High glucose condition increases NADPH oxidase activity in endothelial microparticles that promote vascular inflammation

Felix Jansen1†, Xiaoyan Yang1†, Bernardo S. Franklin2, Marion Hoelscher1, Theresa Schmitz1, Jörg Bedorf3, Georg Nickenig1, and Nikos Werner1*

1Department of Internal Medicine II, University Hospital Bonn, Rheinische Friedrich-Wilhelms University, 53105 Bonn, Germany; 2Institute of Innate Immunity, University Hospital Bonn, Rheinische Friedrich-Wilhelms University, Bonn, Germany; and 3Institute of Pathology, University Hospital Bonn, Rheinische Friedrich-Wilhelms University, Bonn, Germany

Received 9 September 2012; revised 13 December 2012; accepted 10 January 2013; online publish-ahead-of-print 22 January 2013

Time for primary review: 32 days

Aims
Diabetes is a major risk factor for cardiovascular diseases. Circulating endothelial microparticles (EMP) are increased in diabetic patients, but their potential contribution in atherogenesis is unclear. We sought to determine the role of EMP derived under high glucose conditions in the development of atherosclerosis.

Methods and results
EMP were generated from human coronary endothelial cells (HCAEC) exposed to high glucose concentrations in order to mimic diabetic conditions. These EMP were defined as ‘injured’ EMP (iEMP) and their effects were compared with EMP generated from ‘healthy’ untreated HCAEC. iEMP injection significantly impaired endothelial function in ApoE−/− mice compared with EMP and vehicle treatment. Immunofluorescent experiments showed increased macrophage infiltration and adhesion protein expression in atherosclerotic lesions of iEMP-treated ApoE−/− mice compared with controls. To further investigate the underlying mechanism of iEMP-induced vascular inflammation, additional in vitro experiments were performed. iEMP, but not EMP, induced activation of HCAEC in a time- and dose-dependent manner and increased monocyte adhesion. Further experiments demonstrated that iEMP induced activation of HCAEC by phosphorylation of p38 into its biologically active form phospho-p38. Inhibition of p38 activation abrogated iEMP-dependent induction of adhesion proteins and monocyte adhesion on HCAEC. Moreover, we could demonstrate that iEMP show increased NADPH oxidase activity and contain significantly higher level of reactive oxygen species (ROS) than EMP. iEMP triggered ROS production in HCAEC and thereby activate p38 in an ROS-dependent manner.

Conclusion
High glucose condition increases NADPH oxidase activity in endothelial microparticles that amplify endothelial inflammation and impair endothelial function by promoting activation of the endothelium. These findings provide new insights into the pathogenesis of diabetes-associated atherosclerosis.

Keywords
Microparticles • Inflammation • Endothelium • Reactive oxygen species • NADPH oxidase

1. Introduction
Microparticles (MP) are submicron membrane vesicles (0.1–1 μm) shed from the plasma membrane of activated or apoptotic cells. Although MP formation represents a physiological phenomenon, different pathologies are associated with a substantial increase in circulating MP, including autoimmune diseases, malignancies, and inflammatory diseases.1 The levels of endothelial cell-derived microparticles (EMP) are significantly increased in diseases with systemic endothelial damage as seen in hypertension, diabetes mellitus, and coronary artery disease.2 Whether EMP might accelerate disease progression or induce vascular protection is still a matter of debate. Numerous data show that EMP can play a major role in inflammation, thrombosis, and coagulation—all conditions involved in atherogenesis.3–5 However, recent data challenged the presumed deleterious role of EMP and suggest
that EMP are not only a surrogate marker for endothelial health or disease, but display vector function important for the intercellular exchange of biological information.²,⁶ In a number of studies, EMP were shown to promote cell survival, exert anti-inflammatory effects, counteract coagulation processes, or induce endothelial regeneration.⁷–⁹ Taken together, the role of EMP in the maintenance of vascular homeostasis is more complex than initially thought.

EMP including EMP contain a multitude of biological active molecules such as proteins, cytokines, mRNAs, or microRNAs, which can be transferred to target cells and serve as subcellular vectors to propagate bioactive signals. The exact profile of molecules present in EMP depends on the functional state of the releasing cell. Jimenez et al.¹⁰ demonstrated that endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. These findings were confirmed by proteomic analysis revealing that one-third of the proteins found on EMP are specific to the stimulus initiating their release.¹¹ These data indicate that the described paradoxical effects of EMP may vary depending on the environment surrounding microparticles-releasing endothelial cells.

Diabetes mellitus is characterized by increased blood glucose levels and represents a major risk factor for cardiovascular morbidity and mortality. Given the significantly increased EMP levels in plasma of diabetic patients,² a further exploration of their potential impact on vascular health is important and necessary. EMP, derived from glucose-exposed endothelial cells could have their characteristic impact on the endothelium. In this study, we present evidence that EMP from glucose-treated endothelial cells increase vascular inflammation in vitro and in vivo. Activation of the endothelium through reactive oxygen species (ROS) might be a possible pathway.

2. Methods

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

2.1 Cell culture and MP generation

Human coronary artery endothelial cells (HCAEC, PromoCell) were cultured in endothelial cell growth media with endothelial growth media SupplementMix (Promocell) under standard cell-culture conditions (37°C, 5% CO₂). For in vitro experiments, cells of passage 4–7 were used when 70–80% confluent. In order to generate endothelial microparticles from endothelial cells under hyperglycaemic conditions, confluent HCAEC were stimulated with 30 mM glucose for 72 h and then subjected to basal media without growth media for 24 h to generate microparticles as previously described.¹⁰ After starvation, supernatant of apoptotic endothelial cells could have their characteristic impact on the endothelium. In this study, we present evidence that EMP from glucose-treated endothelial cells increase vascular inflammation in vitro and in vivo. Activation of the endothelium through reactive oxygen species (ROS) might be a possible pathway.

2.2 iEMP formation examined by confocal microscope

Glucose-treated HCAEC were labelled with calcine-AM (10 μM, Sigma) and subjected to basal media. Confocal microscope (Leica TCS SP5 AOBS) was used to scan iEMP-releasing process.

2.3 Flow cytometry

MP characterization is analysed by flow cytometry using annexin V-FITC and CD31-PET. To evaluate the size of iEMP and set the gate for MP, nile red particles 0.7–0.9 μm (Spherotech) were used as reference beads. Annexin V positive (AnnV⁺) EMP were enumerated using TrueCOUNT tubes (BD). iEMP/EMP were used at the concentration of 2000 AnnV⁺ MP/μL for all experiments unless indicated otherwise. Detailed flow cytometry analysis is described in the Supplementary material online.

2.4 Electron microscope

Pelleted iEMP was analysed by electron microscope. A detailed protocol is described in the Supplementary material online.

2.5 Animals and procedures

All animal experiments were performed in accordance with the Directive 2010/63/EU of the European Parliament. All animal work was approved and supervised by the regulatory authority of the state of Nordrhein Westfalen and in compliance with German animal protection laws.

Ten-to-twelve-week-old Apolipoprotein E-deficient mice (ApoE⁻/⁻, C57BL/6J genetic background) from Charles River were used for this study. All animals were kept in accordance with standard animal care requirements and maintained in a 22°C room with a 12 h light/dark cycle, and received food and drinking water ad libitum. All mice received a high-fat, cholesterol-rich diet that contained 21% fat, 19.5% casein, and 1,25% cholesterol (Ssniff) for a total of 8 weeks and were injected intravenously twice per week with 1 × 10⁷ Annexin V⁺ iEMP/EMP diluted in 200 μL sterilized PBS. Afterwards, the mice were sacrificed under ketaminehydrochloride (300 mg/kg, Ketanest, Pharmacia) -xylazinehydrochloride (30 mg/kg, Rompun 2%, Bayer) anaesthesia. Respiration rate, muscle relaxation, and different reflexes were used to indicate the adequacy of anaesthesia. Aortic segments, heart, and blood were collected and processed immediately after sacrifice.

To analyse human EMP uptake in vivo, ApoE⁻/⁻ mice were injected intravenously with 1 × 10⁷ EMP/iEMP or vehicle. After 30 min, mice were euthanized under anaesthesia as described earlier. The aorta was isolated free from surrounding tissues and slit opened longitudinally. Human EMP uptake by endothelium was detected by immunofluorescence staining using anti-human CD31 (Santa Cruz). To analyse EMP uptake by endothelial cells, lung lobes were excised, minced, and digested using collagenase (1 mg/mL, Sigma). Cells from lungs were isolated, stained with anti-human CD144-PE and anti-mouse CD31-APC (BD), and analysed with flow cytometry (FACS Calibur, BD). A detailed protocol for EMP uptake in vivo is described in the Supplementary material online.

2.6 Aortic ring preparations and tension recording

Vasodilation and vasoconstriction of isolated aortic ring segments, in response to phenylephrine, carbachol, and nitroglycerin, were determined in organ baths filled with oxygenated modified Tyrode buffer (37°C). A detailed protocol is described in the Supplementary material online.

2.7 Analysis of atherosclerotic plaques

For the detection of atherosclerotic lesions, sections of the aortic root were divided over a series of eight consecutive glass slides. ORO (Sigma) staining was used to detect neutral lipids in the plaque. Haematoxylin and eosin staining was used to analyse necrotic core in the plaques. Plaque macrophages were analysed by Immunofluorescence staining with anti-CD68 mAb (Actris). Vascular wall adhesion molecules were examined by staining with anti-ICAM-1 (R&D), anti-VCAM-1 (BD). A detailed protocol is described in the Supplementary material online.
2.8 Measurement of reactive oxygen species

H$_2$O$_2$ content in MP was determined by 2′, 7′-dichlorofluorescin diacetate (DCF-DA, Sigma) assay. Pelleted MP were diluted in 10 μM DCF-DA and incubated for 45 min at 37°C in dark. Washed with PBS, samples were analysed with flow cytometry (FACS Calibur, BD Biosciences).

Intracellular H$_2$O$_2$ level in MP-treated HCAEC was analysed with DCF-DA. DCF intensity was measured using ELISA Reader and fluorescent microscopy.

To measure cellular ROS production, endothelial cells were washed, trypsinized, pelleted, resuspended in Krebs-HEPES buffer with 100 μmol/L L-012, and immediately placed in a scintillation counter (Lumat LB 9501, Berthold) over 15 min for chemiluminescence analysis.

2.9 NADPH oxidase activity

NADPH oxidase activity was measured by luminescence assay. A detailed protocol is described in the Supplementary material online.

2.10 Western blot and real-time polymerase chain reaction

The detailed protocol is described in the Supplementary material online.

2.11 Immunofluorescence staining in vitro

ICAM and VCAM expression on HCAEC was analysed by Immunofluorescence staining. The detailed protocol is described in the Supplementary material online.

2.12 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Means between two groups were compared with the use of a two-tailed, unpaired Student’s t-test. The one-way ANOVA was used for comparisons of categorical variables. For post-hoc analysis, the Bonferroni test was applied. Statistical significance was assumed when a null hypothesis could be rejected at $P < 0.05$. Statistical analysis was performed using GraphPad Prism 5.

3. Results

3.1 Characterization of iEMP

Microparticles derived from glucose-treated HCAEC were defined as ‘injured’ EMP (iEMP). Compared with HCAEC under normal conditions, glucose-treated HCAEC showed obvious membrane blebbing and vesicle release after starvation in confocal microscope (Figure 1A,a). There was no difference in the morphology of membrane blebbing and microparticles between glucose-treated and non-treated HCAEC under basal medium (data not shown). To characterize iEMP isolated according to our protocol, flow cytometry and electron microscopy were used to investigate size and cellular origin. Cellular origin was explored by investigating iEMP antigen composition. Most of isolated iEMP had a size of <1 μm (Figure 1B and C) and the majority of iEMP externalized phosphatidylserine (PS) and the endothelial cell marker CD31 (Figure 1B). FSC/SSC dot plot analysis of iEMP and the supernatant showed that the supernatant after ultracentrifugation and the washing step contained negligible levels of iEMP (Supplementary material online, Figure S1), suggesting that ultracentrifugation effectively pelleted iEMP. Flow cytometric analysis showed no difference in size, PS/CD31 expression, and the amount of generated MP after serum starvation between iEMP and EMP (data not shown).

3.2 iEMP bind to murine endothelium

Previously, we have demonstrated that EMP are taken up by endothelial cells and influence endothelial cell biology in vitro. In the following experiments, we investigated whether EMP can also bind to endothelial cells in vivo and influence endothelial biology in mice. Following human iEMP-injection into mice, en face immunofluorescence experiments using anti-human CD31 staining on isolated murine aorta revealed the binding of human endothelial microparticles in the murine vessel (Figure 2A). Additional flow cytometry analysis of isolated endothelial cells from murine lung demonstrated the presence of human CD144-positive MP in murine endothelial cells (Figure 2B and C). In accordance with recently published data, these results indicate that human microparticles can be taken up by murine endothelial cells in vivo.

3.3 iEMP impair endothelial function

Diabetes is an established risk factor for accelerated atherosclerosis, with endothelial dysfunction thought to be a key element involved in the pathogenesis of athero-thrombogenic diabetic complications. EMP, produced in response to endothelial cell apoptosis or activation, are significantly increased in diabetic patients, but their potential role in atherogenesis is largely unknown. To evaluate whether iEMP derived under high glucose conditions influence the progression of atherosclerosis, ApoE$^{-/-}$ mice receiving a high-fat, cholesterol-rich diet over 8 weeks were injected intravenously twice per week with $1 \times 10^7$ iEMP/EMP or vehicle. All mice showed no adverse side effects; body weight, blood pressure, and heart rate were similar between groups (Supplementary material online, Figure S2). Compared with vehicle- and EMP-treated mice, iEMP-treated mice displayed a significantly impaired endothelial function as determined by endothelial-dependent relaxation (% of maximal contraction: 91.8 ± 4.4 in iEMP vs. 40.81 ± 6.91 in EMP vs. 41.31 ± 6.42 in control, $P = 0.07$, n = 6–8, Figure 3A). Endothelium-independent vasodilatation was not affected by iEMP or EMP treatment (Figure 3A).

To investigate whether impaired endothelial function might be associated with increased atherosclerotic plaque development, we next assessed the size of atherosclerotic lesions. We found a clear trend towards increased plaque formation in iEMP-treated mice compared with vehicle and EMP treatment (% of total vessel area: 58 ± 4.4 in iEMP vs. 40.81 ± 6.91 in EMP vs. 41.31 ± 6.42 in control, $P = 0.07$, n = 6–8, Figure 3B). Analysis of plaque composition revealed that atherosclerotic plaques in iEMP-treated mice had a significantly larger necrotic core as marker for plaque instability (% of total plaque area: 0.15 ± 0.01 in iEMP vs. 0.08 ± 0.02 in EMP vs. 0.09 ± 0.01 in control, $P < 0.01$, Figure 3C).

3.4 iEMP induce vascular inflammation

Macrophage-mediated vascular inflammation is a trigger of endothelial dysfunction and a critical event in the initiation of atherosclerosis. Therefore, we next quantified macrophage area in the vascular wall as a function of treatment of iEMP/EMP or vehicle. Immunofluorescence staining using antibodies against CD68 revealed a significantly increased macrophage infiltration in the vessel wall of iEMP-treated mice compared with vehicle and EMP treatment (% of total vessel area: 34.1 ± 2.4 in iEMP vs. 23.8 ± 1.5 in vehicle vs. 22.9 ± 2.4 in EMP, $P < 0.05$, n = 6–8, Figure 3D). These findings suggested an iEMP-induced migration of monocytes/macrophages into the vessel.
To assess whether increased inflammatory cell infiltration into the vascular walls of iEMP-treated mice was due to increased levels of circulating proinflammatory cells, we quantified CD11b, CD3, CD4, and CD8 positive cells in the blood using flow cytometry. No difference was detected in the levels of circulating inflammatory cells among the groups (Supplementary material online, Figure S3).

Given the impressive increase of CD68+ macrophage infiltrated area in the vascular wall in iEMP treatment group, we assessed the expression of the proinflammatory adhesion proteins ICAM-1 and VCAM-1 in the vasculature, which play essential roles in monocyte adhesion to the activated endothelium and subsequent migration into the vessel wall. Interestingly, iEMP-treated mice showed significantly increased ICAM-1 expression (% of total vessel area: 40.7 ± 2.7 in iEMP vs. 23.1 ± 2.9 in vehicle vs. 17.9 ± 2.2 in EMP, P < 0.01, n = 6–8, Figure 3E) and VCAM-1 expression (% of total vessel area: 37.7 ± 2.4 in iEMP vs. 23.67 ± 2.7 in vehicle vs. 20.6 ± 2.0 in EMP, P < 0.01, n = 6–8, Figure 3F) compared with vehicle and EMP treatment.

These data indicate that iEMP-induced increased macrophage infiltration is a result of endothelial activation. To further verify whether and how iEMP activate the endothelium and promote monocyte adhesion, additional in vitro experiments were performed.

3.5 iEMP-mediated upregulation of ICAM-1 and VCAM-1 in target HCAEC

We first investigated whether iEMP influence adhesion protein expression in target endothelial cells. Western blot analysis revealed that co-incubation of HCAEC with iEMP but not EMP, increased ICAM-1 and VCAM-1 expression compared with untreated HCAEC (Figure 4A and B). Further experiments showed a dose- and time-dependent upregulation of adhesion protein expression in iEMP-treated HCAEC (Figure 4C and D).

To investigate whether the increased ICAM-1 and VCAM-1 signal in western blot was due to a transfer of adhesion proteins via MP or an upregulation in target HCAEC, real-time RT-PCR experiments were performed. iEMP-treated HCAEC showed a higher expression of ICAM-1 mRNA compared with EMP-treated and untreated HCAEC (Figure 4E). VCAM-1 mRNA was upregulated after iEMP treatment in a similar way (Figure 4E). These results suggest an upregulation of mRNA by iEMP rather than a transfer of adhesion proteins via MP.

Next, we evaluated if upregulation of adhesion proteins might functionally affect monocyte adhesion to endothelial cells. Therefore, calcein AM-labelled monocytes were co-incubated with HCAEC, which had been pre-treated with either iEMP, EMP, or vehicle as...
control for 24 h. iEMP treatment was associated with an increased monocyte adhesion to HCAEC compared with EMP treatment and control (47.05 ± 6.3 vs. 26.93 ± 2.88 vs. 8.4 ± 1.8 P < 0.05, Figure 4F). These data indicate that iEMP-induced upregulation of adhesion proteins might be functionally relevant in terms of enhanced monocyte adhesion.

### 3.6 iEMP-induced upregulation of adhesion proteins is p38 dependent

Next, we aimed to explore a possible pathway by which iEMP promote adhesion protein expression in endothelial cells. Mitogen-activated protein kinases (MAPK) are involved in vascular inflammation. Among those, p38 activation has been described in the context of progression and complications of diabetes. To assess whether p38 is involved in iEMP-induced upregulation of adhesion proteins, we performed western blot analysis on p38 and its active form phospho-p38 (p-p38) at different timepoints after iEMP and EMP stimulation. p38 was activated to p-p38 within 30 min after iEMP-stimulation in target HCAEC (Figure 5A), whereas EMP did not activate p38 (Supplementary material online, Figure S4). ICAM-1 and VCAM-1, which are activated downstream in the p38 cascade, were expressed chronologically after p38 activation (Figure 4D). These data suggest that p38 is actively involved in the iEMP-mediated upregulation of adhesion proteins in HCAEC.

### 3.7 Inhibition of p38 on target HCAEC reduces iEMP-induced ICAM-1 and VCAM-1 expression and subsequent monocyte adhesion

To confirm the possible pathway of p38 activation in iEMP-induced adhesion protein expression, we used the p38 inhibitor SB-203580 for the following experiments. Pre-treatment of HCAEC with SB-203580 (1 μM) followed by iEMP stimulation significantly reduced ICAM-1 and VCAM-1 expression in target cells (Figure 5B). Additional immunofluorescent experiments confirmed that
SB-203580 abrogated iEMP-induced ICAM-1 (9.65 ± 1.09 vs. 5.9 ± 0.56, P < 0.05, Supplementary material online, Figure S5A) and VCAM-1 (30.84 ± 6.2 vs. 10.55 ± 2.85, Supplementary material online, Figure S5B) expression on HCAEC. Subsequently, inhibition of p38 activation using SB-203580 on iEMP target cells showed a significantly reduced monocyte adhesion to HCAEC (25.55 ± 5.68 vs. 7.74 ± 1.8, P < 0.05, Figure 5D). These results confirm the crucial role of p38 activity in the iEMP-mediated activation of target endothelial cells.

3.8 iEMP-induced p38 activation depends on the intra-microparticle ROS content

Next we sought to determine which factor in iEMP might be responsible for p38 activation in endothelial cells. In diabetic conditions, hyperglycaemia-induced overproduction of ROS is causally associated with vascular and multiorgan complications.19 Therefore, we aimed to explore whether ROS might be involved in iEMP-induced p38 activation. Flow cytometry analysis revealed that ROS, as determined by
measuring H$_2$O$_2$ content with 2′,7′-dichlorofluorescin diacetate (DCFDA) assay, is detectable in endothelial microparticles with significantly higher levels in iEMP compared with EMP (4.98 ± 0.18 vs. 3.39 ± 0.26, P < 0.001, Figure 6A). iEMP do not only contain ROS, but also increase ROS production in target HCAEC compared with control and EMP treatment (0.60 ± 0.03 vs. 0.73 ± 0.02 vs. 0.91 ± 0.11, Figure 6B and C). Time-dependent analysis of ROS production in target cells revealed the highest level 30 min after iEMP stimulation (0.12 ± 0.03 vs. 2.2 ± 0.12 vs. 2.0 ± 0.1 vs. 1.7 ± 0.04, P < 0.01, Supplementary material online, Figure S6). Increased ROS production of iEMP target endothelial cells was confirmed additionally using L-012 chemiluminescence (100 ± 7.45 vs. 80.9 ± 17.62 vs. 143.2 ± 10.21, Figure 6D). The use of ROS inhibitors PEG-Catalase and PEG-SOD abolished iEMP-induced ROS production in endothelial cells as demonstrated by L-012 chemiluminescence measurement, indicating that ROS in iEMP might trigger ROS production in endothelial cells (100 ± 11.34 vs. 138.0 ± 8.53 vs. 56.8 ± 5.3 vs. 73.31 ± 5.3, Figure 6E). To determine whether ROS might activate p38 and
therefore mediate the iEMP-induced inflammatory reaction, we measured p38 activation after iEMP treatment using ROS inhibitors. Western blot showed iEMP-dependent p38 activation, whereas inhibition of ROS effectively reduces p38 activation, indicating an ROS-dependent p38 activation in iEMP-treated endothelial cells. In order to look for the source of increased ROS in iEMP, we measured ROS-producing enzymes in iEMP and EMP. We found that NADPH oxidase activity is significantly higher in iEMP compared with EMP (Figure 6H), whereas Xanthine oxidase activity is negligible in iEMP and EMP (data not shown). Moreover, inhibition of NADPH oxidase using VAS2870 significantly decreased iEMP-induced ROS production in target HCAEC as determined by DCF-DA and L-012 assay (Figure 6I and J), indicating that NADPH oxidase in iEMP might increase ROS production in HCAEC.

Taken together, these data suggest that iEMP containing active NADPH oxidase increase ROS production in endothelial target cells activate p38 and further downstream upregulate adhesion protein expression in the signalling cascade. This process leads to increased monocyte adhesion in vitro and in vivo and accelerates atherosclerosis and endothelial dysfunction in mice.

4. Discussion

In this study, we demonstrate that EMP generated from glucose-treated cells, but not from healthy endothelial cells, induce vascular inflammation and impair endothelial function in vivo. Activation of p38 in the endothelium by NADPH oxidase- and ROS-containing iEMP and subsequent upregulation of adhesion proteins appears to be the underlying pathway.

Endothelial function is impaired in hyperglycaemia. However, whether endothelial dysfunction in hyperglycaemia is mainly caused by direct effects of hyperglycaemia or indirectly by other bioactive paracrine mediators is not clear. Increasing data suggest that additional glucose-induced paracrine mediators foster vascular inflammation in diabetic conditions. In this context, elevated levels of circulating EMP are associated with vascular dysfunction in diabetic...
iEMP activate p38 and inhibition of p38 activation reduces iEMP-induced ICAM-1 and VCAM-1 expression and subsequent monocyte adhesion to HCAEC. (A) HCAEC were co-incubated with iEMP for 0, 0.5, 2, 6, 18, and 24 h. p-p38, the phosphorylated and active form of p38 and p38 expression was analysed by western blot. (B–D) HCAEC were pre-treated with the p38 inhibitor SB-203580 (1 μM) or vehicle for 1 h and stimulated with iEMP or vehicle for 24 h. (B and C) ICAM-1 and VCAM-1 expression was detected by western blot. n = 4, **P < 0.01, ***P < 0.001. (D) Monocyte adhesion assay was performed as described in Figure 4E. n = 5–6, *P < 0.05, **P < 0.01, ***P < 0.001.
ROS productions are increased in iEMP and iEMP target HCAEC. Inhibition of ROS in iEMP target HCAEC attenuates p38 activation. (A) Pelleted EMP and iEMP were diluted in PBS and incubated with 2',7'-dichlorofluorescin diacetate (DCFDA, 10 μM) for 45 min. Intra-microparticles H$_2$O$_2$ content was assessed with DCF fluorescence measured by flow cytometry. n = 6, ***P < 0.001. (B and C) HCAEC were stimulated with iEMP, EMP, or vehicle for 30 min and intracellular H$_2$O$_2$ content was assessed with DCFDA (10 μM). (B) Representative fluorescent images of DCF in target endothelial cells. (C) DCF signal is demonstrated as relative fluorescence intensity to nuclear DNA content (DCF:Hoechst ratio), n = 8, *P < 0.05, **P < 0.01. (D) HCAEC in 6-well plates were stimulated with iEMP, EMP, or vehicle for 24 h. ROS productions were determined by L-012 chemiluminescence. n = 6, *P < 0.05, ***P < 0.001. (E) HCAEC were pre-treated with PEG-SOD (100 U/mL), PEG-catalase (200 U/mL), or vehicle for 1 h and stimulated with iEMP or vehicle for 24 h. ROS productions were determined by L-012 chemiluminescence. n = 6, *P < 0.05, ***P < 0.001. (F) HCAEC were pre-treated with PEG-SOD (100 U/mL), PEG-catalase (200 U/mL), or vehicle for 1 h and stimulated with iEMP or vehicle for 30 min. Phospho-p38 and p38 expression was analysed by western blot, *P < 0.05, n = 5. (H) Intra-microparticles NADPH oxidase activity was assessed using lucigenin chemiluminescence, P < 0.01, n = 5. (I) HCAEC were pre-treated with VAS2870 (5 μM) or vehicle for 1 h and stimulated with iEMP or vehicle for 24 h. ROS productions were determined by L-012 chemiluminescence. n = 6, *P < 0.05, ***P < 0.001. (J) HCAEC were pre-treated with VAS2870 (5 μM) or vehicle for 1 h and stimulated with iEMP or vehicle for 30 min and intracellular H$_2$O$_2$ content was assessed with DCFDA (10 μM) ***P < 0.001, n = 8.
Figure 6 Continued.
patients.\textsuperscript{24} The concept that MP can actively modulate disease progression was supported by a recent study, demonstrating that MP derived from vitreous fluid in diabetic patients induced endothelial cell proliferation and formation of new blood vessels, indicating an involvement of microparticles in the progression of diabetic retinopathy.\textsuperscript{25} Recently, we reported that EMP are incorporated by endothelial cells in vitro in an annexin I/phosphatidylserine receptor-dependent manner and protect target cells against apoptosis.\textsuperscript{12} Whether the endothelium in vivo might also be a potential locus for circulating MP uptake is poorly investigated so far. Here, we demonstrate that EMP can be incorporated by murine endothelial cells and affect endothelial biology. These results extend the previously demonstrated findings by Dasgupta et al.,\textsuperscript{13} who showed that the murine endothelium can take up intravenously injected MP from human origin in a developmental endothelial locus-1 (Del-1)-dependent way.

To study the effects of microvesicles in models of atherosclerosis, treatment on ApoE\textsuperscript{-/-} mice with in vitro-generated human micromolecules is a well-established method as demonstrated for exosomes, microparticles, and apoptotic bodies.\textsuperscript{22,23,26} In these studies, microvesicles show various effects depending on the state and origin of the releasing cells. In our study, we show that EMP, but not EMP, induce vascular inflammation, impair endothelial function, and promote atherosclerosis in vivo. Our results broaden previously described findings by Rautou et al.,\textsuperscript{27,28} who have demonstrated in an elegant way that disease severity in patients with atherosclerotic plaques and cirrhosis determines the effects of released MP. Taken together, these findings indicate that the functional state of the releasing cell defines the effect of released MP.

Previous studies showed that in hyperglycaemic context, p38 downstream signalling is well involved in endothelial activation which played a key role in the progression and complications of diabetes.\textsuperscript{17,18} Interestingly, in our study, we found that p38 is activated within 30 min after EMP treatment, followed by upregulated expression of ICAM-1 and VCAM-1 in target HCAEC. Additionally, specific inhibition of p38 activity reduces EMP-mediated effects in terms of upregulating of ICAM-1 and VCAM-1 and enhancing monocyte adhesion. Thus, these findings indicate that overexpression of ICAM-1 and VCAM-1 in EMP target endothelial cells results from p38 activation and are responsible for increased monocyte adhesion. Although most stimuli that activate p38 also activate other MAPK, only p38 is inhibited by the specific anti-inflammatory drug SB203580.\textsuperscript{29} These data support our findings that EMP induce expression of adhesion proteins in target cells in a p38-dependent manner.

In hyperglycaemic conditions, mitochondrial ROS production is increased in endothelial cells and is recognized as a major cause of the clinical complications associated with diabetes.\textsuperscript{19,30} Here, we first show that endothelial microparticles contain ROS with significantly higher levels in MP derived from glucose-treated cells. Additionally, we show increased ROS production in target endothelial cells after stimulation with EMP and an essential involvement of ROS in EMP-induced p38 activation. Upregulation of adhesion proteins and subsequent impairment of endothelial function in vivo after EMP treatment support the concept that MP are not only released in pathological conditions, but can also actively modify disease progression.\textsuperscript{31}

In conclusion, we demonstrate that modified EMP derived under pathological high glucose conditions induce adhesion protein expression in endothelial cells and subsequent monocyte adhesion in a NADPH oxidase-ROS-p38-dependent way. Our findings provide novel insights into the pathophysiological effects of microparticles and their influence on target cells depending on the condition in which they are released. In view of the proinflammatory effects of EMP and the increased levels of EMP in diabetic patients, one may speculate that MP released under pathological high glucose conditions might represent a paracrine mediator transporting proinflammatory messages to target cells and thereby foster vascular inflammation. However, the concept of modified EMP in vascular inflammation is novel and therefore unresolved questions remain: it is unknown whether additional molecules besides NADPH oxidase and ROS in EMP might also be involved in endothelial cell activation. Moreover, it remains to be assessed whether ROS-containing EMP, in addition to endothelial cell damage caused by hyperglycaemia, might induce vascular inflammation as a second hit in diabetic conditions in vivo and thereby promote atherosclerosis.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Conflict of interest:** none declared.

**Funding**

This work was supported by Deutsche Forschungsgemeinschaft (WE4139/1-1).

**References**


