

# The natural cardioprotective particle HDL modulates connexin43 gap junction channels

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## Aims

High-density lipoprotein (HDL) is known for its cardioprotective properties independent from its cholesterol transport activity. These properties are mediated by activation of kinases such as protein kinase C (PKC). Connexin43 (Cx43) is a gap junction protein present in ventricular cardiomyocytes. PKC-dependent phosphorylation modifies Cx43 gap junction channel properties and is involved in cardioprotection. We hypothesized that cardioprotective properties of HDL may be mediated in part by affecting Cx43 gap junction channels.

## Methods and results

Neonatal rat cardiomyocytes were treated with HDL and Cx43 phosphorylation was evaluated by western blotting and immunofluorescence. We found that HDL promoted phosphorylation of Cx43 with a maximal induction at 5 min, which was inhibited by pre-treatment with various PKC inhibitors. Sphingosine-1-phosphate (S1P), a component of HDL, induced effects that were similar to those of HDL. These compounds significantly reduced diffusion of fluorescent dye among cardiomyocytes (~50%) which could be prevented by PKC inhibition. As observed during optical recordings of transmembrane voltage, HDL and S1P depressed impulse conduction only minimally (<5%). Moreover, 5 min of HDL and S1P treatment at the onset of reperfusion significantly reduced infarct size (~50%) in response to 30 min ischaemia in *ex vivo* experiments.

## Conclusion

Short-term treatment with HDL or S1P induces phosphorylation of Cx43 by a PKC-dependent pathway. HDL-induced phosphorylation of Cx43 reduced the diffusion of large tracer molecules between cells, whereas impulse conduction was maintained. Moreover, 5 min treatment with HDL confers cardioprotection against ischaemia/reperfusion injury. These results link Cx43 for the first time to the short-term cardioprotective effects of HDL.

## Keywords

High density lipoprotein • Connexin43 • Phosphorylation • Permeability • Electrical coupling

## 1. Introduction

High-density lipoprotein cholesterol (HDL-C) level correlates inversely with the risk of cardiovascular diseases.<sup>1</sup> The anti-atherogenic effects of HDL have been linked to its capacity to transfer excess cholesterol from peripheral tissues to the liver in a process known as reverse cholesterol transport. More recently, HDL has also been shown to exert anti-inflammatory, antioxidative, anti-apoptotic and vasodilatory functions involving non-cholesterol-dependent actions on endothelial and smooth muscle cells. These properties are mediated by several cellular signaling cascades including PKC, mitogen-activated protein kinase [MAPK: p38-MAPK, extracellular signal-regulated kinase (ERK)1/2 and c-Jun kinase (JNK)] and

phosphatidyl-inositol-3-kinase (PI3K/Akt).<sup>2</sup> However, little information is available about protective effects of HDL on cardiomyocyte function.

Action potential propagation in the hearts depends on electrical coupling of cardiomyocytes by gap junctions (GJ).<sup>3</sup> Moreover, GJ permit intercellular spread of biologically active compounds that include substances deciding over cell survival vs. cell death in various types of cells.<sup>4</sup> GJ channels are formed by members of a family of transmembrane proteins called connexins (Cx). The predominant connexin between ventricular cardiomyocytes is Connexin43 (Cx43). It is typically located at the intercalated disk in the intact ventricular myocardium, but can move to the lateral plasma membranes in response to ischaemia. These changes are accompanied by changes

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in Cx43 phosphorylation. Phosphorylation of Cx43 by different protein kinases, such as PKC or MAPK, has been implicated in connexin expression, trafficking, assembly, degradation and GJ channel gating.<sup>5</sup>

There is increasing evidence that regulation of Cx43 plays an important role in cardioprotection. This is evident from studies aimed at understanding the phenomenon of ischemic preconditioning (IPC), where brief periods of ischaemia and reperfusion in intact hearts can minimize damage from a subsequent prolonged period of ischaemia. The beneficial effects of IPC include a reduction of infarct size and a suppression of ischaemia-associated arrhythmias.<sup>6</sup> Cx43 is known to be essential for the beneficial effects of IPC as shown by the absence of preconditioning effects in heterozygous Cx43 null mice.<sup>7</sup> Whether Cx43 may also be involved in cardioprotection conferred by compounds applied during early reperfusion however remains largely unknown.

In the present study, we investigated whether the protective effects of HDL involve cardiac GJ. We show for the first time that HDL induces the phosphorylation of Cx43 in neonatal rat cardiomyocytes by a PKC-dependent mechanism. HDL induced a large reduction (~50%) in the spread of a large tracer, whereas it had minimal effects (<5%) on impulse propagation. Moreover, 5 min of HDL treatment at the onset of reperfusion limited infarct size induced by an ischaemia/reperfusion protocol *ex vivo*. These effects involved the Sphingosine-1-phosphate (S1P) component of HDL. Our findings suggest that HDL might exert direct protective effects on cardiomyocyte function by inducing PKC-mediated phosphorylation of Cx43, which, in turn, result in limitation of the diffusion of damaging molecules through GJ channels while cardiac conduction is preserved.

## 2. Methods

### 2.1 HDL preparation

High density lipoprotein (HDL) ( $d = 1.063\text{--}1.21$  g/mL) was isolated by cumulative flotation ultracentrifugation from a plasma pool provided by healthy volunteers as describe previously.<sup>8</sup> HDL was subsequently dialyzed against PBS containing EDTA (1 mmol/L) and stored at 4°C. Experiments conformed to Geneva University ethics review board (number 07–037), and are in accordance with the Declaration of Helsinki.

### 2.2 Cell culture

Isolation of neonatal ventricular cardiomyocytes was performed on 1 to 2-day-old Wistar rats euthanized by decapitation. Cells were isolated by digestion with trypsin-EDTA and collagenase. Cultures were used at the third day, after 20–24 h serum starvation.<sup>9</sup> Briefly, after digestion, the cells were pooled in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Groningen, the Netherlands) supplemented with 10% fetal calf serum (FCS, Biochrom AG, Berlin, Germany), penicillin (100 U/mL) and streptomycin (10 µg/mL) and seeded in 150 cm<sup>2</sup> flasks to allow selective adhesion of cardiac fibroblasts. After 1 h, cardiomyocytes were decanted from the plates and seeded in petri dishes. For all experiments described herein, cells were used on the third day of culture after having undergone serum starvation for 20–24 h in DMEM without FCS. Experiments conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1966) and were approved by the cantonal veterinary authorities (number 1012/3134/0-R).

Neonatal rat ventricular myocytes were treated with HDL at the concentration of 400 µg/mL as described in previous reports from our laboratory<sup>10</sup> and incubation times varied from 5 to 60 min. Pre-treatments

with kinase inhibitors were performed 30 min before HDL incubation by using inhibitors of PKC (Chelerythrin 5 µmol/L (Chel.), Sigma-Aldrich; Staurosporine 1 µmol/L (STS); Bisindolylemaleinide I 2 µmol/L (BIMI), Calbiochem), p38-MAPK (SB203580 10 µmol/L, Calbiochem), JNK (SP600125 10 µmol/L, Calbiochem), ERK (UO126 5 µmol/L, Calbiochem), or Akt (LY294002 10 µmol/L, Calbiochem). Neonatal cardiomyocytes were also treated with S1P (Sigma-Aldrich) at the concentration of 0.1 µmol/L during 5 min.

### 2.3 Protein extraction and western blotting

Proteins were extracted from neonatal rat ventricular myocytes exposed to the various experimental protocols in modified RIPA buffer (Tris–HCl pH 8.0 20 mmol/L, NaCl 1 mol/L, NP-40 1%, NaF 50 mmol/L, Na-orthovanadate 10 mmol/L, PMSF 1 mmol/L, EDTA 5 mmol/L, SDS 0.25%, Na-deoxycholate 1%, cocktail of protease inhibitors) as previously described.<sup>11</sup> Western blotting was performed using antibodies against all Cx43 species irrespectively of the phosphorylation sites (BD Transduction Laboratories), Phospho-Cx43 (Ser368) (Cell Signaling), the N-terminal part of Cx43 (Fred Hutchinson Cancer Research Center), Phospho-p38-MAPK (Cell Signaling), Phospho-ERK1/2 (Cell Signaling), Phospho-Akt (Cell Signaling), Phospho c-jun (Santa Cruz Biotechnology), and GAPDH (Chemicon).

### 2.4 Immunofluorescence

Neonatal rat ventricular myocytes were cultured on coverslips for 3 days, treated for 5 min with HDL (400 µg/mL) and then fixed for 5 min in methanol at –20°C. Cells were immunolabelled with phospho-Cx43 (Ser368) antibodies or N-term Cx43 antibodies. Cells were counterstained with Evans blue and nuclei were stained with 4',6-diamidino-2-phenylindole. Staining was examined with a TMD300 microscope (Nikon AG, Küsnacht, Switzerland) equipped with a high-sensitivity Visicam camera (Visitron Systems GmbH, Puchheim, Germany) connected to a personal computer running Metafluor 4.01 software (Universal Imaging, Sunnyvale, CA, USA).

### 2.5 Dye coupling

For the analysis of dye transfer, one neonatal rat ventricular myocyte within a cluster of cardiomyocytes was impaled with a thin tip microelectrode filled with 4% Lucifer yellow dissolved in 150 mmol/L LiCl buffered to pH 7.2. The tracer was allowed to fill the cells by simple diffusion for 3 min. At the end of the injection period, the electrode was carefully removed and all Lucifer Yellow-positive cells, irrespectively of their absolute level of specific fluorescence, were counted immediately using an inverted TMD-300 microscope (Nikon, Egg, Switzerland) equipped with a x40 phase 3 dark medium objective with a numerical aperture of 0.7 (Carl Zeiss) and appropriate filters.<sup>12–14</sup> Neonatal cardiac cells were treated with HDL (400 µg/mL) or S1P (0.1 µmol/L) for 5 min before dye injection. Chelerythrin pre-treatment (5 µmol/L) was performed 30 min before HDL incubation. To estimate the reversibility of HDL or S1P treatments, cells were rinsed during 10 min with PBS before dye injection. Microinjections have been also performed in the presence of 12-O-tetradecanoylphorbol-13-acetate (TPA, 100 nM, 15 min), a PKC activator.

### 2.6 Optical recording of electrical activity

Patterned growth neonatal rat ventricular myocytes preparations (strands measuring 4.2 × 0.6 mm) were obtained using photolithographic techniques as described previously.<sup>15</sup> Strands were kept in culture for 3 days (serum starved for the last 24 h) before being stained for 5 min with the voltage sensitive dye di-8-ANEPPS (135 µmol/L; Biotium). For optical measurements, preparations were mounted in a temperature controlled superfusion chamber on top of an inverted microscope equipped for epifluorescence (Zeiss 135 M) where they were superfused

continuously at 36°C with Hanks' balanced salt solution (HBSS, Sigma) containing (mmol/L) NaCl 137, KCl 5.4, CaCl<sub>2</sub> 1.3, MgSO<sub>4</sub> 0.8, NaHCO<sub>3</sub> 4.2, KH<sub>2</sub>PO<sub>4</sub> 0.5, NaH<sub>2</sub>PO<sub>4</sub> 0.3, and HEPES 10. The fluorescence emitted from the voltage sensitive dye was recorded with high spatio-temporal resolution using a fibre optic recording system described in detail before.<sup>16</sup> All recordings were done at 20× magnification (Fluar 20×, 0.75 N.A., Zeiss, Switzerland) resulting in a spatial resolution of 50 μm. Cardiomyocyte strands were pre-stimulated for 10 s at 2 Hz with an electrode placed next to the individual strands (~1 mm) to obtain steady-state conduction conditions at the time of measurement. The amplitudes of optically recorded action potential upstrokes were scaled to 100% (%APA) and maximal upstroke velocities (dV/dt<sub>max</sub>) were calculated from %APA values and are given in %APA/ms. Under the assumption that action potential amplitudes correspond to 100 mV, %APA/ms translates into V/s. Experiments were performed in triplicate at a given site [control, exposure to HDL (400 μg/mL) or 0.1 μmol/L S1P, washout].

## 2.7 Ex vivo Langendorff perfusion

Animal experimentation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1966) and was approved by the cantonal veterinary authorities (number 1045/3272/0-R). Sprague-Dawley rats were anaesthetized by one intraperitoneal injection of thiopental-natrium (150 mg/kg), and the adequacy of anaesthesia was confirmed with the absence of pedal reflex. The hearts ( $n = 5–6$ /group) were isolated and perfused in a modified Langendorff system under constant pressure (70 mmHg) with gassed (94% O<sub>2</sub>–6% CO<sub>2</sub>) Krebs–Heinseleit buffer solution (NaCl 118 mM, KCl 4.7 mM, MgSO<sub>4</sub> 1.19 mM, KH<sub>2</sub>PO<sub>4</sub> 1.2 mM, CaCl<sub>2</sub> 1.36 mM, NaHCO<sub>3</sub> 25 mM, Glucose 11 mM) at 37°C, as described previously.<sup>17</sup> An intraventricular balloon was introduced in the left ventricle and the left ventricular end-diastole pressure (LVEDP) was fixed to 6 mmHg. The hearts were stabilized for 30 min and subsequently exposed to 30 min of no-flow global ischaemia followed by 60 min of reperfusion. HDL (800 μg/mL) or S1P (0.4 μmol/L) has been added to the perfusion during the first 5 min of reperfusion. The left ventricular developed pressure (LVDP) and the heart rate (HR) were continuously monitored during the experiments. The rate-pressure product was calculated as LVDP×HR. At the end of the reperfusion, the heart was immediately frozen. Frozen hearts were cut into six to seven slices and incubated with triphenyltetrazolium chloride (TTC) to stain viable myocardium. The contrast between TTC-stained and unstained areas was enhanced by immersion in 10% formalin for 1 day. Total myocardial and infarcted areas were measured from computed images using NIH Image software. Infarct areas were normalized to total left ventricular areas and averaged for individual hearts.

## 2.8 Statistical analysis

The data are presented as mean ± SEM. Independent experiments were compared by Student's *t*-test. Differences indicated by an asterisk or a number sign were considered statistically significant at  $P < 0.05$ .

## 3. Results

### 3.1 Effects of HDL and S1P on Cx43 expression and phosphorylation

The effects of HDL treatment on Cx43 expression and phosphorylation were determined by western blotting with three different antibodies. One antibody recognizing all Cx43 isoforms (Figure 1A—upper panel and Figure 1B), a second antibody directed against the phosphorylated Serine 368 (Ser368) of Cx43 (Figure 1A—lower panel and Figure 2A) and a third antibody recognizing the N-terminal part of Cx43 allowing

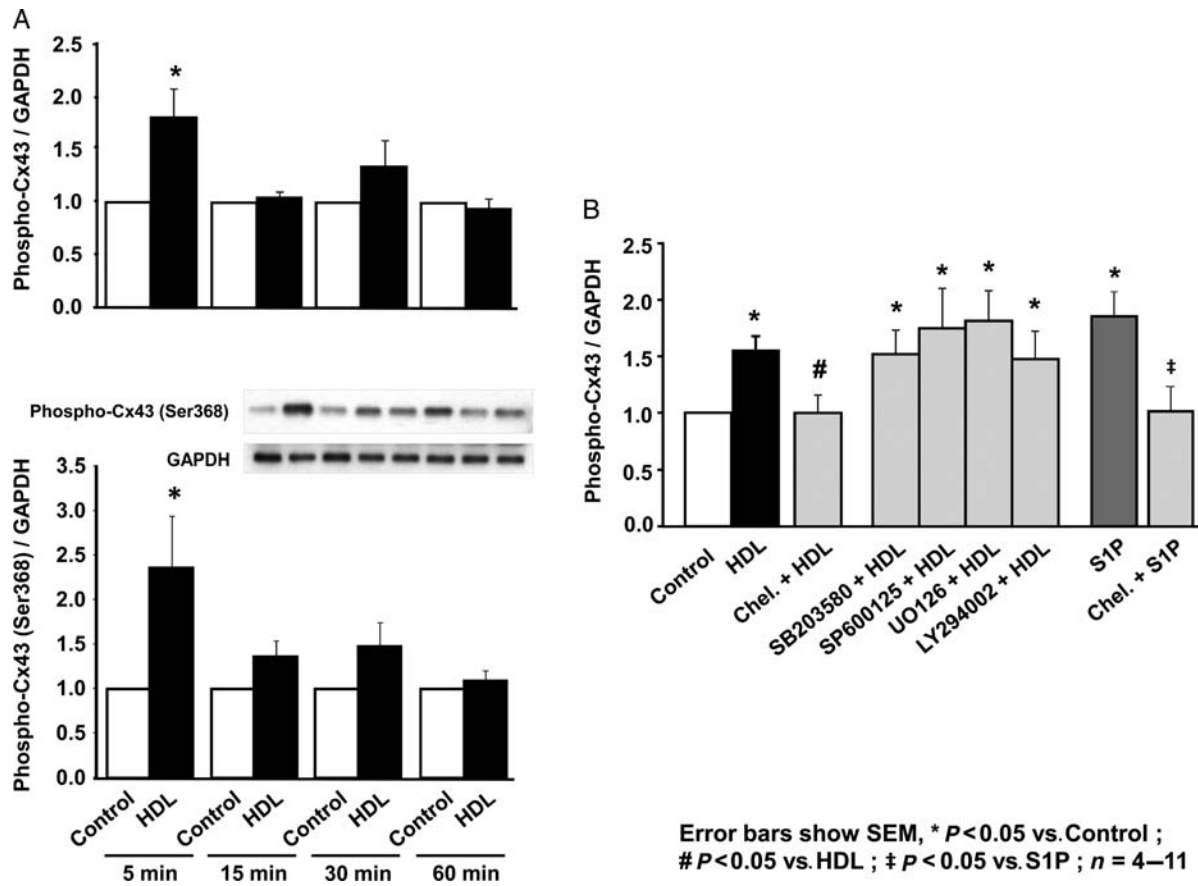
for reliable quantification of the total amount of Cx43 (Figure 2B). We found that treatment with 400 μg/mL HDL induced an increase in Cx43 phosphorylation with the maximal effect after 5 min of incubation (Figure 1A). Pre-treatment of the cells with inhibitors against p38-MAPK (SB203580), JNK (SP600125), ERK1/2 (UO126), or PI3K/Akt (LY294002) pathways did not affect the HDL effect on Cx43 phosphorylation (Figure 1B). The efficiency of these inhibitors was verified by western blotting with antibodies against phospho-p38-MAPK, phospho-c-jun, phospho-ERK1/2, and phospho-Akt. As shown in Supplementary material online, Figure S1, the phosphorylation of these targets by HDL was reversed by pre-treatment with the different inhibitors. Pre-incubation of neonatal rat ventricular cells for 30 min with different PKC inhibitors (chelerythrin: Chel., Staurosporine: STS or Bisindoylmaleinide I: BIM1) blocked HDL-induced phosphorylation of Cx43 (Figures 1B and 2A). Treatment with HDL and PKC inhibitors did however not modify the total amount of Cx43 (Figure 2B). In addition, treatment of neonatal cardiomyocytes with 0.1 μmol/L S1P induced comparable level of Cx43 phosphorylation as HDL treatment (Figures 1B and 2A), without affecting the total amount of Cx43 (Figure 2B). Pre-treatment with the different PKC inhibitors also blocked the effect of S1P on Cx43 phosphorylation (Figures 1B and 2A).

Immunostaining with the different antibodies showed that the Cx43 isoforms are primarily localized at the plasma membrane between cardiomyocytes (Figure 3A). Moreover, we observed increased staining with the Ser368 antibody after HDL treatment, which confirmed the results obtained by western blotting.

### 3.2 Effects of HDL on gap junctional communication

Microinjection of the fluorescent tracer LY revealed that the dye diffused to  $16 \pm 2$  cardiomyocytes (control,  $n = 38$ , Figure 3C) within 3 min. HDL treatment induced a significant decrease in LY diffusion ( $6 \pm 1$  cells,  $n = 42$ ,  $P < 0.01$ ) and pre-treatment with chelerythrin blocked the HDL effects on LY diffusion ( $14 \pm 2$  cells,  $n = 11$ ,  $P < 0.01$  vs. HDL, Figure 3C). Similarly, S1P treatment also reduced spread of the fluorescent tracer ( $9 \pm 1$  cells,  $n = 29$ ,  $P < 0.01$  vs. control, Figure 3C). Moreover, washout of HDL and S1P restored LY diffusion to values close to control ( $16 \pm 3$  cells,  $n = 11$  and  $12 \pm 3$  cells,  $n = 9$ , respectively, Figure 3C). In agreement, incubation of neonatal cardiomyocytes with the PKC activator TPA significantly reduced dye coupling between the cells ( $3 \pm 1$  cells,  $n = 16$ ,  $P < 0.01$  vs. control, Figure 3C).

In contrast to the substantial reduction in LY diffusion, HDL treatment caused an only small and reversible reduction in conduction velocities (Figure 4A, left panel; control:  $327 \pm 4$  mm/s,  $n = 65$ ; HDL treatment:  $310 \pm 3$  mm/s,  $n = 65$ ,  $P < 0.05$ ; washout:  $325 \pm 3$  mm/s,  $n = 46$ ). In parallel, HDL treatment caused a reversible small reduction in maximal upstroke velocities (Figure 4A, right panel; control:  $79.8 \pm 0.2$  %APA/ms; HDL treatment:  $78.0 \pm 0.2$  %APA/ms,  $n = 65$ ,  $P < 0.05$ ; washout:  $80.0 \pm 0.2$  %APA/ms,  $n = 65$ ). As shown in Figure 4B, the effects on conduction velocity were maximal after 5 min of HDL incubation. Moreover, S1P treatment induced also small but significant reductions in conduction velocities (control:  $326 \pm 3$  mm/s; S1P:  $314 \pm 3$  mm/s,  $P < 0.05$ ,  $n = 57$ , Figure 4C, left panel) and maximal action potential upstroke velocities (control:  $80.7 \pm 0.2$  %APA/ms; S1P:  $79.8 \pm 0.2$  %APA/ms,  $P < 0.05$ ,  $n = 57$ , Figure 4C, right panel) similar to that observed during HDL exposure.



**Figure 1** Cx43 phosphorylation in neonatal rat cardiomyocytes after treatment with HDL or S1P. (A) Quantification of Cx43 by western blot showed that HDL (400  $\mu\text{g}/\text{mL}$ ) induced a global transient increase in Cx43 phosphorylation (upper panel), and more particularly phosphorylation of Serine368 (lower panel). Figure shows representative western blots for Cx43 in the absence of HDL or after 5, 15, 30, and 60 min of HDL incubation. Data of six independent experiments are expressed in ratio of phosphorylation status of Cx43 in control conditions. (B) Quantification of phospho-Cx43 after incubation with kinase inhibitors for 30 min and HDL or S1P for 5 min. Inhibition of PKC with Chelerythrin (Chel.) blocked the phosphorylation of Cx43 induced by HDL, whereas the inhibition of p38-MAPK, JNK, ERK1/2, or Akt, respectively by SB203580, SP600125, UO126, or LY294002 did not affect HDL-induced Cx43 phosphorylation. Moreover, treatment with S1P significantly increased Cx43 phosphorylation, an effect blocked by pre-incubation with Chelerythrin. Data of 4–11 independent experiments were normalized to the phosphorylation state of Cx43 measured under control conditions.

### 3.3 Effect of HDL on ischaemia/reperfusion-induced cell death

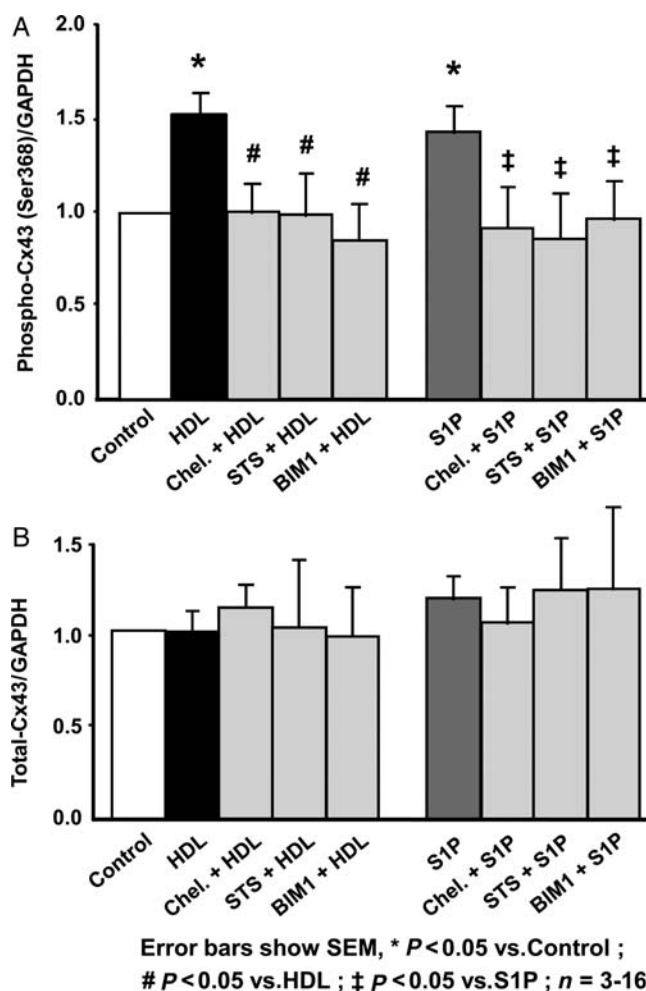
The therapeutic potential of short-term (5 min) treatment of HDL or S1P was determined in isolated heart perfusion experiments *ex vivo* in which hearts were submitted to 30 min of no-flow global ischaemia followed by 60 min of reperfusion (Figure 5A). During the stabilization period, the LVDP was set  $\sim 6$  mmHg for each heart [control:  $6 \pm 1$  ( $n = 5$ ); HDL:  $6 \pm 1$  ( $n = 6$ ); S1P:  $7 \pm 1$  mmHg ( $n = 6$ )]. The LVDP and the HR were monitored and the rate-pressure product (LVDP  $\times$  HR) was calculated. At the end of the stabilization period, LVDP  $\times$  HR was similar between the three experimental groups (control:  $28 \pm 2$ ; HDL:  $29 \pm 1$ ; S1P:  $27 \pm 2$  mmHg  $\times 10^3/\text{min}$ , ns). HDL or S1P was added to the perfusion buffer only during the first 5 min of the reperfusion (Figure 5A). Under control conditions, we observed cell death in  $23 \pm 3\%$  of the total ventricle (Figure 5B). This infarct size was significantly reduced by 5 min of HDL or S1P treatment at the onset of reperfusion. Indeed, cell death was only

observed in  $13 \pm 2\%$  of HDL treated and in  $11 \pm 2\%$  of S1P treated ventricle, ( $P < 0.05$  vs. control, Figure 5B).

## 4. Discussion

Connexins are highly regulated membrane proteins with a C-terminal domain that plays key regulatory roles and provides sites of protein–protein interactions.<sup>18</sup> Cx43 is a phosphoprotein, and its phosphorylation state influences gap junctional intercellular communication (GJIC).<sup>5</sup> In this study, we show that HDL treatment induced an increase in Cx43 phosphorylation. This phosphorylation is maximal after 5 min of incubation with HDL and involves the Serine residue at position 368 in the C-terminal tail of Cx43. Pre-incubation of neonatal cardiomyocytes with inhibitors of MAP kinases or Akt pathways had no effect on HDL-induced phosphorylation of Cx43, whereas three different PKC inhibitors blocked this effect. Although the constituents of the HDL particle that mediate the diverse biological



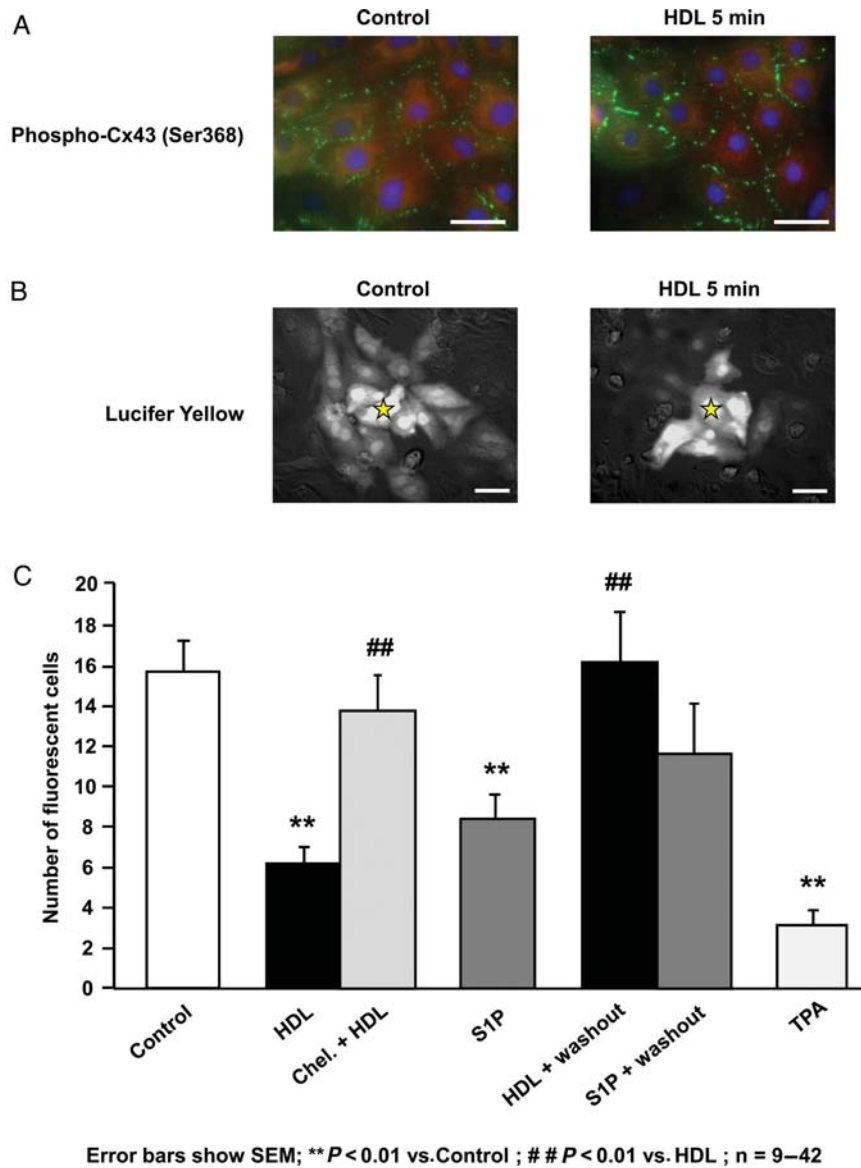


**Figure 2** PKC-dependent effects of HDL and S1P on Cx43 phosphorylation. (A) Quantification of phospho-Cx43 on Serine368 in cardiomyocytes treated with HDL, S1P, Chelerythrin, Staurosporine, or Bisindoylmaleinide I. HDL and S1P treatments induced phosphorylation of Cx43 on Serine368. Moreover, PKC inhibition by Chelerythrin, Staurosporine, or Bisindoylmaleinide I blocked HDL and S1P induced phosphorylation of Cx43. Data of 3–16 independent experiments were normalized to the phosphorylation state of Cx43 measured under control conditions. (B) Quantification of total Cx43 showed that the different treatments did not affect the total amount of Cx43. Data of 3–16 independent experiments were normalized to the amount of Cx43 present under control conditions.

effects remain to be identified, several recent studies have linked the S1P component to the cardioprotective properties of HDL.<sup>19</sup> We observe here that S1P induced comparable levels of Cx43 phosphorylation as HDL treatment in neonatal rat cardiomyocytes. Moreover, S1P-induced phosphorylation of Cx43 was also blocked by the inhibition of PKC, suggesting a common pathway for HDL and S1P. These results demonstrate for the first time that HDL and its component S1P induce phosphorylation of Cx43 by a PKC-dependent mechanism.

Cx43 phosphorylation regulates many aspects of the connexin life cycle, including expression, trafficking, assembly, gating, and degradation.<sup>5</sup> Moreover, the effects of Cx43 phosphorylation on GJIC are strongly dependent on the phospho-amino acid involved. Thus, phosphorylation of Ser368 in Cx43 by PKC has been shown to inhibit GJIC.<sup>20</sup> In this study, we show that HDL limits the spread of large tracer molecules between neonatal rat cardiomyocytes by ~50%. The effects of HDL on GJIC might be due to an action of HDL particles on the cholesterol content of cardiomyocyte plasma

membranes. Indeed, changing the cholesterol content of plasma membranes is known to affect GJIC in neonatal cardiomyocytes.<sup>21</sup> However, this contribution will be minor compared with the effect of HDL on Cx43 phosphorylation, because S1P that is incapable of transporting cholesterol induces a similar effect as HDL on GJIC. Moreover, HDL- and S1P-induced reduction in GJIC can be prevented by inhibition of PKC, and the effects are reversible after the washout of the two compounds. Thus, HDL and S1P reversibly reduce GJ dye permeability between neonatal rat cardiomyocytes in a PKC-dependent manner. As published previously, a reduction in GJ permeability limits the spread of mediators implicated in death pathway during ischaemia and reperfusion.<sup>22</sup> Consequently, limitation of GJIC by HDL may limit the passage of death signals between cardiomyocytes, thus conferring cardioprotection in limiting infarct size. S1P enhanced survival of neonatal cardiomyocytes under hypoxic conditions, and that these anti-apoptotic properties of S1P are mediated by a PKC-dependent mechanism.<sup>23</sup> All together, these studies and our results suggest that limitation of Cx43-mediated



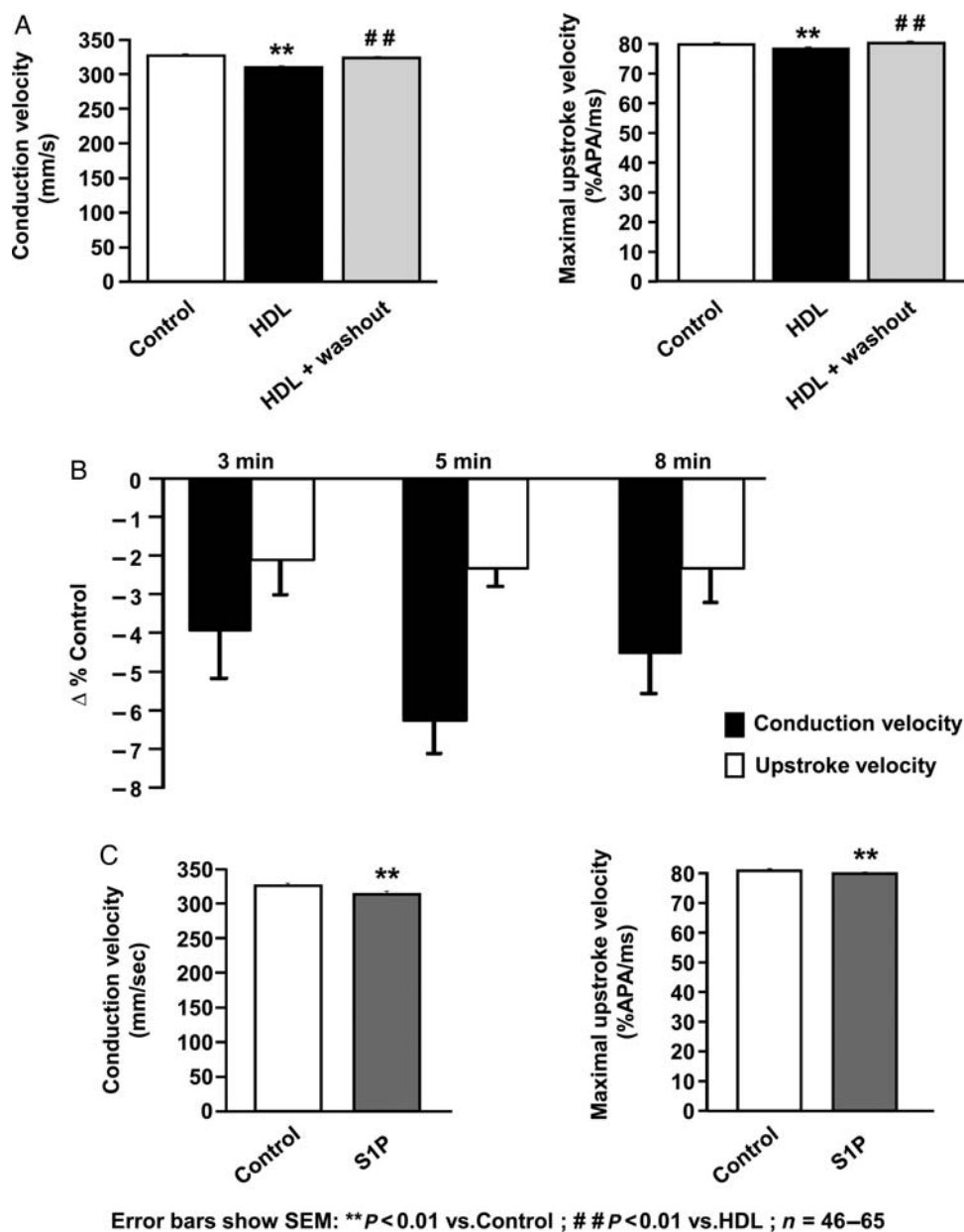
**Figure 3** Cell-to-cell coupling in neonatal rat cardiomyocytes after treatment with HDL or S1P. (A) Immunofluorescent staining of phospho-Cx43 (Ser368) in neonatal rat ventricular cells under control condition or after 5 min HDL incubation. HDL induced an increase in the signal for phosphorylated Cx43 at the plasma membrane. Scale bar represent 50  $\mu\text{m}$ . (B) GJIC was measured by microinjection of LY for 3 min. Images are representative examples of dye diffusion in control cardiomyocyte clusters and after 5 min of HDL incubation. Asterisks indicate the microinjected cells. Scale bar represent 50  $\mu\text{m}$ . (C) LY diffusion is significantly decreased by HDL treatment; an effect that was blocked by PKC inhibition. S1P decreased LY diffusion similar to HDL. Washouts led to total (HDL) or partial (S1P) restoration of dye diffusion. Activation of PKC by TPA induced a significant reduction in dye coupling between cardiomyocytes. Results are presented as mean of 9–42 microinjections for each condition.

GJIC might explain, at least partly, the earlier described anti-death properties of HDL and S1P.

Given its effects on GJ dye permeability, the question arises whether HDL also affects action potential propagation. Indeed, gap junctional conductance ( $G_j$ ) in cardiomyocytes has been shown to be affected by treatment with TPA, a PKC activator.<sup>12</sup> We have therefore evaluated impulse conduction velocities and maximal action potential upstroke velocities in strands of neonatal rat ventricular cardiomyocytes in response to HDL. We show that treatment with HDL or S1P exerts very small though statistically significant effects on impulse conduction velocity (decrease by  $\sim 5\%$ ) and maximal upstroke velocities ( $\sim 2.5\%$ ). At first glance, the combination of

reduced conduction velocities and reduced maximal upstroke velocities suggests that HDL slightly depolarized the preparations and, accordingly, caused a reduction in sodium channel availability. We have, however, observed similar parallel reductions in both conduction velocities and upstroke velocities during progressive gap junctional uncoupling of cardiomyocytes strands with palmitoleic acid before<sup>24</sup> even though these findings contradict theory.<sup>25</sup> Future studies will have to show which of these mechanism(s) underlies the effects of HDL and S1P on impulse conduction and action potential configuration.

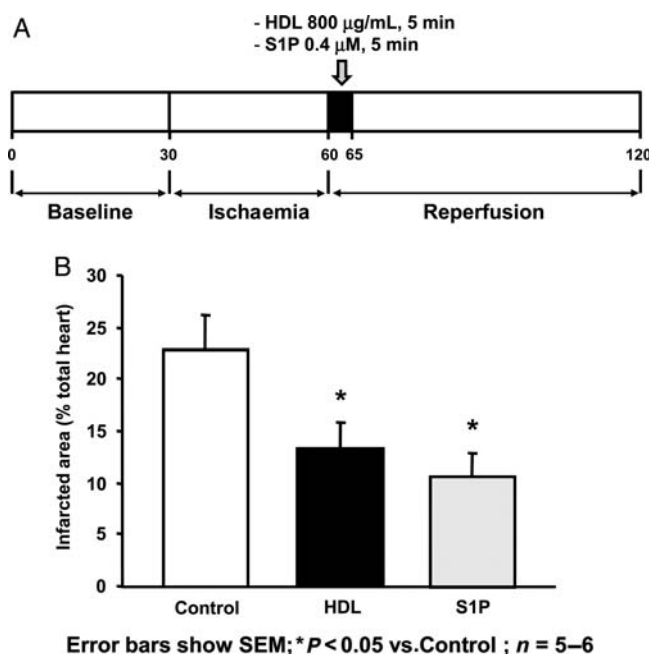
In our *in vitro* studies, the observed effects of HDL and S1P on Cx43 phosphorylation were maximal after 5 min of incubation. HDL and



**Figure 4** Conduction and maximal upstroke velocities in neonatal rat cardiomyocytes after treatment with HDL or S1P. (A) Measurements of conduction velocities (left panel) and maximal action potential upstroke velocities (right panel) in strands of neonatal rat cardiomyocytes in the absence (control) or presence of HDL. Three to eight minutes of HDL incubation led to a slight but significant reduction of both parameters that were completely restored upon washout. Results are presented as mean of 46–65 measurements for each condition. (B) Time-dependent changes in conduction velocities and maximal action potential upstroke velocities during HDL treatment when compared with controls (normalized values). HDL induces a maximal decrease of 6.3% in conduction velocity and 2.3% of maximal upstroke velocity at 5 min of incubation. Results are presented as mean of seven measurements for each condition. (C) Measurements of conduction velocities (left panel) and maximal potential action upstroke velocities (right panel) in strands of neonatal rat cardiomyocytes in the absence (control) or presence of S1P. As for HDL treatment, the incubation with S1P reduces slightly but significantly both parameters. Results are presented as mean of 57 measurements for each condition.

S1P have been studied for cardioprotective properties against ischaemia and reperfusion in *ex vivo* settings, but the precise mechanism of action remains unknown. Depending of the experimental conditions, these compounds have been administrated at variable time points before ischaemia or at the onset of reperfusion. Our *ex vivo* experiments with isolated hearts show that 5 min of treatment with HDL or S1P at the onset of reperfusion are able to reduce the cell death

induced by the 30 min ischaemia and reperfusion by ~50%. In studies performed with the *ex vivo* model of Langendorff perfusion, HDL<sup>26</sup> or synthetic HDL (sHDL)<sup>27</sup> was infused 10 min before ischaemia or during the first 10 min of reperfusion, and induced in both cases a significant improvement of myocardial function at reperfusion. When sHDL was given at the time of reperfusion, myocardial function was already significantly improved after 1 min of reperfusion,



**Figure 5** Infarcted area of isolated hearts submitted to ischaemia/reperfusion protocol and treated with HDL or S1P. (A) Schematic of the study protocol. Isolated hearts were stabilized for 30 min (baseline), followed by 30 min of no-flow global ischaemia and 60 min of reperfusion. HDL (800 µg/mL) or S1P (0.4 µg/mL) was added during the first 5 min of reperfusion. (B) Measurement of infarct size after ischaemia/reperfusion in the absence (control) or in presence of HDL or S1P. Addition of HDL or S1P during the first 5 min of reperfusion significantly reduces cell death induced by ischaemia and reperfusion. Results are presented as mean of 5–6 hearts for each condition.

illustrating the rapid action of sHDL.<sup>27</sup> Moreover, *in vivo* anti-arrhythmic effect of sHDL against ischaemia/reperfusion have been demonstrated in rats where sHDL was given 10 min before the onset of ischaemia.<sup>28</sup> Finally, 2 min of S1P perfusion followed by 5 min of washout before ischaemia has been shown to significantly improve of myocardial recovery at reperfusion and resulted in a significant decrease in the infarct size.<sup>29</sup> Altogether, our study shows that short-term treatment with HDL or S1P at the onset of reperfusion is sufficient to confer significant cardioprotection against ischaemia/reperfusion-induced cell death. In addition, our results link, for the first time, Cx43 gap junction channels to the rapid effect of HDL and S1P treatments in cardioprotection.

In conclusion, we show here that HDL and its constituent S1P induce phosphorylation of Cx43 by a PKC-dependent mechanism. This phosphorylation is associated with a 50% inhibition in the transfer of large molecules between neonatal rat cardiomyocytes. In contrast, impulse propagation remains largely conserved. It therefore appears that HDL, by increasing PKC activity, is ideally suited to counteract spreading depression under pathological conditions: its actions on cardiac GJs composed of Cx43 might limit spread of damaging factors, while impulse conduction remains preserved.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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