Low-density lipoprotein receptor-related protein 1 mediates hypoxia-induced very low density lipoprotein-cholesteryl ester uptake and accumulation in cardiomyocytes

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Aims
The myocardium accumulates intracellular lipids under ischaemic conditions, and myocardial fat deposition is closely associated with cardiac dysfunction. Our aims were to analyse the effect of hypoxia on low-density lipoprotein receptor-related protein 1 (LRP1) expression in neonatal rat ventricular myocytes (NRVM) and cardiac-derived HL-1 cells and the molecular mechanisms involved in this effect, to determine the role of LRP1 in the very low density lipoprotein (VLDL) uptake by hypoxic cardiomyocytes, and to study the effect of hypoxia on lipoprotein receptor expression and myocardial lipid profile in an in vivo porcine experimental model of acute myocardial infarction.

Methods and results
Thin-layer chromatography after lipid extraction showed that VLDL exposure leads to cholesteryl ester (CE) and triglyceride (TG) accumulation in a dose-dependent manner and that hypoxic conditions further increased VLDL-derived intracellular lipid accumulation in HL-1 cells. Knockdown of LRP1 through lentiviral-mediated interfering RNA specifically prevented hypoxia-induced VLDL-CE internalization in HL-1 cells and NRVM. Lipopolysaccharide (LPS)-induced LRP1 overexpression specifically increased VLDL-CE accumulation in NRVM. In addition, using double-radiolabelled [3H]CE-[14C]TG-VLDL, we found that LRP1 deficiency specifically prevented hypoxia-induced VLDL-[3H]CE uptake. Finally, in an in vivo porcine model of infarcted myocardium, ischaemic areas exhibited LRP1 protein up-regulation and intramyocardial CE overaccumulation.

Conclusion
Our results demonstrate that hypoxia increases LRP1 expression through HIF-1α and that LRP1 overexpression mediates hypoxia-induced VLDL-CE uptake and accumulation in cardiomyocytes.

Keywords
LRP1 • Cardiomyocytes • Cholesteryl esters • Hypoxia • HIF-1α

1. Introduction
Cardiomyocytes accumulate intracellular lipids under pathological conditions such as diabetes,1 obesity,2 and ischaemia.3,4 Accumulation of fat in the heart has been associated with cardiac dysfunction and heart failure.5 Remarkably, a high prevalence of fat deposition has been found in areas of chronic myocardial infarction (MI) in humans.6 The patients with fat deposition had larger infarctions, decreased wall thickening, and impaired endocardial wall motion. In agreement, dyslipaemia has been shown to exacerbate the injury in the ischaemic myocardium in different animal experimental models.7 Most of the energy requirements of a normal heart are supplied by fatty acid (FA) oxidation, a process closely coupled to FA uptake since cardiomyocytes have a limited capacity for triglyceride (TG) storage.8 In contrast to a normal heart, the ischaemic heart largely uses carbohydrate as an energetic source to optimize energy production.9 As a consequence, FAs that enter into the ischaemic cardiomyocytes are not used for energy production but are instead converted to TGs that accumulate intracellularly.
FAs are delivered to the heart by two sources, circulating non-esterified FAs bound to plasma albumin and circulating esterified FAs in the form of TG transported in TG-rich lipoproteins, such as very low density lipoproteins (VLDL) and chylomicrons (CM). VLDL and CM-derived TG may be taken up by the heart through at least two pathways: lipoprotein lipase (LpL)-mediated lipolysis and lipoprotein receptor-mediated endocytosis. In addition to acting as a TG source, VLDL is also a major source of cholesterol for the heart, an organ with a minimum capacity for cholesterol synthesis and LDL. However, it is still not clear whether cholesterol accumulates in the ischaemic heart and whether the lipoprotein receptors are involved in cholesterol uptake by the myocardium.

Myocardial cholesterol accumulation from TG-rich lipoproteins contributes to lipotoxic cardiomyopathy and causes systolic and diastolic dysfunction. Moreover, an inverse correlation has been shown between myocardial cholesterol levels and sarco(endo)plasmic reticulum calcium ATPase-2 (SERCA-2) expression. We have previously identified low-density lipoprotein receptor-related protein 1 (LRP1) as a key receptor for selective cholesterol uptake by human vascular smooth muscle cells (VSMC). Interestingly, LRP1 is up-regulated by hypercholesterolaemia through sterol regulatory element-binding protein-2 (SREBP-2) down-regulation and by hypoxia through HIF-1α. The aims of this work were to analyse the effect of hypoxia on LRP1 expression in neonatal rat ventricular myocytes (NRVM) and HL-1 cells and the molecular mechanisms involved in this effect, to determine the role of LRP1 in VLDL uptake by hypoxic cardiomyocytes, and to study the effect of hypoxia on lipoprotein receptor expression and myocardial lipid profile in an in vivo porcine experimental model of acute myocardial infarction (AMI).

2. Methods

An expanded Methods section is available in the Supplementary material online.

2.1 HL-1 cardiomyocyte cell culture

The murine HL-1 cell line was generated by Dr W.C. Claycomb (Louisiana State University Medical Centre, New Orleans, LA, USA) and kindly provided by Dr U. Rauch (Charité-Universitätsmedizin Berlin). These cells show cardiac characteristics similar to those of adult cardiomyocytes.

2.2 NRVM isolation and culture

The study protocols were approved by the animal research committee of our institution (ICCC020/DMAH4711) and they were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. NRVM cultures were prepared from heart ventricles of 3–4-day-old Wistar rats as previously described. Neonates were sacrificed by decapitation and both atria were removed. Cell suspensions were excised and both atria were removed. Cell suspensions were prepared using a neonatal cardiomyocyte isolation system (Worthington). Following sacrifice, hearts from the necrotic border in the ischaemic zone vs. that from the left ventricle free-wall, non-ischaemic/remote myocardium, were used for molecular analysis.

2.3 Generation of LRP1-deficient cardiomyocytes

2.3.1 Design of LRP1 miRNA lentiviral vectors

Three different sequences of miR RNAi (XM_1919_8223; and _8531) were designed to down-regulate LRP1 (accession number: XM_001056970) and cloned into pLenti6.4-CMV-MSGW (Invitrogen). A universal insert-negative control (Invitrogen, pcDNA™6.2-GW/miR-negative control) was also used (Supplementary material online, Figure S1A).

2.3.2 Lentiviral particle production

Lentiviral particle production was performed as previously described with some modifications.

2.3.3 LRP1-deficient cardiomyocytes generation

Lentivirus stocks and a negative control generated from the resulting constructs were titred by blasticidin selection.

2.4 In vivo experimental model of myocardial ischaemia

The pattern of myocardial lipoprotein receptor expression and myocardial neutral lipid accumulation was analysed in the myocardium of a group of male pigs (crossbred commercial pigs: Landrace–Large White). These animals are part of the experimental model used to analyse the molecular and cellular mechanisms involved in cardiac remodelling after AMI. A sham-operated group of animals was used as control. The study protocol was approved by an institutional animal research committee and 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 1996). Twelve hours prior to the experimental induction of acute MI (AMI), a loading dose of clopidogrel (150 mg/kg) was administered to all animals. Haemodynamic parameters, ECG, and body temperature were monitored throughout the experimental procedure to ensure a proper deep plane of anaesthesia. Anaesthesia was induced by the intramuscular injection of Zoletil® (tiletamine and zolazepam) (7 mg/kg), Dormitor® (medetomidin) (7 mg/kg), and atropine (0.03 mg/kg). Animals underwent endotracheal intubation, and anaesthesia was maintained by isofluorane inhalation (1.5–2%). A continuous infusion of amiodarone (300 mg, 75 mg/h) was initiated at the beginning of the procedure in all pigs as a prophylaxis for malignant ventricular arrhythmias. Angiographic images were used to guide the guidewire balloon placement (below the first diagonal branch). The angioplasty balloon (2.5–3 mm) was inflated at nominal pressure (complete occlusion of the LAD was verified by fluoroscopy). Occlusion was maintained for 90 min, and oxygen levels and the ECG were monitored throughout the surgical procedure. The site of occlusion was immediately distal to the origin of the first diagonal branch. At the end of the ischaemic period, animals were sacrificed by an overdose of KCl while anaesthetized. Following sacrifice, heart samples from the necrotic border in the ischaemic septal zone vs. that from the left ventricle free-wall, non-ischaemic/remote myocardium, were used for molecular analysis.

2.5 VLDL preparation

Human VLDL (d1.001–1.019 g/mL) were obtained from pooled sera of normocholesterolaemic volunteers.

2.6 Lipid extraction and determination of cholesteryl ester, free cholesterol, and TG content of cardiomyocytes and myocardium

Cells were exhaustively washed and harvested in NaOH 0.1 M following the VLDL incubation period. In the animal experimental model, one portion of myocardial tissue (5 mg) was also homogenized in NaOH 0.1 M. Lipids were extracted as previously described, and cholesteryl ester (CE), free cholesterol (FC), and TG content was analysed by thin-layer chromatography. See Supplementary material online, Methods for more details.
2.7 Preparation of double-radiolabelled VLDL particles and determination of VLDL uptake by cardiomyocytes

Double-radiolabelled \([^{3}H]CE-^{14}CTG-VLDLs\) were obtained by a previously described method with small modifications.\(^{18}\)

2.8 Small interference RNA-mediated gene silencing of HIF-1\(\alpha\) in HL-1 cells

To inhibit HIF-1\(\alpha\) expression, HL-1 cells were transiently transfected with HIF-1\(\alpha\)-specific small interference RNA (siRNA) acquired from Applied Biosystems (siRNA ID 4390815). An siRNA-random was used as a negative control (Ambion AM 4636) in cellular transfections.

2.9 Constructions for luciferase reporter gene assay and site-directed mutagenesis

The construction and mutation of LRP1 promoter in a luciferase reporter plasmid and the transient transfection assays were performed as previously described in HeLa cells.\(^{19}\)

2.10 Real-time PCR

mRNA expression of LRP1, VLDL receptor (VLDLR), LDLR, SREBP-1, SREBP-2, Bcl-2, and BAX was assessed by real-time PCR.

2.11 Western blot

Proteins were analysed by western blot analysis. See Supplementary material online, Methods for more details.

2.12 Apoptosis detection

Apoptotic cells were visualized using the ApopTag Red In Situ Apoptosis Detection Kit (Chemicon International, S7165) following the manufacturer’s instructions.

2.13 Intracellular calcium handling

To detect changes in intracellular calcium handling, control and LRP1-deficient HL-1 cells previously exposed to VLDL (1.8 mM, 18 h) were loaded with fluo-4 as previously described.\(^{22}\) Calcium was assessed in a 1 × 1 mm\(^2\) of the cell culture, using a resonance scanning confocal microscope (Leica SPS AOBS) with a 10× objective. Cell cultures were loaded with 2.5 μM fluo-4AM for 15 min at RT followed by 30 min of de-esterification. Fluo-4 was excited at 488 nm, with the laser intensity set to 20%. To induce Ca\(^{2+}\) overload, Ca\(^{2+}\) was increased to 5 mM, and mannitol 2.2% (v/v) was added to maintain normo-osmolarity (312 mOsm). Cells were subjected to electrical field stimulation using square pulses of 5 ms duration and with the voltage set at 1.5 times the stimulation threshold. The electrodes were at a distance of 100–200 μm from the imaging field. Calcium transient amplitude was evaluated at 0.5 Hz.

2.14 Statistical analysis

Continuous variables are expressed as mean ± SEM.

3. Results

3.1 Effect of hypoxia on LRP1, VLDLR, and LDLR mRNA expression in NRVM and HL-1 cells

The effect of hypoxia on lipoprotein receptor expression was analysed at the mRNA level by real-time PCR and at the protein level by western blot analysis. As shown in Figure 1A, hypoxia increased LRP1 mRNA (from 1.6-fold at 4 h to 2.7-fold at 16 h) and VLDLR mRNA (from 2.8-fold at 4 h to 4-fold at 16 h) and decreased LDLR mRNA expression (from 10% at 4 h to 52% at 16 h) in a...
time-dependent manner in HL-1 cells. As shown in Table 1, hypoxia significantly reduced SREBP-1 and SREBP-2 mRNA expression levels to ~60%. In agreement with real-time PCR results, western blot analysis showed that hypoxia (24 h) strongly up-regulated LRP1 and VLDLR protein expression in HL-1 (Figure 1B) and NRVM (Supplementary material online, Figure S1) cardiomyocytes. In contrast, hypoxia down-regulated LDLR protein expression in both HL-1 (Figure 1B) and NRVM cardiomyocytes (Supplementary material online, Figure S1).

3.2 Role of HIF-1α on LRP1 up-regulation induced by hypoxia in HL-1 cells

In order to investigate the involvement of HIF-1α in LRP1 up-regulation by hypoxia, we inhibited HIF-1α expression by nucleofection with specific siRNA-HIF-1α. siRNA-HIF-1α but not siRNA-random completely prevented the 3.8-fold increase in HIF-1α protein levels induced by hypoxia in HL-1 cells (Figure 2A, left panel). Hypoxia did not exert an effect on LRP1 protein expression (Figure 2A, right panel) in HIF-1α-deficient cells but it significantly increased LRP1 expression in control and siRNA-random-treated HL-1 cells. As shown in Figure 2B, real-time PCR results showed that siRNA-HIF-1α efficiently prevented LRP1 and VLDLR mRNA overexpression induced by hypoxia. In contrast, HIF-1α inhibition did not show any significant effect on the down-regulatory effect of hypoxia on LDLR expression. Previous studies from our group showed that human LRP1 promoter contains two HRE-binding sites (HRE-1 from −1072 to −1069 and HRE-2 from −695 to −692). In order to investigate the role of HRE-1 and HRE-2 sites in the hypoxic modulation of the LRP1 promoter, we performed transient transfection of HL-1 cells using LRP1 promoter constructions with the two HRE sites (from −1305 to +341), without the HRE-1 site, without the HRE-2 site, and without any HRE site. As shown in Figure 2C, hypoxia (24 h) increased the transcriptional activity of the wild LRP1 promoter by 2.4-fold and that of the HRE-1-or HRE-2-mutated LRP1 promoter by 1.7-fold and 1.6-fold, respectively. Hypoxia had no effect on the construction without HRE sequences. These results demonstrate that HIF-1α mediates the induction of LRP1 transcription in hypoxic HL-1 cells.

3.3 Effect of VLDL on intracellular lipid accumulation in normoxic and hypoxic HL-1 cells

As previously reported in NRVM cardiomyocytes,22 VLDL increased both intracellular CE (from 6.7 ± 2.2 to 77.6 ± 8.8 μg CE/mg cell protein) and TG (from 2.5 ± 0.2 to 69.2 ± 3.0 μg TG/mg cell protein) content of HL-1 cells (Figure 3A) in a dose-dependent manner. Hypoxic conditions further increased VLDL-derived intracellular CE (to 110.5 ± 1.6 μg CE/mg cell protein) and TG accumulation (to 80.5 ± 3.9 μg TG/mg cell protein). However, in contrast to the effect of hypoxia per se on the TG content of NRVM,22 hypoxia per se had no effect on the TG content of HL-1 cells. In agreement with these data, confocal microscopy (Figure 3B) showed higher Dil-VLDL uptake in hypoxic than in normoxic HL-1 cells. As shown in Figure 3C, apoptosis was undetectable in normoxic and hypoxic HL-1 cells unexposed or exposed to VLDL, although it was clearly detected in the positive control (HL-1 treated with H₂O₂).

3.4 Effect of LRP1 deficiency in VLDL-CE and VLDL-TG intracellular accumulation induced by hypoxia in HL-1 cells and NRVM cardiomyocytes

In order to know the involvement of LRP1 on VLDL uptake and VLDL-derived intracellular lipid accumulation, three miR RNAi (RNAi1919, RNAi8223, and RNAi8531) (Supplementary material online, Figure S2A) were tested to target LRP1. RNAi1919 and RNAi8531 showed similar ability to reduce LRP1 mRNA expression in hypoxic NRVM and HL-1 cardiomyocytes (Supplementary material online, Figure S2B). We selected RNAi8531 (RNAi-LRP1) to knock-down LRP1 expression.

RNAi-LRP1 treatment significantly prevented the hypoxia-induced LRP1 protein up-regulation in both HL-1 (Figure 4A) and NRVM cardiomyocytes (Supplementary material online, Figure S3A) (either unexposed or exposed to VLDL). In contrast, VLDLR or LDLR expression was unaltered in hypoxia subjects subjected to RNAi-LRP1 treatment. This treatment did not alter the low levels of LRP1 protein expression in normoxic cells.

Both control and RNAi-LRP1-treated HL-1 cells showed undetectable levels of intracellular CE (Figure 4B, left panel) and TG (Figure 4B, right panel) in the absence of VLDL. However, NRVM cardiomyocytes (control or RNAi-LRP1 treated) showed basal levels of intracellular TG that were increased 1.3-fold by hypoxia (Supplementary material online, Figure S3B, right panel). In the presence of VLDL, the intracellular lipid profile in LRP1-deficient cells was different from that in control cells when exposed to hypoxia. LRP1 deficiency almost completely prevented hypoxia-induced VLDL-derived CE overaccumulation in HL-1 cells (Figure 4B, left panel) and NRVM cardiomyocytes (Supplementary material online, Figure S3B, left panel). Nevertheless, LRP1 deficiency did not show any effect on the hypoxia-induced VLDL-derived TG accumulation in HL-1 cells (Figure 4B, right panel) or NRVM cardiomyocytes (Supplementary material online, Figure S3B, right panel). These results suggest that LRP1 up-regulation is essential for intracellular VLDL-derived CE accumulation in hypoxic cardiomyocytes.

In order to explore the role of LRP1 in VLDL-CE accumulation through a different approach, we attempted to overexpress LRP1. Previous studies showed that LRP1 may be up-regulated by lipopolysaccharides (LPS) in neurons26 and by angiotensin II in human VSMC.27 Here, LPS that significantly induced LRP1 protein expression in NRVM cardiomyocytes (Supplementary material online, Figure S3C) specifically increased intracellular VLDL-derived CE accumulation by 1.6-fold (Supplementary material online, Figure S3D). However, LPS did not alter the intracellular VLDL-TG accumulation (Supplementary material online, Figure S3D) in these cells. Therefore, these results

| Table 1 Effect of hypoxia on SREBP-1 and SREBP-2 mRNA levels in HL-1 cells |
|-----------------|-----------------|
| Normoxia | 3.50 ± 0.22 | 2.93 ± 0.18 |
| Hypoxia | 1.30 ± 0.13* | 1.02 ± 0.09* |

HL-1 cells were exposed to normoxia or hypoxia for 24 h. SREBP-1 and SREBP-2 mRNA expression levels were analysed by real-time PCR. Data were processed with specially designed software based on threshold cycle (Ct) values, normalized to ARBP mRNA levels, and expressed as mean ± SEM of three experiments performed in duplicate.

*P < 0.05 vs. normoxic HL-1 cells.
confirm that LRP1 when overexpressed contributes to VLDL-CE uptake by cardiomyocytes.

3.5 Effect of LRP1 deficiency in \([^{3}\text{H}]\)CE-\([^{14}\text{C}]\)TG-VLDL uptake in hypoxic HL-1 cells

A more sensitive lipid uptake analysis was carried out with radiolabelled VLDL. The involvement of LRP1 in \([^{3}\text{H}]\)CE and \([^{14}\text{C}]\)TG uptake was analysed by determining \([^{3}\text{H}]\) and \([^{14}\text{C}]\) counts per minute (c.p.m.) associated with control and LRP1-deficient HL-1 cells. The specific activity of double-radiolabelled VLDL was 439 ± 53.5 c.p.m. \([^{3}\text{H}]\)/μg VLDL prot and 87 ± 15 c.p.m. \([^{14}\text{C}]\)/μg VLDL prot. As shown in Figure 5A, the increased \([^{3}\text{H}]\)CE uptake induced by hypoxia (2.3-fold) in control HL-1 cells was completely prevented in LRP1-deficient cells. In contrast, the increased \([^{14}\text{C}]\)TG uptake induced by hypoxia (1.3-fold) was similar in control and LRP1-deficient cells (Figure 5B). The \([^{3}\text{H}]\)/\([^{14}\text{C}]\) ratio in double-radiolabelled VLDL was 5 ± 0.8. As shown in Figure 5C, the low CE/TG uptake (1.04 ± 0.19) in normoxic HL-1 was increased by 2-fold by hypoxia in control but not in LRP1-deficient cells. In order to know whether increased VLDL-CE uptake was an unspecific
Figure 3 Effect of VLDL on intracellular lipid accumulation in normoxic and hypoxic HL-1 cells. (A) HL-1 cells were exposed to increasing VLDL concentrations under normoxic or hypoxic conditions as explained in Methods. Thin-layer chromatography showing intracellular CE, TG, and FC bands, and line graphs showing their quantification in normoxic (dotted line) and hypoxic (continuous line) HL-1 cardiomyocytes. Results were expressed as micrograms of cholesterol or TG per milligram of protein and shown as the mean of three experiments performed in duplicate (deviations <5% from the mean do not appear in the computer-originated graphs). *P < 0.05 vs. normoxic HL-1; #P < 0.05 vs. VLDL-unexposed HL-1. (B) HL-1 cells were incubated with DiI-VLDL (1.8 mM) for 24 h. Cells were then exhaustively washed, fixed, and photographed using confocal microscopy. (C) Confocal microscopy detection of cell death in control or VLDL (1.8 mM)-exposed HL-1 cells, using the ApopTag Red In Situ Apoptosis Detection Kit. Scale bar 20 μm.
Figure 4 Effect of RNAi-LRP1 on lipoprotein expression and VLDL-derived intracellular lipid profile in normoxic and hypoxic HL-1 cells. HL-1 cells were exposed to VLDL (1.8 mM) under normoxic or hypoxic conditions as explained in Methods. (A) Western blot analysis and bar graphs showing the quantification of LRP1, VLDLR, and LDLR. Unchanged levels of β-tubulin are shown as a loading control. Results are expressed as mean ± SEM of three experiments performed in triplicate. (B) Thin-layer chromatography showing CE, TG, and FC bands, and histograms with their quantification. Results are expressed as micrograms per milligram of protein and shown as mean ± SEM of three experiments performed in triplicate. (C) The ratio between Bcl2 and BAX mRNA expression was considered the cell survival ratio. Data are shown as a percentage of control cells and expressed as mean ± SEM of four experiments performed in duplicate. (D) Representative western blot showing caspase-3 (CPP32) protein expression. Unchanged levels of β-tubulin are shown as a loading control. *P < 0.05 vs. normoxic cells; #P < 0.05 vs. VLDL-unexposed cells; †P < 0.05 vs. control cells.
effect due to starved conditions, we analysed the uptake of L-[3H]-glutamine in our experimental settings. Our results showed that neither hypoxia nor RNAi-LRP1 altered L-[3H]-glutamine uptake in HL-1 cells (Figure 5D), suggesting that the observed changes in VLDL uptake are unrelated to starvation.

3.6 Effect of LRP1 deficiency on cell survival and calcium handling

To test the viability of the cell culture, we evaluated the balance between intracellular mRNA levels of an apoptotic marker (BAX) and a survival marker (Bcl2). VLDL or hypoxia significantly decreased Bcl2/BAX ratio (Figure 4C) but not caspase-3 (CPP32) expression (Figure 4D), suggesting that VLDL and hypoxia did not induce cellular apoptosis, although they may reduce the survival capacity of HL-1 cells in agreement with previous results in NRVM cardiomyocytes.22 RNAi-LRP1 treatment did not influence HL-1 cell survival.

As shown in Supplementary material online, Figure S4, VLDL-exposed HL-1 cells either unstimulated or stimulated (centre panel) were more prone to present waves and irregular responses compared with VLDL-unexposed HL-1 cells (left panel), as previously shown in NRVM cardiomyocytes.22 The percentage of waves increased in calcium-overloaded unstimulated (centre panel, up) but not in stimulated HL-1 cardiomyocytes (centre panel, down).

VLDL-exposed LRP1-deficient HL-1 cells (right panel), either unstimulated or stimulated, showed a reduced percentage of waves and irregular responses compared with control HL-1 cells (centre panel). However, the percentage of not responding cells was much higher in LRP1-deficient cells (right panel) than in control cells (centre panel). In agreement, the percentage of not responding was increased, whereas the percentage of waves was reduced in calcium-overloaded-LRP1-deficient HL-1 cells under stimulation.

3.7 Effect of acute ischaemia induced by coronary balloon occlusion on lipoprotein receptor pattern and neutral lipid profile in the porcine myocardium

In order to test whether the findings obtained in vitro could be translated to the ischaemic heart, we used a porcine experimental model of acute ischaemia. First, LRP1 and VLDLR expression was determined in non-ischaemic (remote myocardial zone) and ischaemic (peri-necrotic myocardium or penumbra) regions in the myocardium of infarcted and sham-operated animals (Figure 6A) by western blot analysis. LRP1 protein expression was significantly increased in the ischaemic myocardium (5.68-fold vs. sham; \(P < 0.05\)) (Figure 6A, left panel). Although to a minor extent, VLDLR protein was also significantly increased in the ischaemic zone (2.15-fold vs. sham; \(P < 0.05\))
We also evaluated the effect of ischaemia on myocardial CE, TG, and FC content analysed using thin-layer chromatography after lipid extraction (Figure 6B). Both CE (Figure 6B, left panel) and TG (Figure 6B, right panel) were strongly up-regulated in the ischaemic area of the myocardium (2-fold and 2.84-fold increase, respectively, vs. sham; \( P < 0.05 \)), whereas no differences were observed in the neutral lipid content of the non-ischaemic area and sham.

4. Discussion

Our results demonstrate that (i) hypoxia up-regulates LRP1 and VLDLR whereas down-regulates LDLR in cardiomyocytes, (ii) hypoxia up-regulates LRP1 expression through HIF-1\( \alpha \) in HL-1 cells, and (iii) LRP1 overexpression mediates hypoxia-induced VLDL-CE uptake and accumulation in cardiomyocytes. In addition, both LRP1 expression and CE levels are up-regulated in the ischaemic myocardium of an \emph{in vivo} porcine model of AMI.

The current study shows that hypoxia up-regulates LRP1 through HIF-1\( \alpha \) in HL-1 cells, as previously shown in human VSMC.\(^{19} \) In agreement with previous results,\(^{28} \) we also found that HIF-1\( \alpha \) is key for the hypoxic modulation of VLDLR in HL-1 cells. Therefore, LRP1 and VLDLR share a common mechanism of transcriptional activation by hypoxia and this may explain the huge myocardial LRP1 and VLDLR up-regulation that we have found in the porcine model of AMI. In contrast to the clear role of HIF-1\( \alpha \) on LRP1 and VLDLR overexpression, this transcription factor does not seem to play a role in LDLR down-regulation. In fact, LDLR down-regulation may be explained by the SREBP-1 and SREBP-2 decay in hypoxic HL-1 cells, as previously reported in hypoxic human VSMC.\(^{19} \)

Niu et al.\(^{29} \) demonstrated that cholesterol uptake from remnants of TG-rich lipoproteins was strongly reduced by suramin (an unspecific inhibitor of the LDL receptor superfamily) in a perfused heart model. In the present study, we found that LRP1 is crucial for selective CE uptake and accumulation in hypoxic cardiomyocytes. These results are in agreement with the role of LRP1 mediating CE selective uptake from lipoproteins\(^{18,30} \) due to LRP1 capacity to move to caveolae, patches of the sarcolemmal membrane that act as acceptors of CEs.\(^{31} \) In addition, LRP1 and proteoglycans, which are up-regulated by hypoxia,\(^{32} \) cooperate to mediate ligand uptake.\(^{33} \) In contrast to the strong impact of LRP1 inhibition on VLDL-CE uptake in hypoxic cardiomyocytes, LRP1 deficiency did not exert any significant effect on VLDL-TG uptake or VLDL-TG accumulation. These results show that VLDL-CE uptake occurs via a different mechanism than VLDL-TG uptake. Previous studies showed that CD36 is involved in myocardial VLDL-TG uptake.\(^{14} \) CD36 is up-regulated by hypoxia\(^{34} \) and it may thus participate in the increased FA uptake and TG accumulation under ischaemia. A recent study points to VLDLR as an essential receptor for TG accumulation in hypoxic HL-1 cells and in the ischaemic myocardium.\(^{28} \) VLDLR closely interacts with LpL,\(^{11} \) which plays a major role in VLDL uptake by the heart.\(^{14,28} \) Moreover, LpL and VLDLR activities correlate, have a similar tissue-specific expression and distribution,\(^{13,15} \) and are increased by hypoxia in the cardiomyocyte.\(^{22,28,35} \) Therefore, it is plausible that LpL and VLDLR participate in hypoxia-induced VLDL-CE accumulation. Further experiments aimed at establishing the precise contribution of LpL, VLDLR, and LRP1 in myocardial VLDL-CE uptake in pathophysiological situations will be extremely relevant for preventing the deleterious effects of TG and CE delivery to the heart.

Our data show that increased intracellular neutral lipid accumulation does not induce cardiomyocyte apoptosis in short incubation periods. Similar results have been previously reported in lipid-loaded human VSMC\(^{19} \) and HL-1 cells.\(^{28} \) However, hypoxia and lipids probably induce cell death at longer exposure periods. Indeed, in the...
context of diabetes/obesity, a strong TG accumulation has been correlated with cell death26 and diastolic dysfunction in animal models26,27 and also in type 2 diabetes mellitus patients.28 In the context of ischaemia, TG accumulation promotes cardiac ER stress.28 The deleterious effects of myocardial lipids both in TG and in ischaemia have been exclusively ascribed to TG, assuming that these are the only neutral lipids accumulated in cardiomyocytes. It has been reported that TG-rich lipoproteins such as VLDL deliver both TG and CE to cardiomyocytes.14,28 and that hypoxia increases both VLDL-TG and VLDL-CE delivery to neonatal cardiomyocytes.22 In the present study, we show that myocardial neutral lipid includes both TG and CE in a porcine model of acute ischaemia. Therefore, we consider that this assumption should be revised and that most of the deleterious effects of neutral lipid deposition ‘lipotoxic effects’ may not only be caused by TG but also by myocardial CE. Relevant studies have shown that myocardial cholesterol contributes to lipotoxic cardiomyopathy15,16 and that it is the cause of systolic and diastolic dysfunction.17 In fact, an inverse correlation between myocardial cholesterol levels and SERCA-2 expression has been shown.17 Our group has recently demonstrated that SERCA-2 plays a crucial role in the hypoxic potentiation of calcium handling disturbances induced by VLDL.22 Taken together, these results suggest that LRPI may be a target in order to prevent ischaemic myocardial functional alterations associated with intracellular cholesterol accumulation in cardiomyocytes. Results from the present study show that although LRPI inhibition reduces VLDL-induced waves and irregular responses, LRPI deficiency increases the percentage of inactive cells and worsens HL-1 resistance to calcium overload. These results are in agreement with the pivotal role of LRPI as a signal-transducing receptor that mobilizes calcium intracellular release.39 Indeed, the impairment of LRPI cholesterol carrier function without altering LRPI essential signal transduction is the better strategy to prevent hypoxia-induced CE accumulation. Cardiomyocytes in the peri-infarct zone become markedly overloaded with neutral lipids31 that are key determinants for the transition from reversible to irreversible acute myocardial injury and for arrhythmias.30 Therefore, our results should be considered for designing efficient strategies to prevent cardiac alterations derived from myocardial cholesteryl accumulation.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References

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