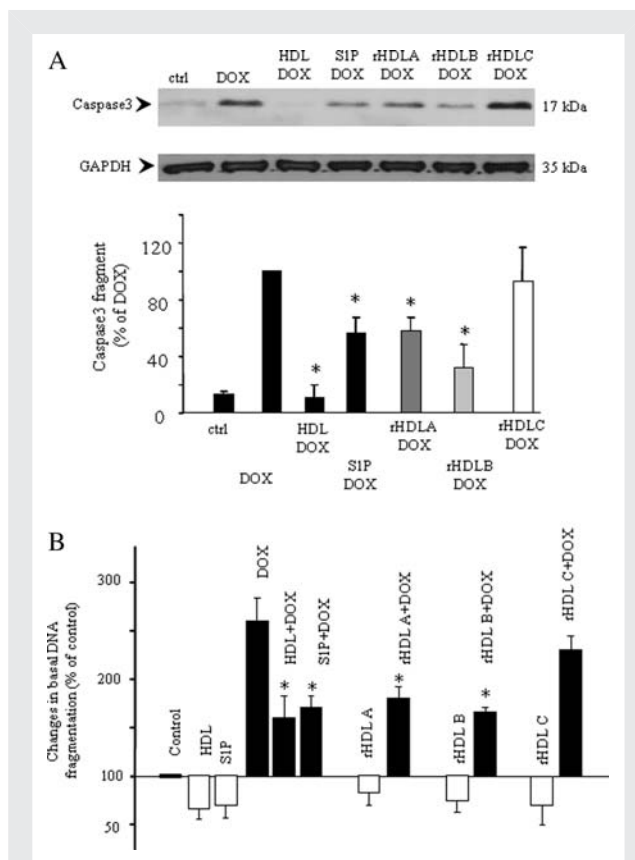


Figure 3 Inhibition of DOX-induced caspase3 activation and DNA fragmentation by HDL, rHDL and S1P. (A and B) Cardiomyocytes were pretreated or not (control, ctrl) with HDL (400 $\mu\text{g}/\text{mL}$), S1P (1 μM), or three different rHDLs containing rHDL A (S1P, 1 μM), rHDL B (S1P, 1 μM +apoAI, 100 $\mu\text{g}/\text{mL}$), rHDL C (apoAI, 100 $\mu\text{g}/\text{mL}$) for 30 min prior to the incubation with DOX (0.5 μM) for 8 h (A) or 24 h (B). Caspase3 activation (A) was analysed as described in Figure 1 and DNA fragmentation (B) as described in Figure 2. Representative blots are shown. * $P < 0.05$ compared with DOX values ($n = 3-4$).

affinity agonist of S1P1, S1P3, S1P4, and S1P5 receptors) had no effect either on basal or DOX-induced caspase3 activation (Figure 4B). JTE013 also abolished the inhibitory action of HDL and S1P on DOX-induced DNA fragmentation, whereas VPC23019 had no effect (Figure 4C). Likewise, SEW2871 and FTY720 did not affect basal or DOX-induced DNA fragmentation.

Thus our results indicate that in DOX-treated cardiomyocytes, HDL and S1P exert their anti-apoptotic action essentially through the receptor S1P2.

3.4 Critical role of Stat3 and ERK1/2 in the anti-apoptotic effect of HDL and S1P

We have previously reported that in ventricular cardiomyocytes, ERK1/2 is critical for HDL- and S1P-induced Stat3 activation.²⁹ Thus, we investigated the role of ERK1/2 and p38 MAPK in the anti-apoptotic responses induced by HDL and S1P using specific inhibitors of the ERK1/2 and p38 MAPK signalling pathways, U0126 and SB203580, respectively. U0126 strongly reduced the

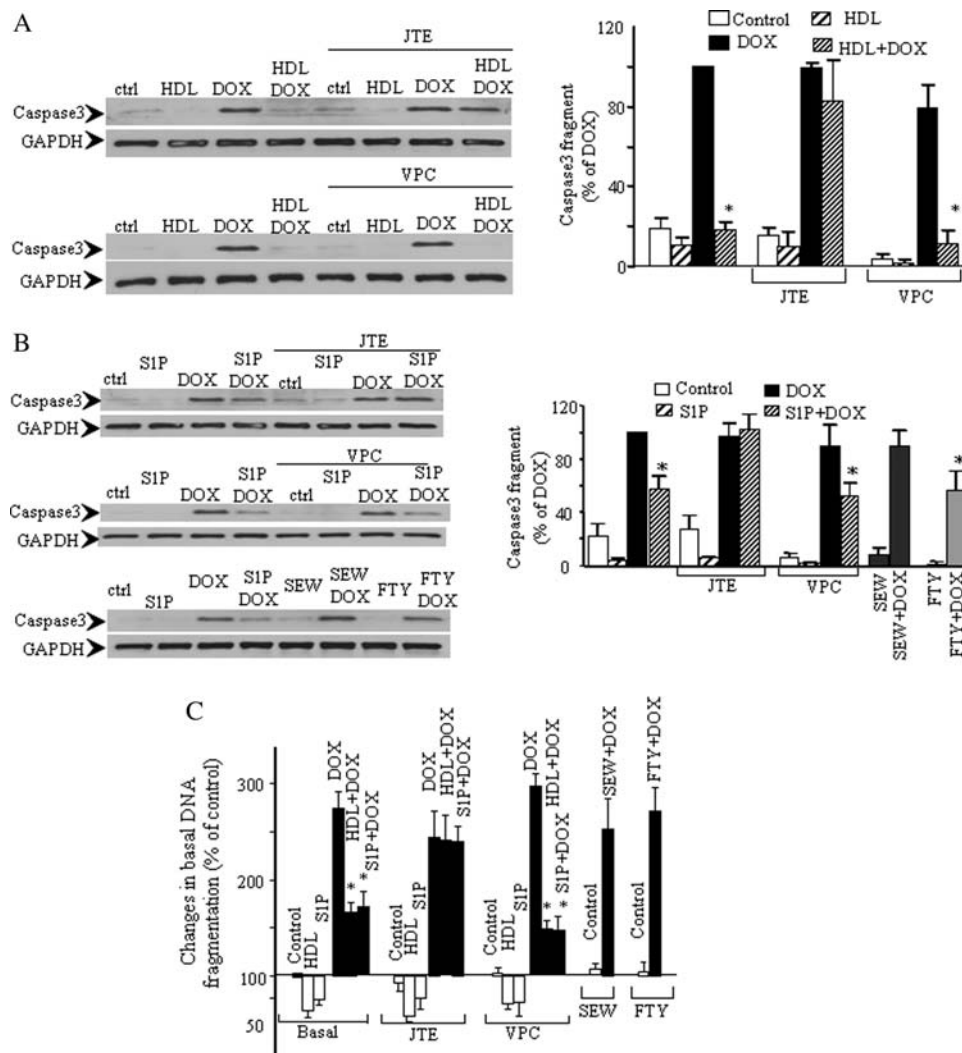


Figure 4 HDL and S1P inhibit DOX-induced caspase3 activation and DNA fragmentation through S1P2. (A) Cardiomyocytes were untreated (basal) or incubated (20 min) with JTE013 (JTE, 5 μ M; S1P2 antagonist) or VPC23019 (VPC, 5 μ M; S1P1/S1P3 antagonist). A second pre-incubation period (30 min) \pm HDL was followed by addition of DOX (0.5 μ M) for 8 h. Caspase3 activation was subsequently analysed in cellular extracts. (B) The same experimental procedure as for (A), except S1P replaced HDL (top two panels). For panel 3, cells were pre-incubated with S1P, SEW2871 (SEW, 1 μ M; S1P1-specific receptor agonist), FTY720 (FTY, 1 μ M; high affinity agonist of S1P1, S1P3, S1P4, and S1P5 receptors) or non-treated (control, ctrl) prior to addition of DOX (8 h) then analysis of caspase3 activation. (C) Cells were pre-incubated (20 min) with JTE, VPC, SEW, FTY or untreated (basal) prior to addition of HDL or S1P (30 min) (to non-treated or JTE and VPC treated samples only). Subsequently DOX (0.5 μ M) was added for 24 h before analysing measuring histone-associated DNA fragments. * P < 0.05 compared with corresponding DOX values (n = 3–5).

anti-apoptotic effect of HDL and S1P on DOX-induced caspase3 activation (Figure 5A and B) and DNA fragmentation (Figure 5C), whereas SB203580 had no significant effect. We had previously shown that LY 294002, a specific inhibitor of the Akt pathway, had no effect on HDL or S1P-induced activation of Stat3.²⁹ In the present study, we confirmed that the protective effects of HDL and S1P against DOX-induced caspase-3 activation and DNA fragmentation are maintained in the presence of the inhibitor. The data are presented in Supplementary material online, Figures S1 and S2. It suggests that ERK1/2 is principally involved

in the protective influence of HDL and S1P against apoptosis in cardiomyocytes exposed to DOX.

To evaluate the role of Stat3 in the anti-apoptotic effect of HDL and S1P, we employed the Stat3 pathway inhibitor AG490.¹⁸ As shown in Figure 6A, the inhibitor decreased HDL- and S1P-induced Stat3 phosphorylation. In parallel, there were reductions in the ability of HDL and S1P to counter DOX-induced caspase3 activation and DNA fragmentation (Figure 6B and C).

Our results show that Stat3 is implicated in the protective effect of HDL and S1P against DOX-induced apoptosis.

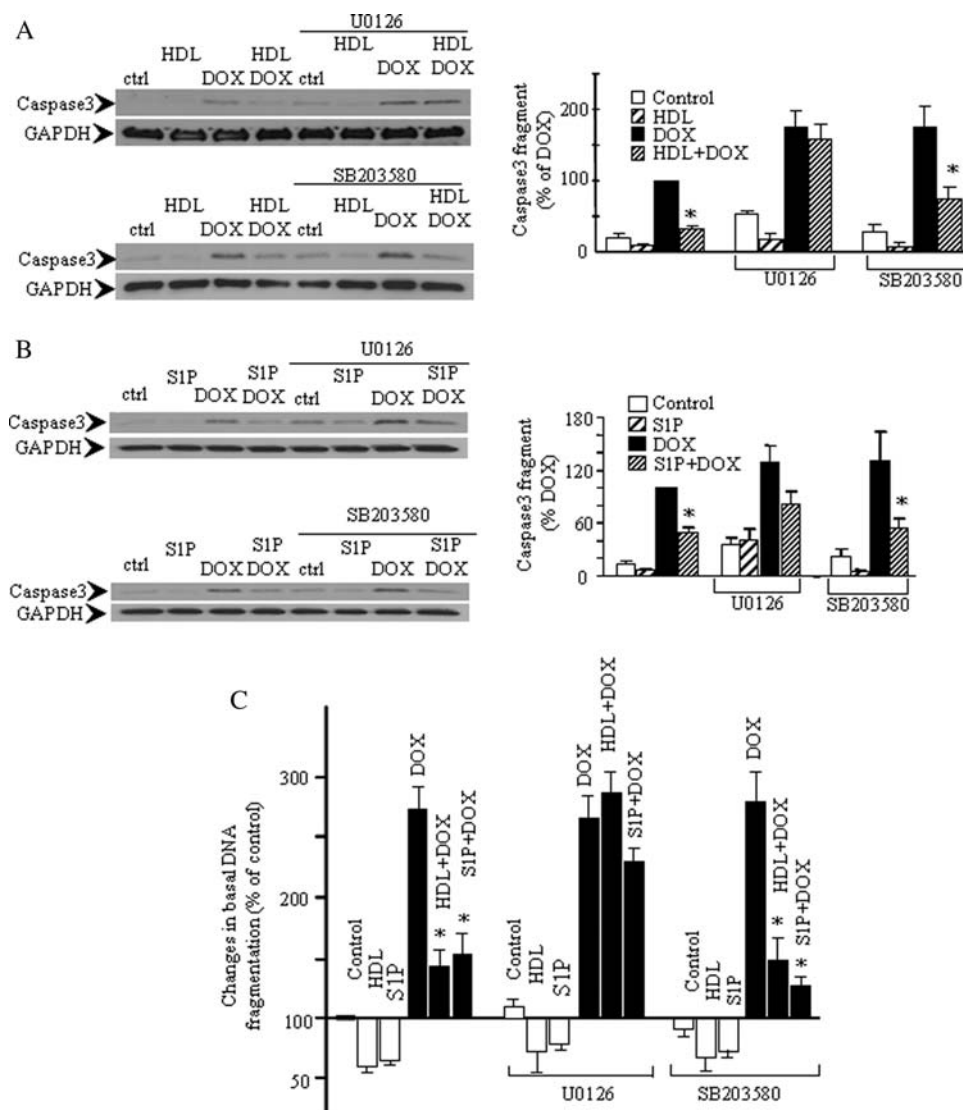


Figure 5 ERK1/2 plays a role in the inhibitory effect of HDL and S1P on DOX-induced activation of caspase3 and DNA fragmentation. Cardiomyocytes were incubated (30 min) with U0126 (10 μ M) or SB203580 (10 μ M), prior to stimulation (30 min) with HDL (400 μ g/mL) or S1P (1 μ M). Subsequently, DOX (0.5 μ M) was added and caspase3 activation (A and B) or DNA fragmentation (C) analysed after 8 h (A and B) or 24 h (C). * $P < 0.05$ compared with corresponding DOX values ($n = 4-5$).

4. Discussion

This is the first demonstration, to our knowledge, of the ability of HDL to reduce toxicity in ventricular cardiomyocytes by counteracting DOX-induced apoptosis. The anti-apoptotic action appears to be mediated essentially through the S1P component of HDL. Indeed, S1P and rHDLs containing S1P had a strong inhibitory action on DOX-induced caspase3 activation and apoptotic DNA fragmentation with, consequently, improved cell viability, comparable to HDL isolated from human plasma. In contrast, rHDL containing apoA1 but no S1P did not significantly affect DOX-induced apoptosis.

Our studies further reveal that these anti-apoptotic activities are mediated by Stat3 and involve ERK1/2. Both the ERK1/2 antagonist, U0126 and the Stat3 pathway inhibitor, AG490, blunted the anti-

apoptotic effects of HDL and S1P on DOX-induced caspase3 activation and DNA fragmentation. Our previous studies²⁹ demonstrated that the ERK1/2 pathway inhibitor, U0126, suppressed HDL- and S1P-induced Stat3 activation in cardiomyocytes, indicating that ERK1/2 activation occurs upstream of that of Stat3. In that study,²⁹ we also analysed the influence of the PI3K/Akt pathway inhibitor, LY 294002 and showed that it had no impact on HDL or S1P activation of Stat3. It was confirmed in the present study with respect to the protective effects of HDL and S1P against DOX-induced apoptosis (see Supplementary material online, Figures S1 and S2). The results accord with several studies reporting that Stat3 plays a role in the survival of cardiomyocytes in pathophysiological situations such as myocardial ischaemia, reperfusion injury, and DOX treatment.³⁰⁻³²

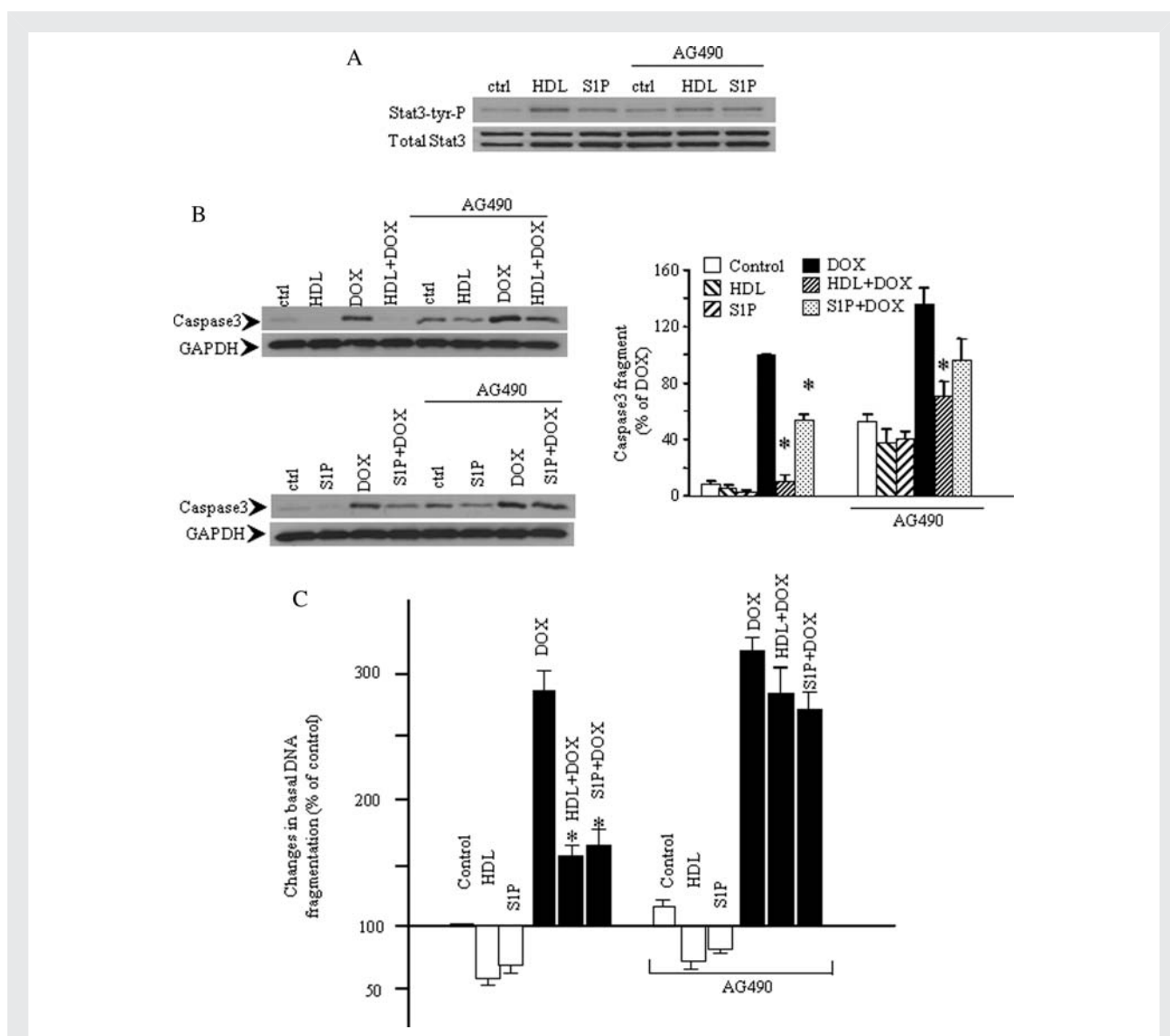


Figure 6 Pharmacological inhibition of Stat3 reduces the inhibitory effect of HDL and S1P on DOX-induced caspase3 activation and apoptotic DNA fragmentation. Cells were incubated (16 h) with the Stat3 inhibitor AG490 (50 μ M) prior to stimulation with HDL (400 μ g/mL) or S1P (1 μ M). (A) After HDL or S1P stimulation (90 min), Stat3 tyrosine phosphorylation (Stat3-P) was analysed in cellular extracts by western blotting. A representative blot is shown. (B) After stimulation (30 min) with HDL or S1P, cells were incubated with DOX (0.5 μ M, 8 h), followed by analysis of caspase3. Representative blots are shown at the top of (B) (ctrl = control). (C) Cells were incubated (16 h) with the Stat3 inhibitor AG490 (10 μ M) prior to stimulation with HDL or S1P. Thereafter, cells were incubated with DOX (0.5 μ M, 24 h) and DNA fragmentation analysed. * $P < 0.05$ compared with values from corresponding DOX cells ($n = 3$).

Also in agreement with our data, S1P and S1P associated with HDL have been shown to activate potent intracellular signalling pathways involved in cardiac remodelling.^{12–15} Conversely, the HDL structural peptide, apoA1, appears to confer cardioprotection primarily by influencing reverse cholesterol transport and reducing atherosclerosis as shown by studies using apoA1 and mimetic peptides³³ or rHDL containing apoA1.^{34,35} In this context, it has been established that increases of HDL in statin-treated patients correlate in part with an inhibitory effect on atheroma progression.³⁶ Interestingly, one study reported that rats treated with DOX showed a drop in serum HDL.³⁷

Extracellular S1P functions as a ligand for a family of plasma membrane receptors designated S1P1 to S1P5. S1P1, S1P2, and S1P3 subtypes are expressed in the cardiovascular system.³⁸ Using various, well-characterized agonists and antagonists of the S1P receptor subtypes including two potent and specific inhibitors of S1P1/S1P3 and S1P2, respectively, our results clearly designate S1P2 as the receptor principally mediating the effects of HDL/S1P on apoptosis induced by DOX in cardiomyocytes. At the present time, there are few studies concerning the role of S1P receptors in cardiomyocytes. In rat-ventricular cardiomyocytes, S1P-induced hypertrophic and cardioprotective effects during hypoxia were found to occur through

the S1P1 receptor.^{28,39} In contrast, Theilmeier *et al.*¹⁵ reported that HDL and its constituent S1P protect the heart against I/R injury via an S1P3 receptor mediated pathway. More recently, Means *et al.*⁴⁰ showed that both S1P2 and S1P3 receptors contribute to protect cardiomyocytes from I/R damage *in vivo*. The same authors found that in cultured mouse cardiomyocytes, S1P acts predominantly through the S1P2 receptor, a result which is consistent with our findings. While our data provide no evidence for the involvement of S1P1 or S1P3 receptors, we cannot presently exclude that another receptor, perhaps activated through the apoA1 component of HDL, might be involved in the anti-apoptotic action of HDL, possibly by facilitating the action of S1P. Further studies are required to define the respective roles of the receptors S1P1, S1P2, and S1P3. Such studies should also address the influence of other receptors, such as the HDL binding scavenger receptor, SR-B1, that are involved in other intracellular pathways mediating specific functions.

In summary, the present study demonstrates that in ventricular cardiomyocytes: (i) HDL counteracts DOX-induced apoptosis mainly via its S1P constituent and the receptor S1P2; (ii) that Stat3 activation involving ERK1/2 plays an essential role in the anti-apoptotic effect of HDL. The study thus reveals pathways that could be explored as targets to limit cardiomyocyte death and possible heart failure. The results also underline that the clinical relevance of HDL goes beyond facilitated removal of cholesterol from the blood vessel wall. Strategies designed to increase HDL levels in general and their S1P content in particular may improve the prognosis of the myocardium at risk in DOX-treated patients. Infusion of rHDL has already been shown to increase transiently serum HDL levels. Whether such treatment concomitantly with DOX therapy could limit the cardiotoxic effects of the drug merits consideration.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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