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Membrane raft–lysosome redox signalling platforms in coronary endothelial dysfunction induced by adipokine visfatin

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Aims The adipokine visfatin, produced during obesity, has been reported to participate in the development of cardiovascular disease associated with metabolic syndrome. The present study was designed to test a hypothesis that visfatin causes coronary endothelial dysfunction through lysosome trafficking and fusion to cell membranes, membrane raft (MR) clustering, and formation of redox signalosomes.

Methods and results By using confocal microscopy, it was found that visfatin, but not adiponectin, stimulated NADPH oxidase (NOX) subunits, gp91^{phox} aggregation in MR clusters and p47^{phox} translocation to these MR clusters in bovine coronary arterial endothelial cells (CAECs), leading to activation of NOX with a 2.5-fold increase in $O_2^{\cdot-}$ production. A signalling lipid, ceramide, was found to be enriched in such membrane MR–NOX complexes of CAECs. Lysosomal fluorescent dye (FM1-43) quenching and de-quenching revealed that visfatin induced the fusion of lysosomes to cell membranes and incorporation of acid sphingomyelinase and its product, ceramide, in such MR–NOX signalling platforms. Functionally, visfatin significantly attenuated endothelium-dependent vasodilation in small coronary arteries (by 80%), which was blocked by lysosomal function inhibitor and MR disruptors.

Conclusion These results suggest that lysosome-associated molecular trafficking and consequent ceramide accumulation in cell membrane may mediate the assembly of NOX subunits and their activation in response to adipokine visfatin in CAECs, thereby producing endothelial dysfunction in coronary circulation.

Keywords Lipid rafts • Transmembrane signalling • Coronary artery • Oxidative stress • Sphingolipids

1. Introduction

The adipokine visfatin has been reported to be an injurious factor leading to vascular dysfunction in obesity. This adipokine was first identified as a pre-B-cell colony-enhancing factor and plays roles in the early development of B lymphocytes.[1] Later, it was isolated and named visfatin from visceral adipose tissue with insulin-like function.[2] Despite obstacles involving non-repeatable results in early studies,[3–5] recent studies have confirmed that visfatin is, indeed, an adipokine which may be involved in the development of various obesity-associated pathological changes.[6–13] Evidence has been accumulated that implicates visfatin as a nicotinamide phosphoribosyltransferase that regulates nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylase activity when it works as a dimer.[13] Most recently, visfatin has been reported to be increased in the blood of individuals with chronic kidney disease[8,11] and foetal growth retardation.[14] The visfatin plasma level is inversely associated with flow-mediated vasodilation.[7,11] Moreover, increased plasma levels of visfatin have been detected in the abdominal and epicardial adipose tissues in patients with coronary artery diseases,[9,15] which importantly participated in the development of coronary atherosclerosis.[15,16] Although it has been suggested that visfatin is a potential risk factor for endothelial injury under certain pathological conditions, so far there has been no direct evidence confirming that visfatin causes coronary endothelial damage and how it does so.

Our previous studies have demonstrated that membrane rafts (MRs, formerly lipid rafts) clustering on the arterial endothelial cells (ECs) is an important pathogenic mechanism resulting in endothelial dysfunction. This MR clustering has been considered as an initiating mechanism in endothelial injury in response to damaging

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factors such as death receptor ligands, inflammatory factors, or irradiation.[17–20] It has been known that MR clustering recruits or aggregates redox signalling molecules such as NADPH oxidase (NOX) subunits, gp91^{phox}, p47^{phox}, and Rac GTPase, resulting in the formation of a membrane signal amplification platform that activates and enhances production of superoxide ($O_2^{\cdot-}$), which could be inhibited by an MR disruptor, methyl-β-cyclodextrin (MCD), or a cholesterol depletion reagent, filipin. Such MR signalling platforms with $O_2^{\cdot-}$ production has been referred to as MR redox signalling platforms, which are now taking centre stage in studies on endothelial impairment induced by death receptors, as suggested by an editorial commentary of *Hypertension*.[17,21–24] The formation of MRs in the EC membrane has been reported to be associated with the production of ceramide via lysosomal acid sphingomyelinase (ASMase). This ASMase is translocated onto the plasma membrane via membrane proximal lysosome trafficking and fusion upon stimulation of death receptors.[22,25–28] The question remained whether this MR clustering and redox signalling mechanism is also involved in the action of visfatin on endothelial function.

In the present study, the hypothesis being tested was that the early action of visfatin causing endothelial dysfunction is due to lysosomal ASMase activation, the formation of MR redox signalling platforms and consequent local oxidative stress. To test this hypothesis, we first tested whether visfatin induces coronary endothelial dysfunction in an isolated coronary arterial preparation. Then, we determined whether visfatin-induced endothelial dysfunction is due to its stimulatory effect on MR clustering that results in the aggregation and assembling of NOX subunits, leading to $O_2^{\cdot-}$ production, using confocal co-localization of NOX subunits in MRs and $O_2^{\cdot-}$ quantitation by electron spin resonance (ESR) spectrometry. Finally, we explored the mechanisms by which visfatin induces MR clustering through the translocation and activation of ASMase via lysosome fusion by specific fluorescence quenching and de-quenching methods.

2. Methods

2.1 Isolated small coronary artery tension recording

Fresh bovine hearts were obtained from a local abattoir. Small coronary arteries (~200 μm ID) were prepared for tension recording in a Multi Myograph 610 M (Danish Myo Technology, Aarhus, Denmark) to evaluate the endothelium-dependent vasodilator response as we described previously.[29] Since blood concentrations of visfatin range from 35–50 ng/mL in normal subjects to 60–80 ng/mL in obese subjects,[8,30] 100 ng/mL of visfatin was used in our experiments. In various investigations, the doses of visfatin have been reported to be ranging from 5 to 500 ng/mL.[31,32] Detailed protocols are presented in Supplementary material online.

2.2 Confocal analysis of MR clusters and its co-localization with NOX subunits, ceramide, or ASMase in coronary arterial ECs

For microscopic detection of MR platforms, coronary arterial ECs (CAECs) were grown on glass cover slips and then treated with 2 μg/mL visfatin (BioVision, Mountain View, CA, USA) for 4 h and then clustering of MRs was detected. Additional groups, MCD (Sigma, St Louis, MO, USA; 1 mM, 30 min), amitriptyline (Sigma; 1 mg/mL, 30 min), or vacuolin-1 (Sigma; 10 μM, 1 h), were added to pre-treat the cells before visfatin stimulation. Detection of MR clusters was performed as described previously.[17,25,27,33,34] Detailed MR cluster detection and dual staining methods are presented in Supplementary material online.

2.3 Quenching and de-quenching of FM1-43 for detection of lysosome fusion in CAECs

As we and others described previously,[25,35,36] CAECs were loaded with 8 μM FM1-43 in 1640 medium with 10% FBS for more than 2 h at 37°C. After washing with FBS-free medium, a low laser power (λ excitation = 488 nm) was used to avoid possible fluorescent bleaching. For FM1-43 quenching experiments, after cells were loaded with 8 μM FM1-43 for 2 h, 1 mM BPB was added in the extracellular medium before observations. For FM1-43 de-quenching experiments, cells were loaded simultaneously with 8 μM FM1-43 and 1 mM BPB for 2 h.

2.4 Assay of ASMase activity and liquid chromatography–electrospray ionization tandem mass spectrometry for quantitation of ceramide

The activity of ASMase was assessed by incubation of CAEC homogenates with its substrate, *N*-methyl-[^{14}C]-sphingomyelin, and then radiospectrometry of the product, ^{14}C-choline (see detail inSupplemental material online). Separation, identification, and quantitation of ceramide in ECs were performed by liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS) as we described previously.[29] Detailed protocols are presented in Supplemental material online.

2.5 NOX Assay

A dihydroethidium (DHE)-based fluorescence spectrometric assay was used as we described previously.[17] Briefly, 20 μg proteins of CAECs were incubated with 100 μM DHE and 0.5 mg/mL salmon test DNA (binds ethidium to amplify fluorescence signal) in 200 μL of PBS. NADPH (1 mM) was added immediately before recording ethidium fluorescence by a fluorescence microplate reader (FLX800, Bio-Tek). The ethidium fluorescence increase (arbitrary unit) was used to represent NOX activity.

2.6 ESR measurement of NOX-dependent $O_2^{\cdot-}$ production

For detection of NOX-dependent $O_2^{\cdot-}$ production, proteins from CAECs were extracted using sucrose buffer and resuspended with modified Krebs–Hepes buffer containing deferoximine (100 μM, Sigma) and diethyldithiocarbamate (5 μM, Sigma). The NOX-dependent $O_2^{\cdot-}$ production was examined by addition of 1 mM NADPH as a substrate in 50 μg protein and incubation for 15 min at 37°C in the presence or absence of SOD (200 U/mL), and then supplied with 1 mM $O_2^{\cdot-}$ specific spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Noxygen, Elzach, Germany). The mixture was loaded in glass capillaries and immediately analysed for $O_2^{\cdot-}$ production in a Miniscope MS200 ESR spectrometer (Magnettech Ltd, Berlin, Germany). The results were expressed as the fold changes of control.

2.7 Statistics

Data are presented as means ± SE. Significant differences between- and within-multiple groups were examined using ANOVA for repeated measures, followed by Duncan's multiple-range test. A value of $P < 0.05$ was considered statistically significant.

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3. Results

3.1 Visfatin but not adiponectin impaired endothelium-dependent vasodilation to bradykinin

In endothelium-intact coronary arterial preparation, bradykinin (BK) produced a concentration-dependent vasodilation with a maximal reduction of vessel tension by 77.2 ± 5%. Incubation of these small coronary arteries with visfatin (100 ng/mL for 1 h) significantly inhibited the vasodilator response of coronary arteries to BK as shown by a down-shift of vasodilatory curve. However, adiponectin (ADP, 1 μg/mL) had no effect on BK-induced vasodilation (*Figure 1A*). As shown in *Figure 1B*, visfatin-induced impairment of vasodilation was blocked when the arterial endothelium was pre-treated by an MR disruptor, MCD or filipin. Similarly, inhibition of ASMase, a determining enzyme for MR clustering, by amitriptyline or silencing of ASMase gene was found to substantially block visfatin-induced impairment of coronary vasodilation (*Figure 1C*).

3.2 Detection of visfatin-induced MR clustering in EC membrane

To explore the mechanism by which visfatin induces impairment of endothelium-dependent vasodilation, fluorescent confocal microscopy was conducted to detect MR clusters. As shown in *Figure 2A*, under resting conditions, MRs were found to be homogenously distributed throughout the EC membrane as indicated by weak diffusing green fluorescence with some random punctuate stainings (control). After visfatin incubation, however, green fluorescent patches were detected in the EC membrane (shown by label of visfatin). *Figure 2B* summarized the effects of different inhibitors on MR clustering in the EC membrane by counting these positive percentages of MR clusters or patches. As shown in *Figure 2C*, under resting conditions, only 22.8% of the cells displayed intense MR clusters, whereas ADP did not alter MR clustering (28.5%). 65.9% of ECs displayed MR clusters or patches after they were treated with visfatin, which was similar to FasL, a classical stimulator of MR clustering (73.1%). The use of different disruptors of MRs such as MCD or filipin attenuated visfatin-induced clustering of MR. In addition, the ASMase inhibitor, amitriptyline, also restrained visfatin-induced MR clustering by 45.3%.

3.3 Visfatin-induced NOX subunits aggregation in MR clusters

Our previous studies demonstrated that MR clustering in response to death receptor activation leads to NOX aggregation and assembling, thereby activating this enzyme. The present study also examined whether visfatin-induced MR clustering leads to the assembling of NOX subunits to produce activity. Indeed, a membrane NOX subunit, gp91^{phox}, and a cytosolic subunit, p47^{phox}, were found to be co-localized with a relatively specific MR marker, CTX-B. As shown in *Figure 3A* and *B*, co-localization was significantly increased when ECs were treated with visfatin, indicating the formation of complex of MR-NOX. Such co-localization of an MR marker with NOX subunits was blocked by the MR disruptor, MCD, or by the ASMase inhibitor, amitriptyline. In addition, given that ASMase as a key enzyme for MR clustering is a lysosomal enzyme, we tested whether visfatin-induced formation of MR–NOX complex is associated with lysosome trafficking or fusion as we reported previously.[25,28] It was found that the lysosome fusion inhibitor, vacuolin-1, markedly reduced co-localization of NOX subunits with MR markers. Another additional experiment showed that in caveolin-1 siRNA-transfected cells, gp91^{phox} and p47^{phox} were still strongly co-localized with CTX-B after visfatin stimulation, which indicated that such visfatin-induced MR cluster formation was not a caveolae-related raft. Data from a co-localization coefficient analysis are summarized in *Figure 3C*; visfatin indeed caused the aggregation of NOX subunits in MR areas, and therefore, MR clustering may provide physical force to assemble these NOX subunits.

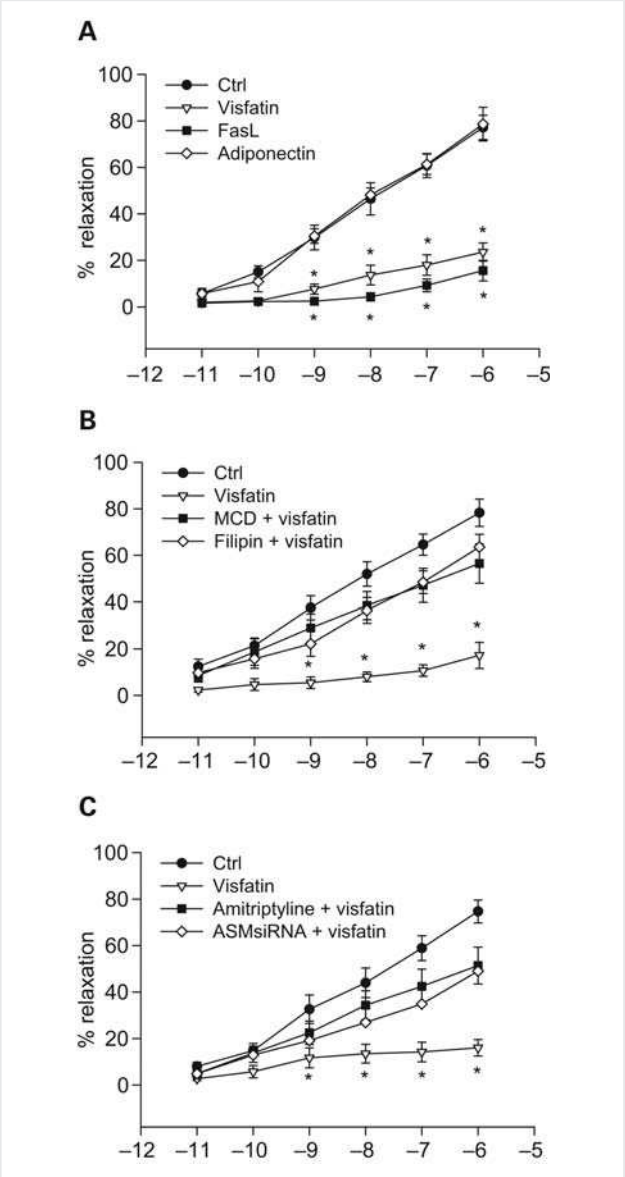

Figure 1 Concentration–response curves of endothelium-dependent vasodilation induced by BK in the freshly isolated small coronary arteries. (*A*) The response of arteries pre-treated with visfatin (100 ng/mL for 1 h), ADP (1 μg/mL for 1 h), or FasL (10 ng/mL for 20 min). (*B*) The response of arteries in the presence of MR disruptor MCD (1 mM for 20 min) or filipin (1 μg/mL for 20 min) before visfatin administration. (*C*) The response of arteries in the presence of ASMase inhibitor amitriptyline or after ASMase gene silencing prior to visfatin stimulation. $n = 6$ cow hearts, $^*P < 0.05$ vs. control group.

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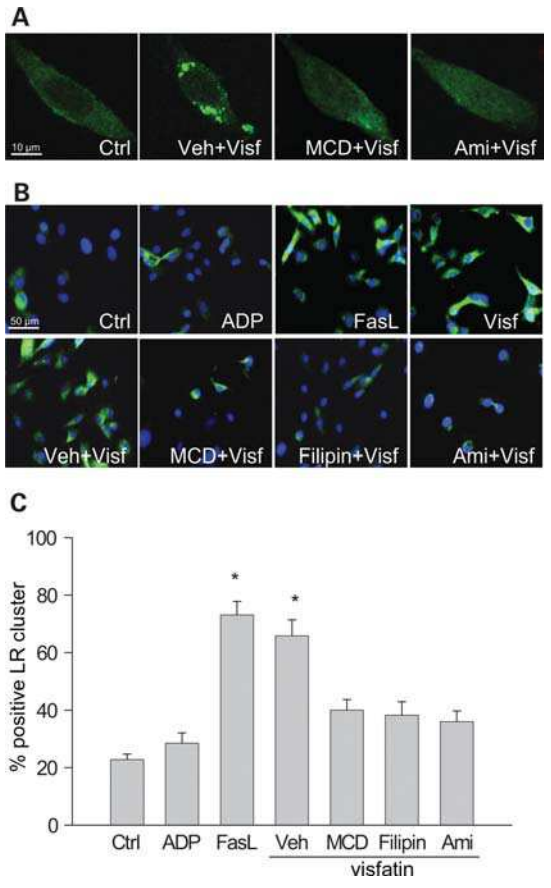

Figure 2 Visfatin-induced MR clustering on CAEC membrane. (*A*) Representative confocal fluorescence images for MR clusters on the CAEC membrane induced by visfatin or revised by MR disruptor, MCD or ASMase inhibitor, amitriptyline. (*B*) Summarized images showing the MR clusters after administration of ADP, FasL, visfatin, MCD + visfatin, filipin + visfatin, and Ami (amitriptyline) + visfatin. (*C*) Summarized quantitative percentage of cells with MR clusters in different treatments. $n = 6$ batches of cells, $^{*}P < 0.05$ vs. control group.

3.4 Visfatin increased NADPH activity and NOX-dependent $O_2^{\cdot-}$ production in CAECs

To further provide functional evidence that the aggregation and assembling of NOX subunits activate $O_2^{\cdot-}$ production, we performed two series of experiments to detect NOX activity and $O_2^{\cdot-}$ production. As shown in Supplementary material online, *Figure S1*, visfatin induced a significant increase in NADPH activity and NOX-dependent $O_2^{\cdot-}$ production (*Figure 3D*) in CAECs, and such an increase in NADPH activity and $O_2^{\cdot-}$ production could be completely blocked by the MR disruptor, MCD, ASMase inhibitor or ASMase siRNA, or lysosome fusion inhibitor, vacuolin-1. It is clear that NOX assembling in MR clusters or platforms is activated and that this activation is dependent upon the translocation of ASMase from lysosomes and consequent MR clustering.

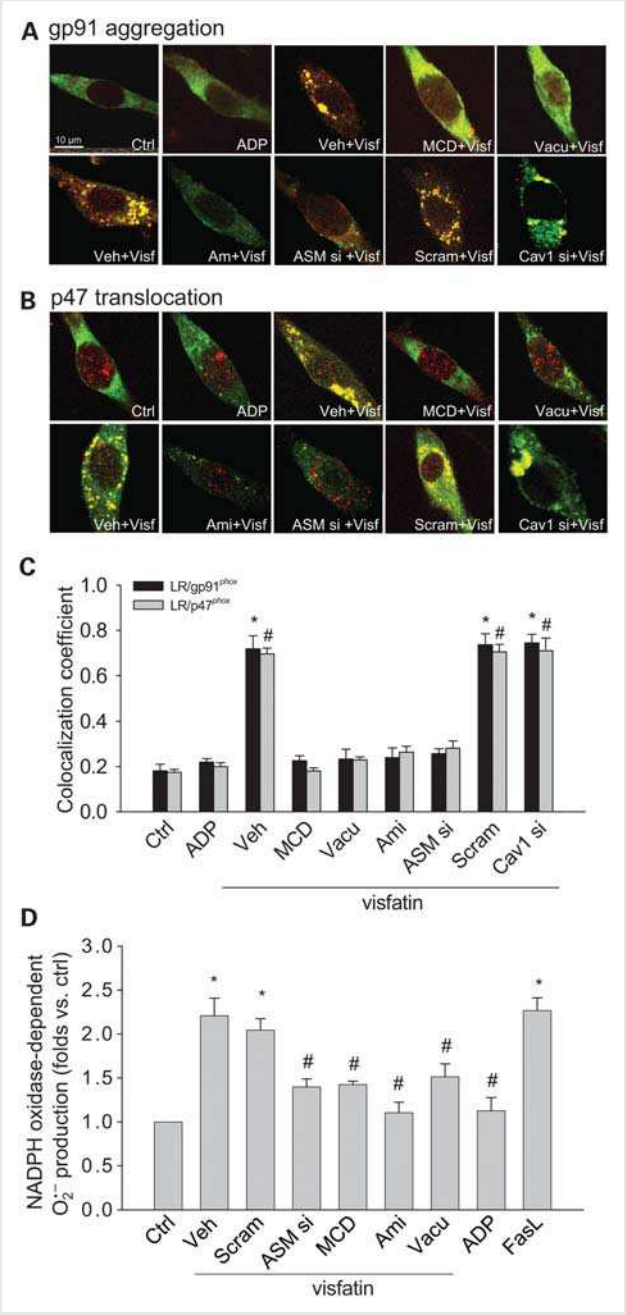

Figure 3 Co-localization of MRs and gp91^{phox} (*A*) or p47^{phox} (*B*) detected by confocal microscopy in intact and permeabilized CAECs after treatment of ADP, visfatin, MCD + visfatin, vacuolin-1 + visfatin, Ami (amitriptyline) + visfatin, ASMase siRNA + visfatin, scrambled sRNA + visfatin, and caveolin-1 siRNA + visfatin. Only overlaid images were presented. Yellow spots in overlaid images indicate co-localization of the MR marker, GM1 and gp91^{phox} or p47^{phox}. (*C*) Summarized co-localization coefficient data showing co-localization levels of MRs and gp91^{phox} or p47^{phox} after different stimulations. $n = 6$ batches of cells, $^{*}P < 0.05$ vs. gp91^{phox} control group; $^{\#}P < 0.05$ vs. p47^{phox} control group. (*D*) Summarized data depicting changes in NOX-dependent $O_2^{\cdot-}$ production in CAECs with different treatments. $n = 6$ batches of cells, $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. vehicle group.

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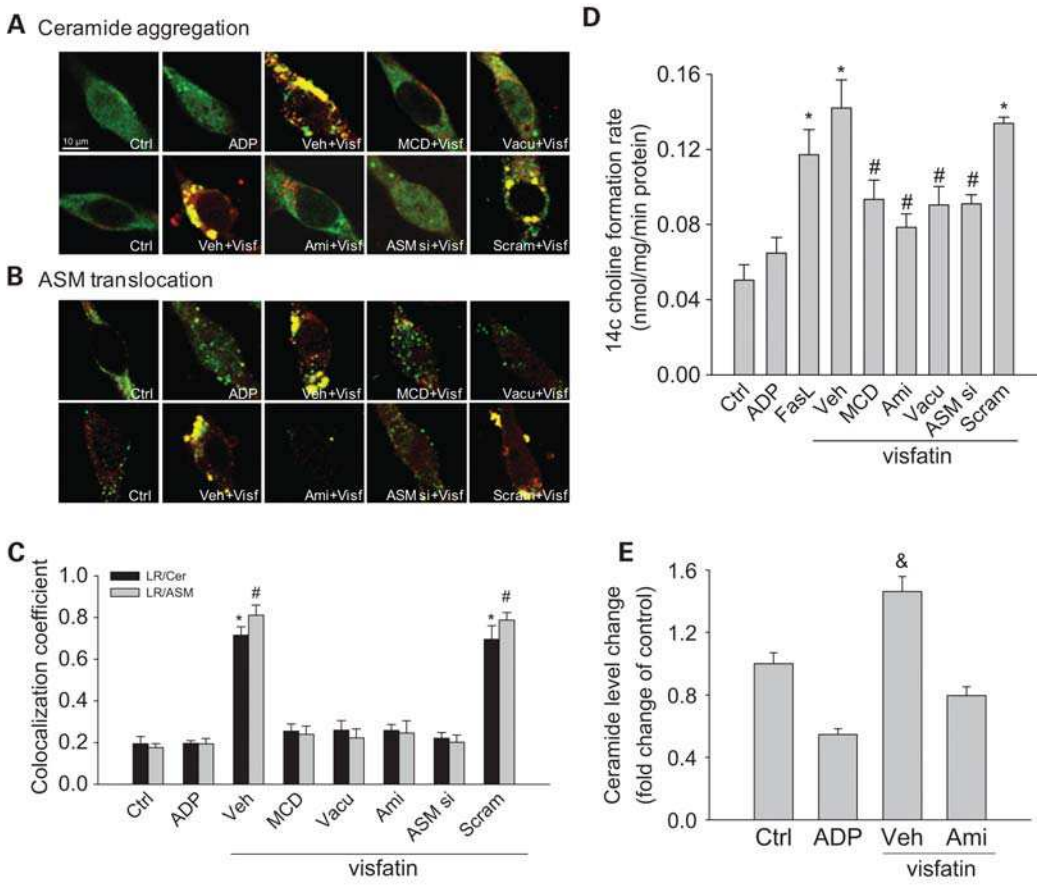

Figure 4 Co-localization of MRs and ceramide (*A*) or ASMase (*B*) detected by confocal microscopy in intact and permeabilized CAECs after treatment of ADP, visfatin, MCD + visfatin, vacuolin-1 + visfatin, Ami (amitriptyline) + visfatin, ASMase siRNA + visfatin, and scrambled sRNA + visfatin. Only overlaid images were presented. Yellow spots in overlaid images indicate co-localization of MRs and ceramide or ASMase. (*C*) Summarized co-localization coefficient data showing co-localization levels of MRs and ceramide or ASMase after different stimulations. $n = 6$ batches of cells, $^{*}P < 0.05$ vs. ceramide control group; $^{\#}P < 0.05$ vs. ASMase control group. (*D*) Effects of adipokines on the activity of ASMase in CAECs with or without inhibitors, MCD, Ami (amitriptyline), Vacu (vacuolin-1), and ASMase siRNA. FasL was used as the positive control. $n = 6$ batches of cells, $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. vehicle group. (*E*) Effects of adipokines on the production of ceramide in CAECs analysed by LC–ESI-MS. $n = 6$ batches of cells, $^{\&}P < 0.05$ vs. control group.

3.5 Visfatin-induced ASMase translocation and ceramide production in MR formation via confocal microscopy

The next question we addressed was how visfatin stimulates MR clustering. To answer this question, we detected ASMase translocation to MR of the membrane from lysosomes and local ceramide production, which have been reported as critical mechanisms for MR clustering in endothelial and other cells. As shown in *Figure 4A*, visfatin, but not ADP, markedly increased ceramide production locally in MR clusters, as shown in yellow spots or patches stained by CTX-B (green fluorescence) and anti-ceramide antibody (red fluorescence). In the presence of the MR disruptor MCD, ASMase inhibitor amitriptyline, or ASMase siRNA, such visfatin-induced local production of ceramide in MR clusters was abolished. In addition, lysosome fusion inhibitor, vacuolin-1, also blocked the production of ceramide.

It was also found that this ceramide production was derived from ASMase activation since ASMase was also co-localized in the same area of the cell membrane, within MRs. Similarly, MR disruption, ASMase inhibition, or silencing and lysosome fusion inhibition blocked the occurrence of ASMase in MR clusters (*Figure 4B*). The results from co-localization coefficient analysis are shown in *Figure 4C*. It is clear that visfatin increased co-localization of ceramide with the MR marker-ganglioside GM1 or local ASMase. This co-localization of ceramide or ASMase with the MR markers was attenuated by MCD, amitriptyline, ASMase siRNA, or vacuolin-1.

3.6 ASMase activity and ceramide production in CAECs with treatment of adipokines

We also analysed the activity of ASMase and tested the effect of adipokines on ASMase activity. In these experiments, the metabolite of sphingomyelin via ASMase, [^{14}C]-choline phosphate, was measured. As shown in *Figure 4D*, visfatin significantly increased the ASMase activity by three-fold, but ADP had no effect on ASMase activity. After pre-treatment of ECs with the MR blocker—MCD, ASMase inhibitor—amitriptyline, ASMase siRNA, or lysosome fusion

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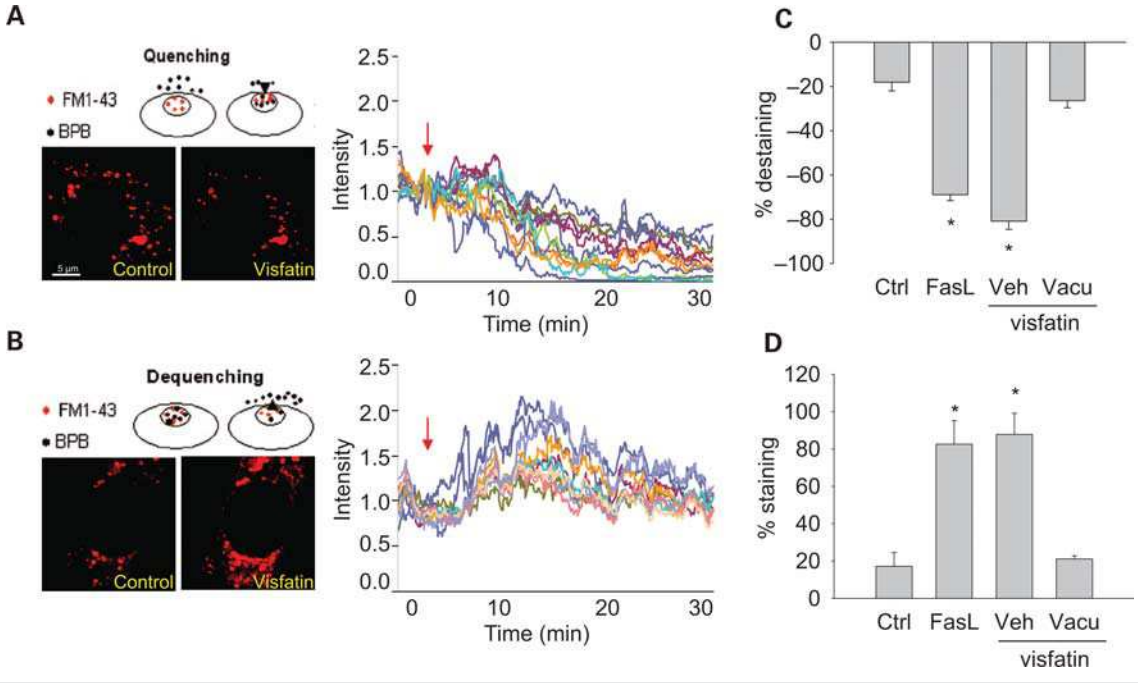

Figure 5 Visfatin-induced lysosome fusion. (*A* and *B*) Representative fluorescent images and normalized fluorescent intensity trace for quenching (upper panel) and de-quenching (lower panel) of FM1-43 by BPB to monitor lysosome fusion to cell membrane induced by visfatin. The arrow indicated the beginning of treatments. (*C* and *D*) Summarized results showing changes in FM1-43 fluorescence in the vehicle or vacuolin-1 (10 μM for 1 h)-pre-treated group of cells normalized by the fluorescence obtained before treatment. $n = 80$ puncta from five batches of cells, $^*P < 0.05$ vs. control group.

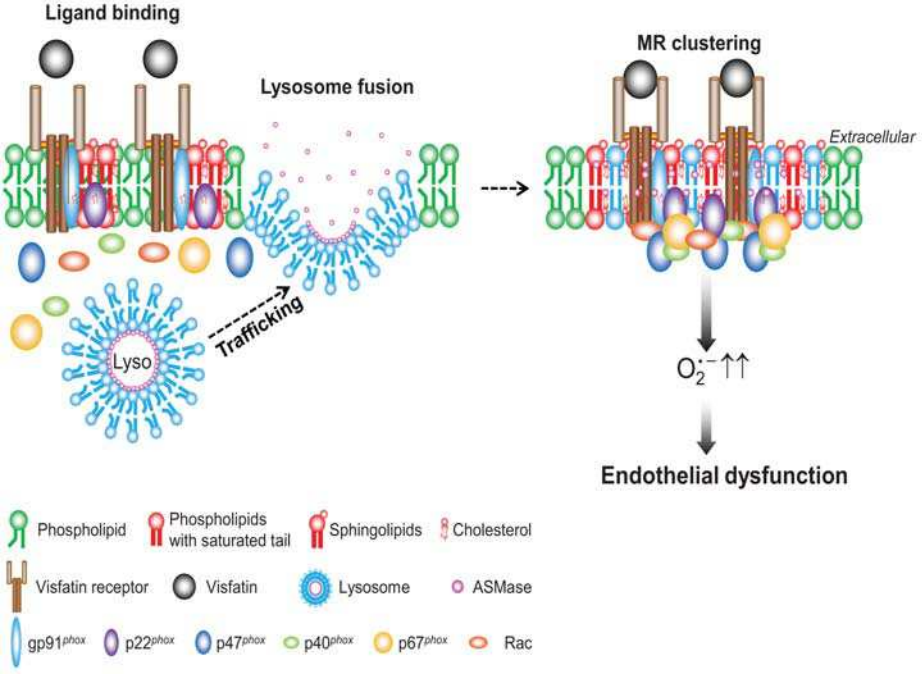

Figure 6 Diagram of visfatin-induced MR clustering that results in the formation of MR redox signalling platforms in CAECs.

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inhibitor—vacuolin-1, the increase in ASMase activity induced by visfatin was significantly attenuated. As shown in *Figure 4E*, LC–ESI-MS analysis also demonstrated that visfatin, but not ADP, increased ceramide production in CAECs. When these cells were pre-treated with the ASMase inhibitor, amitriptyline, visfatin failed to increase ceramide production. This is consistent with the results obtained above by co-localization of ceramide in MR clusters.

3.7 Visfatin induced lysosome fusion

Given that ASMase is located on the lysosome membrane, we performed additional experiments to provide direct evidence that visfatin induced lysosome fusion, which leads to the translocation and activation of ASMase on the cell membrane. In these experiments, both FM1-43 quenching and de-quenching were observed to indicate lysosome fusion. As shown in *Figure 5A*, FM1-43 quenching by BPB and consequent lysosomal fluorescence reduction were observed in CAECs, when these cells were treated with visfatin. Since BPB was added in the bath solution, it could not have access to quench FM1-43 in lysosomes under resting conditions. Only if lysosome fuses to the cell membrane, BPB enters lysosomes to quench FM1-43. The typical FM1-43 quenching curves recorded from a number of cells or lysosomes show decrease in FM1-43 fluorescence intensity. In de-quenching experiments, both FM1-43 and BPB were loaded into lysosomes together and therefore there was low FM1-43 fluorescence detected due to quenching of BPB under control conditions. When visfatin induced lysosome fusion to cell membrane, BPB as a fast diffusing fluorescence dye diffused out of cells and thereby caused de-quenching of FM1-43 leading to gradual increases in fluorescence in lysosomes (*Figure 5B*). These results are summarized in *Figure 5C* and *D*. Visfatin and a positive lysosome fusion compound, FasL, produced both FM1-43 quenching and de-quenching by more than 80%. In the presence of vacuolin-1, visfatin-induced FM1-43 quenching and de-quenching or lysosome fusion was significantly reduced.

4. Discussion

The present study demonstrated that the adipokine visfatin induced lysosome fusion and consequent translocation of ASMase, which resulted in ceramide production. As a fusagen, local ceramide induced MR clustering, leading to the formation of MR signalling platforms in the EC membrane. These signalling platforms recruit and assemble NOX subunits to form an active redox signalling complex within MR clusters. It is the redox signalling platform formation and $O_2^{\cdot-}$ production that mediate visfatin-induced reduction in NO bioavailability and impairment of endothelium-dependent vasodilation.

In functional studies, we found that visfatin, but not ADP, remarkably impaired the endothelium-dependent vasodilator response of these coronary arteries to BK. This impairing effect of visfatin on endothelium-dependent vasodilation is similar to our previous results obtained with death receptor agonists, FasL and TNF-α.[21,25] In previous studies, visfatin was also reported to induce endothelial dysfunction in cultured cell preparations[32] and in some obese patients.[7] However, a recent study has reported that visfatin produced vasodilation in rat aorta.[37] The reason for such discrepancy is unknown. It is possible that the doses used in our studies were different from those in the previous report. There are also possible differences existing between species or vascular beds since, in this study, small bovine small coronary arteries were used compared with rat aorta from the previous study.

One of the interesting findings is that the damaging effect of visfatin on BK-induced endothelium-dependent vasodilation was blocked by the MR disruptor, MCD or filipin, indicating that endothelial MR clustering is involved in the action of visfatin, which is similar to the action of death receptor activation by FasL, TNF-α, endostatin.[17,28,33] It seems that visfatin acts like an inflammatory or death receptor ligand, which leads to the formation of MR signalling platforms and causes endothelial injury. This view is further supported by our findings that the inhibitor of ASMase, amitriptyline, or ASMase gene silencing almost completely abolished visfatin-induced impairment of endothelium-dependent vasodilation. Since ASMase is an enzyme that serves as an initiating factor to activate MR clustering and thereby results in MR-associated transmembrane signalling, the inhibition of visfatin action by its inhibitor or gene silencing further links the action of visfatin to MR clustering and consequent formation of the MR signalling platforms or signalosomes in coronary arterial ECs.

To further address the role of endothelial MR clustering or MR signalling platforms in mediating the action of visfatin and to explore related cell and molecular mechanisms mediating the formation of such visfatin-induced MR signalling platforms, a series of experiments in primary cultures of bovine CAECs were performed. Confocal microscopy showed that increased formation of MR clusters in the cell membrane of CAECs after visfatin stimulation could be blocked by pre-treatment of the cells with MR disruptors, ASMase inhibitor, or siRNAs. These results provide direct evidence that visfatin indeed stimulates the formation of MR platforms in the endothelial membrane, which is dependent upon the ASMase activity. Furthermore, it was found that visfatin-induced MR clustering led to the aggregation and translocation of NOX subunits and, thereby, assembled and activated this redox signalling enzyme in the EC membrane. Double staining of CAECs for MR marker (such as GM1 labelled by CTX-B) and NOX subunits (labelled by their antibodies) showed that visfatin, but not ADP, enhanced co-localization of these components which were originally speared across the cell membrane or in the cytosol. When MR disruptors, ASMase inhibitors or siRNA were used, the co-localization of NOX subunits with MR marker was abolished. These findings demonstrate, for the first time, that visfatin stimulates MR clustering and drives NOX assembly in the endothelial membrane.

The next question was whether assembled NOX within MR clusters is activated to produce $O_2^{\cdot-}$, thereby damaging endothelium-dependent vasodilation because $O_2^{\cdot-}$ has been reported to be an endothelial injurious factor under different pathological conditions.[17,33,38] As measured by ESR analysis, visfatin indeed increased $O_2^{\cdot-}$ production in CAECs, which was blocked or attenuated by MR disruptors, ASMase inhibitors, or siRNAs. It is obvious that MR clustering-associated assembly of NOX in the cell membrane leads to activation of this $O_2^{\cdot-}$-producing enzyme. Increased $O_2^{\cdot-}$ production interacts with nitric oxide (NO) to reduce its bioavailability, resulting in reduction or impairment of endothelium-dependent vasodilation. This $O_2^{\cdot-}$ and NO interaction has been reported as an important mechanism in mediating endothelial dysfunction induced by many different cytokines, death receptor ligands, and inflammatory factors.[17,21–24] It is possible that MR clustering may be an initiating event when visfatin acts on ECs or the intact endothelium in arteries.

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In additional experiments, the mechanism by which visfatin induces MR clustering and leads to the formation of MR-NOX signalling platform or signalosomes was explored. As discussed above, ASMase is capable of producing ceramide, and the latter may cause MR clustering.[34] This study provided several lines of evidence to support this view for the role of ASMase–ceramide in mediating the effect of visfatin. First, the ASMase inhibitor or siRNA was found to block visfatin-induced endothelial dysfunction or the corresponding reduction in the formation of MR-NOX signalling platforms. Secondly, ceramide as a membrane fusagen was produced increasingly when ECs were stimulated by visfatin as shown locally by confocal microscopy or in total by LC–ESI-MS analysis. Thirdly, confocal microscopy demonstrated that ASMase was translocated into the MR area of the EC membrane, which may produce ceramide locally to initiate or promote MR clustering, resulting in the activation of NOX and redox signalling or regulation. Finally, the biochemical analysis of ASMase activity showed that ASMase was activated by visfatin. All of these results further emphasize that ASMase is an important enzyme mediating the action of visfatin to cause MR clustering and lead to the formation of MR–NOX redox signalling platforms in coronary arterial ECs. It is the MR redox signalling platform that produces $O_2^{\cdot-}$, reducing NO bioavailability and ultimately impairing endothelium-dependent vasodilation.

Another important question addressed was how ASMase, a lysosome enzyme, could be translocated into the MR clusters and mediate such important transmembrane signalling when CAECs were stimulated by visfatin. On the basis of previous reports that lysosome movement is importantly involved in ASMase translocation in different cells in response to a variety of stimuli,[25,39] the present study provides evidence demonstrating that visfatin stimulates lysosome trafficking and fusion to membranes and results in lysosomal ASMase translocation and activation in MR clusters. For example, we found that an inhibitor of the lysosome fusion inhibitor, vacuolin-1, was shown by confocal microscopy to inhibit MR clustering and co-localization of MR markers with NOX subunits, indicating that lysosome fusion to cell membrane does participate in the formation of MR redox signalling platforms. This lysosome fusion inhibitor also blocked visfatin-induced $O_2^{\cdot-}$ production, adding evidence that such lysosome fusion links membrane redox signalling mediated by $O_2^{\cdot-}$. Moreover, lysosome fusion inhibition blocked the translocation of ASMase into the MR clusters in the EC membrane and thereby blocked ceramide production locally and globally. This suggests that lysosome fusion is a driving force for ASMase translocation and activation, but not the other way around. Finally, we directly examined the effect of visfatin on lysosome fusion by the measurement of FM1-43 quenching and de-quenching by BPB, which is a commonly used, accurate method to detect lysosome fusion to the cell membrane. It was found that visfatin indeed increased FM1-43 quenching and de-quenching, indicating that this adipokine increases lysosome fusion. This effect is similar to our previous results obtained by FasL stimulation or direct activation of ASMase.[25,27,28] Although the present study did not attempt to explore how visfatin stimulates lysosome fusion, it is well known that an increase in intracellular Ca^{2+}, SNAREs, and triggering ceramide itself may be involved in such lysosome fusion, which will be another full study to be reported in the near future.

In summary, the present study demonstrated that the adipokine visfatin induced MR clustering and thereby resulted in the formation of MR redox signalling platforms in CAECs. As shown in *Figure 6*, this transmembrane redox signalling mechanism associated with MR clustering may mediate the detrimental action of visfatin to induce endothelial dysfunction. When visfatin acts on ECs, lysosomes are stimulated to traffic and fuse into the cell membrane, resulting in the activation of ASMase, production of ceramide, and formation of MR–NOX redox signalling platforms. This redox signalling platform contains a variety of signalling molecules which interact to form signalosomes, which produces $O_2^{\cdot-}$ and induces endothelial dysfunction. This MR–NOX redox signalling platform is a critical molecular mechanism mediating visfatin-induced endothelial dysfunction such as impairment of endothelium-dependent vasodilation.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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