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Reconstituted high-density lipoprotein suppresses leukocyte NADPH oxidase activation by disrupting lipid rafts

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Abstract
Reconstituted discoidal high-density lipoprotein (rHDL) has potent vascular protective actions. Native HDL suppresses cellular generation of reactive oxygen species, whereas this antioxidant effect of rHDL is less clear. This study examined the effects of rHDL on NADPH oxidase, a major source of cellular superoxide generation, in both leukocytes and human umbilical vein endothelial cells. Superoxide was measured with lucigenin-enhanced chemiluminescence. Expression of NADPH oxidase sub-units was determined by real-time PCR. Pre-treatment of HL-60 cells with rHDL (10 and 25 μM) for 1 h significantly reduced phorbol 12-myristate 13-acetate-stimulated superoxide production. Treatment with rHDL for up to 24 h did not change the mRNA expression of NADPH oxidase sub-units. In HL-60 cells, depletion of cholesterol from the plasma membrane by methyl-β-cyclodextrin mimicked the effect of rHDL, whereas cholesterol repletion blunted the effects of rHDL. Treatment with rHDL induced disruption of the lipid raft structures and blunted PMA-induced redistribution of p47phox into lipid rafts. In contrast, treatment of endothelial cells with rHDL for up to 18 h had no effect on either basal or tumour necrosis factor-α-stimulated NADPH oxidase activity, but markedly suppressed the cytokine-induced expression of proinflammatory adhesion molecules. The results suggest that rHDL inhibits NADPH oxidase activation in leukocytes, probably by interrupting the assembly of NADPH oxidase sub-units at the lipid rafts. This effect may contribute to the vascular protective actions of rHDL against inflammation-mediated oxidative damage.

Keywords: *High-density lipoprotein, lipid raft, NADPH oxidase, superoxide*

Introduction

Epidemiological evidence shows that the plasma concentration of high-density lipoproteins (HDL) is inversely related to the risk of cardiovascular disease [1]. The mechanisms of the protective actions of HDL are not fully understood. The best known anti-atherogenic function of HDL particles is attributable to their ability to promote the efflux of cholesterol from cells. However, there is mounting evidence that HDL has other effects that are independent of the cholesterol transporting functions, including antioxidant, anti-inflammatory and anti-thrombotic properties [1–3].

For example, our previous studies have shown that HDL inhibited cytokine-induced expression of proinflammatory adhesion molecules in human vascular endothelial cells *in vitro* [4]. Moreover, in humans with genetically low HDL cholesterol, low-dose endotoxin induces inflammatory responses and thrombin generation that are enhanced as compared to normal controls [5]. There is evidence that HDL may also have prominent antioxidant effects [3]. Incubation with HDL prevented chemically [6] or cell-induced [7] oxidative modifications of low-density lipoproteins. Moreover, inverse relationships between HDL and systemic oxidative stress or the level of circulating oxidized low-density lipoproteins

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have been reported in humans [8,9]. The antioxidant effects of HDL are thought to result mainly from antioxidant enzymes associated with HDL particles. For example, HDL-associated paraoxonases are able to hydrolyse oxidized fatty acids and thereby reduce the accumulation of oxidized lipids in low-density lipoproteins [3]. Over-expression of human paraoxonase has been shown to reduce oxidized low-density lipoproteins levels *in vivo* in a mouse model of metabolic syndrome [10]. Other enzymes, such as platelet-activating factor acetylhydrolase and lecithin-cholesterol acyltransferase, may also be involved in the protective effects against low-density lipoproteins oxidation [3]. In addition, the ability of HDL particles to extract lipid peroxidation products from oxidized lipoproteins or plasma membranes may facilitate the clearance of the detrimental lipid peroxides [3].

Several lines of evidence however suggest that HDL may directly modulate the generation of reactive oxygen species from cellular sources. For instance, treatment of polymorphonuclear neutrophils with native HDL significantly decreased reactive oxygen species release induced by oxidized low-density lipoproteins [11] or by direct cell–cell contact with activated T lymphocytes [12]. In human umbilical vein endothelial cells, it was shown that pre-treatment with HDL acutely reduced the levels of reactive oxygen species release stimulated by oxidized low-density lipoproteins or H_2O_2 [13]; this inhibitory effect of HDL on cellular reactive oxygen species generation has also been observed *in vivo* after ischemia/reperfusion in kidneys [13]. Recently, we demonstrated that systemic administration of reconstituted HDL (rHDL) containing isolated apolipoprotein (apo) A-I and 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) completely abolished the increase in superoxide production induced by a periarterial collar in the carotid arteries of rabbits [14] and this effect was accompanied by reduced vascular inflammation. All these results indicate that modulating cellular reactive oxygen release may contribute to the observed beneficial effects of HDL.

In this study, we investigated the effects of rHDL on the activities of phagocytic and non-phagocytic NADPH oxidases, which represent a major enzymatic source of reactive oxygen species in vascular tissues [15,16], as there is little information about the effects of rHDL on NADPH oxidase functions. Moreover, we tested the hypothesis that interruption of NADPH oxidase assembly at the membrane lipid rafts may be involved in the effects of rHDL.

Materials and methods

Reagents

The following reagents used in the study were obtained from Sigma-Aldrich Australia (Castle Hill, New South Wales, Australia): cholesterol, NADPH (reduced β-nicotinamide adenine dinucleotide phosphate), 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC), methyl-β-cyclodextrin, phorbol 12-myristate 13-acetate (PMA), tumour necrosis factor-α (TNF-α), xanthine and xanthine oxidase.

Cell culture

The HL-60 human myelocytic cells were cultured in RPMI 1640 supplemented with 10% foetal calf serum, 2 mM l-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. Because undifferentiated HL-60 cells did not exhibit PMA-induced oxidative burst, HL-60 cells was differentiated by incubation with 1.5% dimethylsulphoxide for 3–4 days before experiments. Human umbilical vein endothelial cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in EGM-2 BulletKit (Lonza Group Ltd, Basel, Switzerland) containing 5% serum.

Preparation of reconstituted HDL

Discoidal rHDL containing human apolipoprotein A-I complexed to PLPC in a molar ratio of 1:100 was prepared by cholate dialysis method as described previously [17]. The resulting rHDL was dialysed extensively against sterile phosphate-buffered saline before use. The concentrations of rHDL given in the text refer to the final concentrations of apolipoprotein A-I.

Chemiluminescence measurement of superoxide

NADPH oxidase activity was assessed by measuring superoxide with lucigenin-enhanced chemiluminescence using a Topcount microplate scintillation counter (PerkinElmer, Waltham, MA) running in single-photon-count mode, as described previously [18]. Briefly, HL-60 cells were resuspended in a Krebs-HEPES (in mM: NaCl 98.0, KCl 4.7, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, d-glucose 11.1 and Hepes-Na 20.0) based assay solution containing 5 μM lucigenin; 200 μl of aliquots of the cell suspension ($\sim 10^6$ cells) were dispensed into a 96-well plate (OptiPlate-96, Packard) in triplicate for luminescence measurement. NADPH oxidase was activated by PMA of 100 ng/ml. Endothelial cells (40 000 per well) were cultured in OptiPlates for 48 h. Before experiments, cells were pre-incubated with diethyldithiocarbamic acid (3 mM) in Krebs-HEPES solution for 45 min to inactivate endogenous superoxide dismutase. The endothelial NADPH oxidase was stimulated with 100 μM NADPH and the chemiluminescence was detected with 5 μM lucigenin. We measured NADPH oxidase in intact endothelial cells because this method was more sensitive and more reproducible

than using homogenates under our experimental conditions. In both HL-60 and endothelial cells, the rHDL was removed from the medium before cell stimulation by washing the cells with fresh Krebs-HEPES solution. In a cell-free assay system, superoxide was generated by mixing xanthine (100 μM) with xanthine oxidase (0.03 U/ml) in the Krebs-HEPES solution as previously described [19].

To quantitate the chemiluminescence signal (count per second), we performed parallel chemiluminescence and cytochrome c reduction assays, using xanthine/xanthine oxidase as the source of superoxide. The reaction was carried out in 200 μl of Krebs-HEPES solution containing 5 μM lucigenin or 140 μM cytochrome c. Reduced cytochrome c was determined by ΔA_{550} with an extinction coefficient of 21.1 mM^{-1} cm^{-1}. Under such an experimental condition, 1000 CPS is equivalent to a rate of superoxide generation of 1.4 pmol/min.

Cholesterol depletion and repletion

Cholesterol depletion and repletion experiments were carried out as described previously [20]. Methyl-β-cyclodextrin was dissolved in the culture medium to a final concentration of 1.5%. Cholesterol depletion was performed by incubating cells in the cyclodextrin containing medium for 1 h at 37°C. To replenish cellular cholesterol after rHDL treatment, cells were incubated in the presence of a cholesterol/cyclodextrin mixture at 37°C for another hour after rHDL. A stock solution of cholesterol/cyclodextrin (0.4 mg/ml cholesterol) was prepared by vortexing 10 ml of 10% cyclodextrin mixed with 200 μl of cholesterol solution (20 mg/ml in ethanol) at 40°C. This stock was diluted 1:25 in the culture medium in final experiments.

Real-time PCR

Cells were washed with PBS and collected in 0.5 ml TriReagent (Ambion, Austin, TX). The total RNA was extracted according to the manufacturer's instruction. Total RNA was reverse-transcribed to cDNA using random hexamer and TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) at 48°C for 30 min followed by 95°C for 5 min. The real-time PCR reactions were performed in the 7300 real-time PCR system (Applied Biosystems) using the TaqMan Universal PCR master mix and the pre-designed gene-specific probe and primer sets (TaqMan Gene Expression Assays, Applied Biosystems). The 18s RNA was used as the housekeeping gene.

Western blot

Western blot for various NADPH oxidase sub-units in HL-60 cells was performed as described previously [21]. The primary antibodies (rabbit anti-human Nox2, p47phox and p67phox) were purchased from Upstate (Millipore Australia, NSW, Australia).

Lipid raft labelling

Membrane lipid rafts were localized with the Vybrant® Lipid Raft Labelling Kit (Molecular Probes, Inc., Eugene, OR) according to manufacturer's protocol. Briefly, HL-60 cells were incubated with Alexa Fluor-conjugated cholera toxin sub-unit B, followed by cross-linking the labelled lipid rafts into distinct patches with a rabbit anti-cholera toxin B antibody. After labelling, cells were washed and fixed in cold methanol for 10 min. In our preliminary test, we found that this fixation procedure did not change the pattern of the labelled lipid rafts. In some experiments, the fixed cells were double-labelled for p47phox (goat anti-p47phox, 1:50) (from Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescent images were taken with an inverted fluorescence microscope (IX81F, Olympus Australia Pty Ltd, Victoria, Australia) and analysed with the Cell® imaging system (Olympus).

Lipid raft isolation

Lipid rafts were isolated according to the methods described by Guichard et al. [22]. Cells were homogenized at 4°C in buffer A (20 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) in a Dounce homogenizer, after which 80% sucrose stock in buffer A was added to the cell lysate to give a final concentration of 40% sucrose. The mixture was then layered under a sucrose step gradient (30 and 5% in buffer A, 3 ml each) and centrifuged at 200 000 g for 20 h at 4°C. After centrifugation, eight fractions were collected from the top of the tubes. To define the fractions containing lipid rafts, we performed dot blot analysis of ganglioside GM1 using Horseradish peroxidase-conjugated cholera toxin β-sub-unit (Sigma) according to Ilangumaran et al. [23].

Data and statistics

Data were presented as mean ± standard error of the mean (S.E.M.). The mean data were analysed with one-way analysis of variance (one-way ANOVA) followed by Newman-Keuls *t*-test (for multiple comparisons) unless indicated otherwise. A *p*-value of less than 0.05 was regarded as statistically significant. All *n* numbers shown are the number of independent experiments.

Results

Effects of rHDL on superoxide generation by xanthine and xanthine oxidase

We firstly characterized the effect of rHDL on superoxide generation by a cell-free assay system containing xanthine and xanthine oxidase using lucigenin-mediated chemiluminescence [19,21]. Surprisingly, we found that inclusion of the rHDL preparation in the assay mixture artificially enhanced the chemiluminescence signal (Figure 1A). As previous studies have shown that phospholipids may interfere with the chemiluminescence reaction [24], thus we tested the effect of PLPC. We found that PLPC had a similar effect on the chemiluminescence signal as rHDL (Figure 1B), whereas apolipoprotein A-I had a minor effect (Figure 1C), supporting that this artificial effect of rHDL was likely to originate from the phospholipids constituent. Because of this inference between phospholipids and the chemiluminescence reaction, in the following experiments, we completely removed these agents before the chemiluminescence assay by washing the cells with the Krebs-HEPES solution after the period of pre-incubation.

Effects of rHDL on NADPH oxidase activity and expression in leukocytes

We then analyzed the effects of rHDL on NADPH oxidase activity in leukocytes using the neutrophil-like cell line HL-60. The basal rate of superoxide generation in PMA-stimulated HL-60 cells was 0.8 ± 0.3 pmol/min/million cells ($n = 3$). As shown in Figure 2A, pre-treatment of HL-60 cells with rHDL (2–25 μM) for 1 h concentration-dependently reduced PMA-induced superoxide release detected by chemiluminescence. The effects of rHDL at 10 and 25 μM were not different. This PMA-induced chemiluminescence signal was totally abolished by the NADPH oxidase inhibitor diphenyleneiodonium (DPI, 1 μM). Then we examined whether apolipoprotein A-I or PLPC had any effects on leukocyte NADPH oxidase activation. In contrast to rHDL,

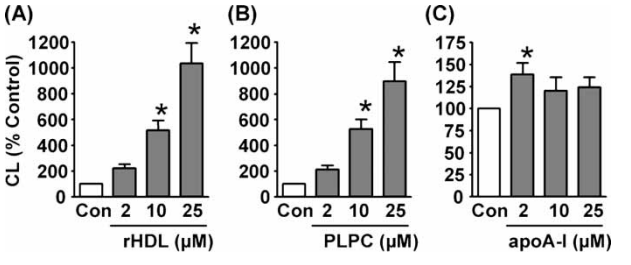

Figure 1. Effects of reconstituted discoidal high-density lipoprotein (rHDL), 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC), apolipoprotein A-I (apoA-I) on superoxide generation by xanthine (100 μM) plus xanthine oxidase (0.03 U/ml) detected by lucigenin-enhanced chemiluminescence (CL). Saline was used as control (Con). Data are mean ± SEM. * $p < 0.05$ vs Con, $n = 5$–6.

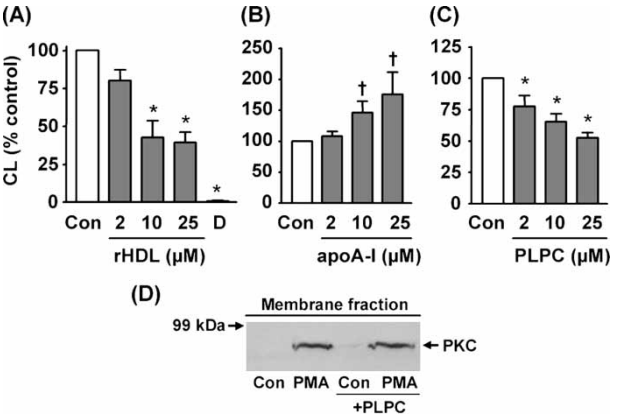

Figure 2. Effects of (A) rHDL, (B) apoA-I and (C) PLPC on phorbol 12-myristate 13-acetate (PMA, 100 ng/ml)-stimulated superoxide release (detected by chemiluminescence, CL) in HL-60 cells. The NADPH oxidase inhibitor diphenyleneiodonium (DPI, 1 μM) was used as a positive control. * $p < 0.05$ vs control (Con); † $p < 0.05$ vs control by unpaired t-test, $n = 3$–6; (D) Western blot showing the effect of PLPC (25 μM) on membrane translocation of PKC following PMA treatment (example from two independent experiments).

purified apolipoprotein A-I (2–25 μM) appeared to have an enhancing effect on PMA-stimulated superoxide production in HL-60 cells (Figure 2B). Interestingly, we found that pre-treatment of the cells with PLPC (2–25 μM) also inhibited PMA-induced NADPH oxidase activity in a concentration-dependent manner (Figure 2C). This effect of PLPC was similar to that of rHDL. It is technically difficult to exactly match the concentration of free PLPC with that contained in rHDL particles, because purified PLPC without the protein moieties has limited solubility in the aqueous phase. It has been shown that phospholipids may modulate NADPH oxidase function by suppressing protein kinase C activation in human monocytes [25], therefore we also measured the effect of PLPC on membrane translocation of protein kinase C (PKC) following PMA treatment. As shown in Figure 2D, PLPC had little effect on PMA-induced PKC activation in HL-60 cells.

Next we examined whether rHDL can modify the expression of NADPH oxidase sub-units in HL-60 cells. As shown in Figure 3A, treatment with rHDL (10 and 25 μM) for 24 h did not significantly change the mRNA levels of the NADPH oxidase sub-units Nox2, p22phox, p47phox and p67phox. To clarify whether rHDL had any effects on the protein levels of NADPH oxidase, we performed western blot analysis. Consistent with our previous observations [21], differentiation with dimethylsulphoxide upregulated expressions of p47phox and p67phox but not Nox2 in HL-60 cells (Figure 3B). Treatment with rHDL alone (25 μM) for 72 h did not significantly change the protein levels of Nox2, p47phox or p67phox in undifferentiated HL-60 cells (Figure 3B). Then we co-incubated the cells with dimethylsulphoxide and rHDL for 72 h. Interestingly, we observed

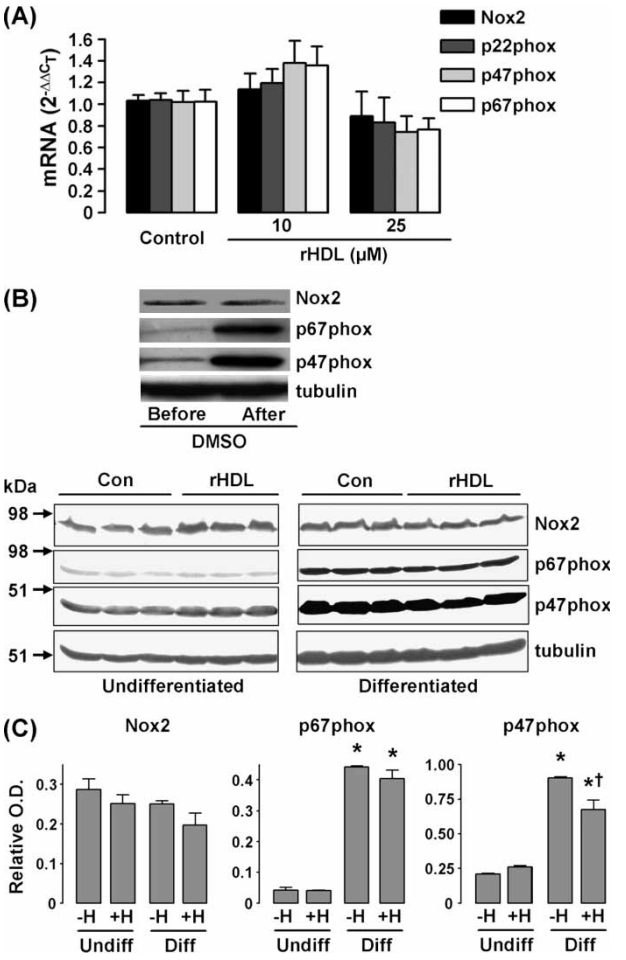

Figure 3. Effects of rHDL on mRNA and protein levels of NADPH oxidase sub-units in HL-60 cells. (A) Differentiated HL-60 cells were treated with rHDL for 24 h and the mRNA levels of various sub-units were measured by real-time PCR ($n=3$); (B) Effects of dimethylsulphoxide-induced differentiation (above) and rHDL (25 μM) (below) on NADPH oxidase sub-units expression in undifferentiated and differentiated HL-60 cells as indicated. In the latter experiments, rHDL was co-administered with dimethylsulphoxide (1.5% v/v); (C) Bar graphs of the densitometry data of the western blots. The results are all normalized to β-tubulin and expressed as the relative optical density (O.D.). * $p<0.01$ vs undifferentiated cells; † $p<0.01$ vs cells without rHDL (H) treatment, one-way ANOVA, $n=3$.

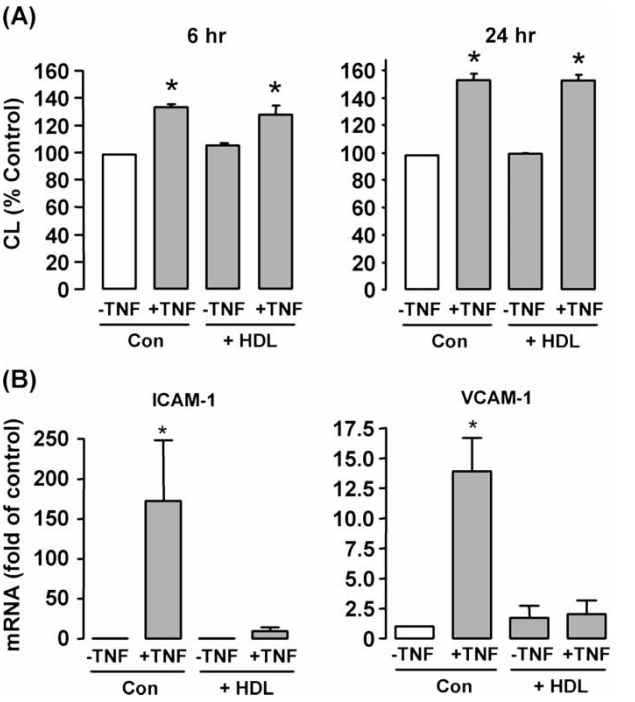

Figure 4. Effects of rHDL on (A) NADPH oxidase activity (NADPH-stimulated superoxide) and (B) adhesion molecule expression in human umbilical vein endothelial cells without and with tumour necrosis factor-α (TNF, 20 ng/ml) stimulation. Cells were incubated with saline (Con) or rHDL (25 μM) for 6 (for adhesion molecule expression) and 24 h. TNF was added 30 min after HDL. * $p<0.05$ vs -TNF, $n=3$.

that co-treatment with rHDL slightly but significantly blunted the upregulation of p47phox, but not p67phox, during dimethylsulphoxide-induced differentiation (Figure 3B and C).

Effects of rHDL on NADPH oxidase activity in endothelial cells

The basal rate of NADPH-stimulated superoxide generation in endothelial cells was 8.7 ± 2.7 pmol/min/million cells ($n=3$). In contrast to HL-60 cells, we found that treatment with rHDL (25 μM) in endothelial cells for 6 and 24 h had no effect on the basal NADPH oxidase activity (Figure 4A), which was also abolished by 1 μM DPI (7.3 ± 2.6% of control, $p<0.05$, $n=3$). Treatment of endothelial cells with TNF-α (20 ng/ml) significantly increased the NADPH oxidase activity at 6 and 24 h; however, pre-treatment with rHDL had no effect on TNF-α-stimulated NADPH oxidase activity (Figure 4A). To confirm that the concentration of rHDL used in endothelial cells was biologically relevant, we treated the endothelial cells with TNF-α for 6 h in the absence and presence of rHDL. As shown in Figure 4B, TNF-α-induced expressions of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were all markedly suppressed by rHDL at 25 μM.

Role of cholesterol mobilization in the effects of rHDL

Next we tested the hypothesis that the inhibitory effect of rHDL on phagocytic NADPH oxidase activity in HL-60 cells was related to HDL-mediated cholesterol mobilization from the plasma membrane. We first examined the effects of cholesterol depletion using methyl-β-cyclodextrin. As shown in Figure 5A, treatment with 1.5% cyclodextrin for 1 h significantly reduced PMA-induced superoxide release to a similar level as that in cells treated with rHDL at 10 and 25 μM. Then we examined whether rHDL-induced inhibition of NADPH oxidase in HL-60 cells can be reversed by repletion of membrane cholesterol. As shown in Figure 5B, cholesterol repletion with a cholesterol/cyclodextrin mixture (final concentration

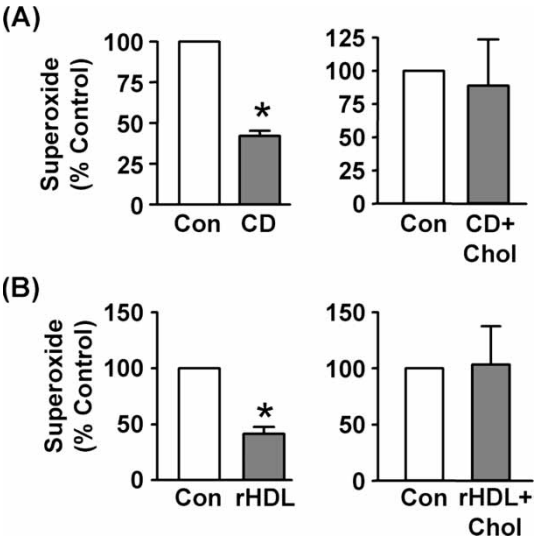

Figure 5. Effects of (A) cholesterol depletion by 1.5% cyclodextrin (CD) and CD plus cholesterol (Chol) repletion ($n = 3$–4) and (B) rHDL (25 μM) and rHDL plus cholesterol repletion ($n = 4$–8) on the rate of PMA-stimulated superoxide production in HL-60 cells. Cholesterol repletion was performed by treating the cells with a cholesterol/cyclodextrin mixture (final concentration of cholesterol = 16 μg/ml) for another hour following treatment with CD or rHDL. * $p < 0.05$ vs control (Con), unpaired t-test.

of cholesterol = 16 μg/ml) for another hour after rHDL (25 μM) treatment restored PMA-induced NADPH oxidase activation. Moreover, cholesterol repletion also blunted the effects of cyclodextrin (Figure 5A).

HDL interrupted lipid raft structures

In leukocytes, there is evidence that the cholesterol-enriched membrane lipid rafts are important in facilitating NADPH oxidase activation and superoxide production [26]. Therefore we examined whether interference with lipid rafts might be involved in the effects of rHDL on NADPH oxidase activation in HL-60 cells. Fluorescent labelling using cholera toxin B revealed that patches of lipid rafts can be readily detected in the majority of the non-stimulated HL-60 cells (Figure 6A), whereas the characteristic fluorescent patches found in control cells could not be seen in almost all cells treated with methyl-β-cyclodextrin (Figure 6B). Treatment with rHDL of 25 μM increased the proportion of cells without typical lipid raft patches (from $27.6 \pm 3.8\%$ in control cells to $70.5 \pm 3.5\%$ in rHDL-treated cells, $p < 0.01$, $n = 4$; Figure 6C).

HDL suppressed redistribution of NADPH oxidase sub-units into the lipid rafts

To clarify whether rHDL has any effect on redistribution of NADPH oxidase sub-units into the lipid rafts, we performed sucrose density gradient cell fractioning to isolate lipid rafts. As shown in Figure 7A, the lipid raft marker ganglioside GM1 was enriched in fractions 3–5. To confirm the enrichment of lipid rafts in these fractions, we performed western blotting for caveolin-1. We found that caveolin-1 signal was observed in fraction 3. Western blot analysis of NADPH oxidase sub-units revealed that in resting HL-60 cells, p47phox and p67phox were mainly located in fractions 7 and 8 (cytosol plus the detergent-soluble membranes). Unexpectedly, we could not observe translocation of p47phox toward fractions 3–5 in PMA-stimulated cells (Figure 7A). We reasoned that maybe only a minor proportion of the pool of p47phox in fractions 7/8 was mobilized by PMA. To test this possibility, we combined the GM1-rich fractions of 3–5 and the protein was concentrated by trichloroacetate precipitation. As shown in Figure 7B, the level of p47phox in these fractions (as normalized to total p47phox in fraction 7) was enhanced by PMA and this response was attenuated by rHDL and cyclodextrin. Taken together, these data suggested that PMA-induced mobilization of p47phox toward the lipid raft-containing fractions was blunted by rHDL.

To further corroborate the Western blot results, we performed immunofluorescence experiments to detect p47phox localization in relation to lipid rafts in dimethylsulphoxide-differentiated HL-60 cells. As shown in Figure 8A–D, p47phox was present in a diffused manner in resting cells (Figure 8C). Following stimulation with PMA, p47phox immunoreactivity aggregated into clusters as demonstrated by the fluorescent patches (Figure 8D, arrows). Using

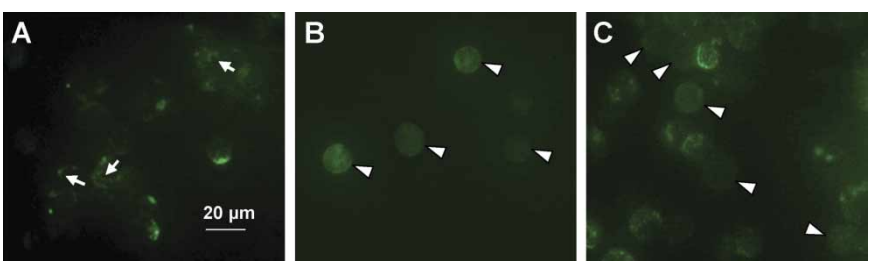

Figure 6. Lipid raft labelling of HL-60 cells. (A) Membrane lipid rafts were labelled with the cholera toxin sub-unit conjugated with the Alexa Fluor dye (green). After cross-linking with the anti-cholera toxin antibody, the labelled lipid rafts form distinct patches (arrows). (B) Treatment of the cells with 1.5% methyl-β-cyclodextrin disrupted the lipid rafts, resulting in a diffuse pattern of fluorescence (arrow heads). (C) Treatment with rHDL of 25 μM increased the number of cells without typical lipid raft patches (arrow heads).

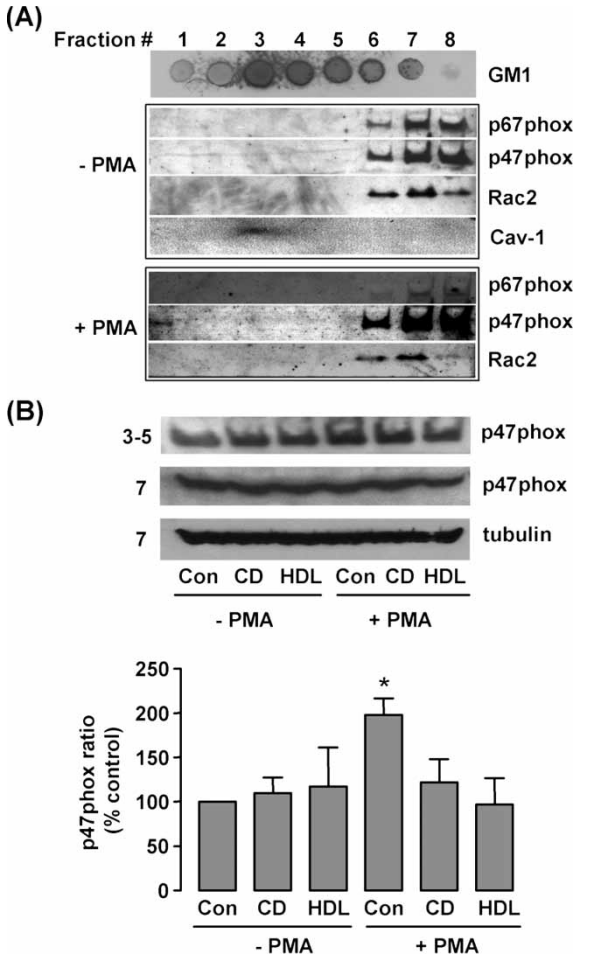

Figure 7. Effects of rHDL on redistribution of NADPH oxidase sub-units into lipid rafts. (A) Lipid rafts were isolated by sucrose density gradient cell fractioning. The lipid raft marker ganglioside GM1 was detected with Horseradish peroxidase-conjugated cholera toxin β-sub-unit using dot blot. P47phox, p67phox and caveolin-1 (Cav-1) were detected with Western blotting in different cell fractions; (B) Effects of rHDL (25 μM) and cyclodextrin (1.5%) on PMA-induced redistribution of p47phox in fractions 3–5 in treated and untreated cells. The fraction numbers are marked at the left of the blots. The bar graph shows quantitative data of the level of p47phox in fractions 3–5 normalized to the total p47phox in fraction 7 (expressed as% of control, $^\star p < 0.05$ vs control, $n = 3$).

double labelling, we observed that these p47phox-enriched patches colocalized with lipid rafts in PMA-stimulated cells, whereas this colocalization was not obvious in resting cells (Figure 8E and F). Moreover, we found that this PMA-induced colocalization of p47phox and lipid rafts was blunted in rHDL-treated cells and almost abolished in cyclodextrin-treated cells (Figure 8G and H).

Discussion

Previous evidence showing that HDL regulates superoxide species production from phagocytic or non-phagocytic NADPH oxidases was obtained with native HDL preparations derived from human plasma [11–13,27]. In the present study, we provided evidence that NADPH oxidase function can also be modulated by an artificially reconstituted HDL preparation, which has been shown to have multiple vascular protective actions such as anti-inflammatory [17] and anti-atherosclerotic [14] effects, as well as an enhanced re-endothelialization process after inflammatory vascular injury [28]. We found that pre-incubation with rHDL inhibited PMA-induced NADPH oxidase activation in HL-60 cells in a concentration-dependent manner. The effective concentration of rHDL (10–25 μM) was similar to that of native HDL used in previous studies [11,12]. This effect of rHDL was specific for the phagocytic NADPH oxidase, because a similar effect was not observed in vascular endothelial cells.

The mechanisms of the inhibition of leukocyte NADPH oxidase activity by HDL are poorly understood. Chander and Kapoor [29] reported that HDL had superoxide scavenging properties as measured in several cell-free assays. In the present study, however, we found that both the rHDL preparation and PLPC markedly enhanced the chemiluminescence signal induced by superoxide generated by xanthine plus xanthine oxidase. This effect of rHDL or PLPC is likely to be an artifact because of the interference between phospholipids and the chemiluminescence reaction [24]. Although this phenomenon prevented us from determining the superoxide scavenging activity of rHDL, our data indicate that the inhibitory effect of rHDL on NADPH oxidase function is not attributable to a direct scavenging of superoxide, because in all cell-based experiments, rHDL was removed before chemiluminescence measurements.

On the other hand, we have demonstrated that cholesterol depletion from the plasma membrane by methyl-β-cyclodextrin mimicked the effect of rHDL on NADPH oxidase activation. This effect of cyclodextrin on leukocyte NADPH oxidase has also been reported by other groups [26,30]. In a mouse microglia cell line and HL-60 cells, the membrane-bound sub-units Nox2 and p22phox have been identified in the cholesterol-enriched lipid rafts in the membrane [26]. In human neutrophils, treatment with interleukin-8 primed the NADPH oxidase for an enhanced superoxide production upon activation and this priming process was associated with recruitment of the cytosolic sub-units p47phox and Rac2 into the lipid rafts [22]. Moreover, disruption of the lipid rafts by cholesterol depletion compromised the activation of NADPH oxidase in leukocytes [26,30]. These studies suggest that the lipid rafts in the cell membrane have an important role in facilitating the assembly and activation of NADPH oxidase. Consistent with this notion, we have found that the inhibitory effect of rHDL on leukocyte NADPH oxidase was reversed by cholesterol repletion, which also blunted the effect of cyclodextrin. To further clarify the mechanisms by which rHDL inhibited

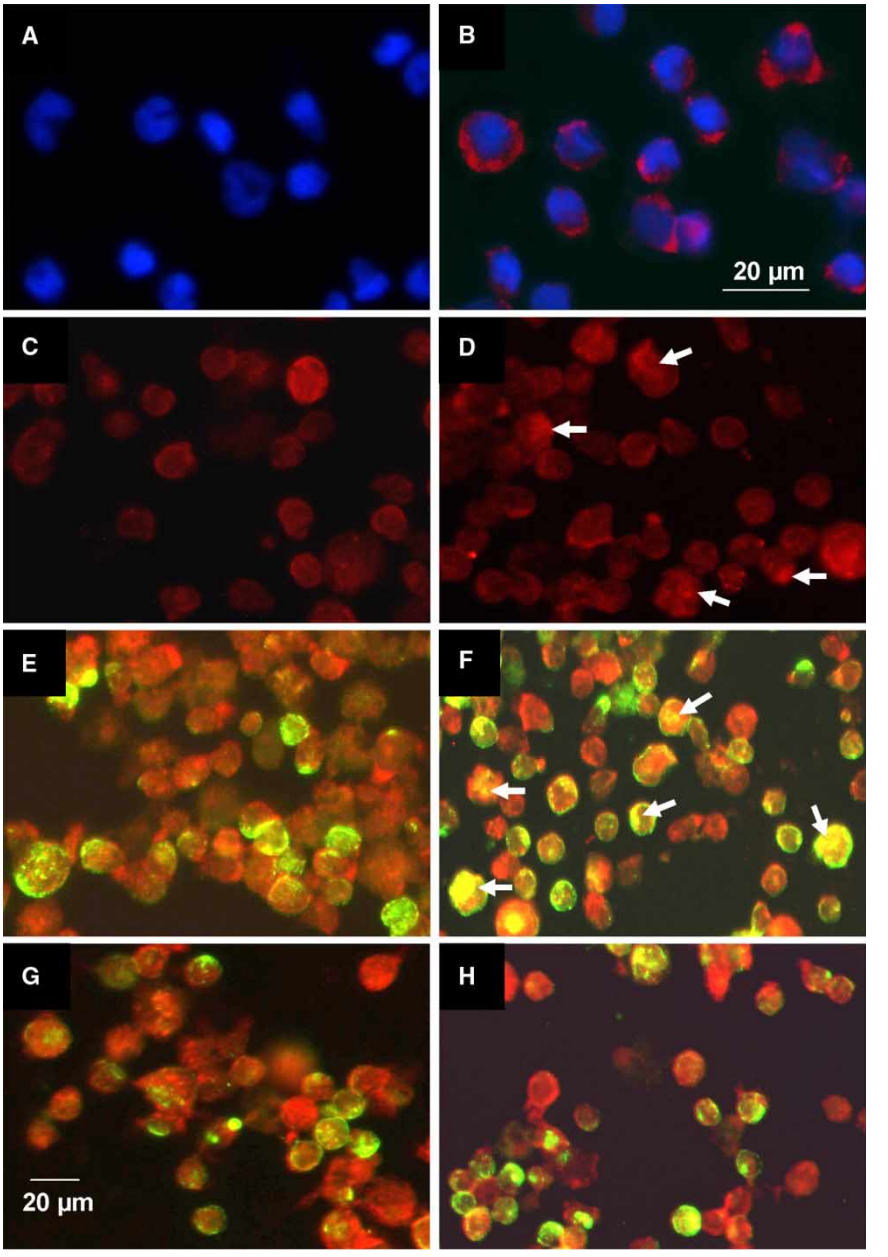

Figure 8. Immunofluorescence labelling of p47phox and lipid rafts in dimethylsulphoxide-differentiated HL-60 cells. (A and B) P47phox was labelled with a Cy3-conjugated secondary antibody (red in B), but not in negative control slides (A) in which normal goat IgG was used instead of the primary antibody. Nuclears were counter stained with DAPI (blue). (C and D) p47phox was present in a diffused manner in resting cells (C). Following stimulation with PMA, p47phox immunoreactivity aggregated into clusters as demonstrated by the fluorescent patches (D, arrows). (E and F) Merged images of double labelling of p47phox (red) and lipid rafts (green) showing that, as compared to resting cells (E), PMA stimulation induced colocalization of p47phox and lipid rafts (yellow colours indicated by arrows in F). This PMA-induced colocalization of p47phox and lipid rafts was blunted in rHDL-treated cells (G) and almost abolished in cyclodextrin-treated cells (H). Experiments performed in duplicate.

NADPH oxidase activation in leukocytes, we carried out sub-cellular fractioning experiments, in which we demonstrated that PMA-induced mobilization of p47phox toward the lipid raft-containing fractions was blunted by rHDL. Moreover, we carried out immunofluorescence double labelling experiments. We found that PMA stimulation induced p47phox colocalization with lipid rafts and this PMA-induced colocalization of p47phox and lipid rafts was blunted in rHDL-treated cells and almost abolished in cyclodextrin-treated cells. Taken together, these data are consistent with our cholesterol depletion experiments showing that PMA-induced NADPH oxidase activation in HL-60 cells requires intact lipid rafts, supporting that rHDL-induced suppression of NADPH oxidase activation may involve altered redistribution of NADPH oxidase sub-units into the lipid raft sites.

We also examined whether rHDL affected expression of NADPH oxidase sub-units in HL-60 cells.

The real-time PCR experiments demonstrated that rHDL alone did not have any significant effects on NADPH oxidase at the mRNA level. Together with the fact that, in the functional studies with superoxide generation, the cells were only pre-treated with rHDL for 1 h followed by acute PMA stimulation, these results suggested that the inhibitory effect of rHDL on leukocyte NADPH oxidase activation was unlikely to involve altered expressions of NADPH oxidase sub-units. Moreover, we found that treatment with rHDL for 72 h did not change the protein levels of Nox2, p47phox or p67phox in undifferentiated HL-60 cells. On the other hand, we observed that rHDL co-treatment slightly blunted the upregulation of p47phox expression during HL-60 cell differentiation induced by dimethylsulphoxide. This may be related to an inhibitory effect of HDL on leukocyte differentiation.

In contrast to leukocytes, rHDL did not have any effect on NADPH oxidase-derived superoxide production in vascular endothelial cells, either under basal conditions or after stimulation with the pro-inflammatory cytokine TNF-α, although rHDL at the same concentration markedly suppressed ICAM-1 and VCAM-1 expressions induced by TNF-α. This effect of rHDL seems to be discrepant to previous observations that native HDL or rHDL prevented reactive oxygen species formation induced by various stimuli in endothelial cells [13,31,32]. It is noted that, however, in these studies the source of reactive oxygen species was not fully characterized and enzymes other than NADPH oxidase, such as nitric oxide synthase [31], may be involved in these experimental conditions. The mechanisms underlying the differential effects of rHDL on leukocyte and endothelial NADPH oxidases are unclear and this may be related to the expression and sub-cellular localization of different isoforms of NADPH oxidase sub-units. In contrast to leukocytes, in which Nox2 is the predominant isoform of the Nox sub-unit, in endothelial cells, a major source of superoxide is the Nox4 type NADPH oxidase [33,34]. Recent studies have revealed that these two isoforms of Nox have distinct characteristics. For example, several lines of evidence have suggested that Nox4 does not require the cytosolic sub-units for superoxide production [35,36]. In addition, Nox4 is mainly located in an intracellular compartment that colocalizes with endoplasmic reticulum [37], rendering it insensitive to structural alterations of the plasma membrane. On the other hand, Nox2 is also expressed in endothelial cells and is responsible for superoxide generation [38]. However, the sub-cellular localization of the endothelial Nox2 is significantly different from that of the neutrophil oxidase, in that the endothelial Nox2 predominantly exists in intracellular compartments but not in the plasma membrane [39]. Moreover, there is evidence that the activation of NADPH oxidase in endothelial cells might not require the assembly of different sub-units in the plasma membrane as in leukocytes [40].

We also found that PLPC had a similar effect as rHDL on leukocyte NADPH oxidase activation, whereas the purified apolipoprotein A-I had an enhancing effect. Our previous studies have shown that small unilamellar vesicles of PLPC had anti-inflammatory effects both in cultured endothelial cells [41] and *in vivo* [14]. Interestingly, in neutrophils, unsaturated long chain phospholipids such as dioleoyl phosphatidylcholine and dilinoleoyl phosphatidylcholine, in micromolar concentrations have been shown to inhibit PMA-induced superoxide production [42]. Tonks et al. [25] further demonstrated that pre-incubation of human monocytes with dipalmitoyl phosphatidylcholine inhibited NADPH oxidase activation; and this effect was not attributable to a direct modulation of NADPH oxidase assembly and function, but appeared to be due to a suppression of protein kinase C activation. To examine whether PLPC may modulate PKC activation in HL-60 cells, we measured membrane translocation of PKC following PMA treatment. However, we found that PLPC had little effect on PMA-induced PKC activation. Thus, the inhibitory effect of PLPC on PMA-induced superoxide production in HL-60 cells requires further investigation. On the other hand, the mechanism of the observed enhancing effect of apolipoprotein A-I on superoxide release from HL-60 cells is unknown. We cannot totally exclude the possibility that this effect is caused by other constituents contained in the apolipoprotein A-I preparation.

In summary, our results suggest that inhibition of NADPH oxidase activation in leukocytes, probably through cholesterol depletion and interruption of the assembly of NADPH oxidase sub-units at the lipid rafts, may be a mechanism by which the reconstituted HDL exerts protective effects against vascular inflammation.

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