Effects of oxidative stress versus cytokine-dependent inflammation on the release of endothelial microparticles

James M. Heilman[1], William S. Evans[1], Steven J. Prior[1,2]

[1]Department of Kinesiology, University of Maryland School of Public Health, College Park, MD, USA
[2]Baltimore Veterans Affairs Geriatric Research, Education and Clinical Center and Research and Development Service, Baltimore, MD, USA

ABSTRACT

Endothelial microparticles (EMPs) are released by endothelial cells in response to apoptosis and/or endothelial cell activation. As such, EMPs are considered markers of endothelial dysfunction and atherosclerosis that may be detected before clinical manifestation of vascular diseases. Even though certain mechanisms of EMP release are fairly well-known, EMP release following common stressors such as inflammation and oxidative stress remains less clear. **PURPOSE:** Determine the effects of lipopolysaccharide-induced cytokine-dependent inflammation (LPS) and hydrogen peroxide-induced oxidative stress (H_2O_2) on activated and apoptotic EMP release in cultured endothelial cells. **METHODS:** Three lots of passage 4-5 human umbilical vein endothelial cells (HUVECs) were incubated with either 1 µg/mL LPS for 24h or 400 µM H_2O_2 for 1 hour in endothelial growth medium 2 (EGM2). Each condition was performed in each cell lot and processed in duplicate. After 1 hour of H_2O_2 incubation, old medium was removed and new EGM2 was added to the cells, then collected after 24 hours. This was done because H_2O_2 incubation for long periods will kill the cells and degrade EMPs. Endothelial microparticles were isolated from the medium of each condition and stained with fluorescent conjugated antibodies for flow cytometry to quantify $CD31^+42b^-$ (apoptosis) and $CD62E^+$ (endothelial cell activation) EMPs. Analysis of variance was used to test for differences among H_2O_2, LPS, and control conditions. **RESULTS:** There was a significant increase in the amount of $CD31^+42b^-$ EMPs per µL of media following the H_2O_2 condition compared with the LPS condition (5.00 ± 1.84 vs. 1.31 ± 0.64, $P < 0.05$); however, there were no statistically significant differences between conditions and control. **CONCLUSION:** Microparticle release occurs in quiescent and stimulated endothelial cells. H_2O_2-induced oxidative stress enhances generation of apoptotic EMPs compared with cytokine-dependent LPS inflammation. As such, oxidative stress may be a more potent, specialized stimulus for endothelial cell dysfunction at the onset of chronic vascular disease compared to a cytokine-dependent stimulus, which instead works primarily through endothelial cell activation.

INTRODUCTION

- Accumulation of endothelial microparticles (EMPs) in plasma is associated with endothelial dysfunction. As such, it is important to understand the mechanisms of their release in response to different stimuli.
- Even though vascular inflammation results in increased EMP counts, there are multiple signaling cascades for the release of EMPs. As such, we sought to determine whether or not EMP release is associated with specific inflammation signaling mechanisms.
- Cytokine-dependent inflammation occurs as part of the immune response. Cytokines are known to cause proliferation and stimulation of immune cells, resulting in an immune response. Lipopolysaccharide (LPS) is commonly associated with this due to its presence on gram-negative bacteria, which implicates it as an inflammatory stimulant.
- Oxidative stress, in contrast, is brought on by the accumulation of reactive oxygen species (ROS) and causes significant cellular damage. This type of inflammation is greatly dependent on the presence of H_2O_2.

Purpose: Determine how LPS-induced cytokine-dependent inflammation and H_2O_2-induced oxidative stress affect the release of activated and apoptotic EMPs in cultured endothelial cells.

Hypotheses: Both cytokine-dependent inflammation and oxidative stress will result in EMP release in cultured endothelial cells. Oxidative stress will result in a significant amount of apoptotic EMP release due to its potent effect on tissues and its known role in the development of atherosclerosis.

METHODS

Cell Culture:
- Three lots of passage 3-4 male and female human umbilical vein endothelial cells (HUVECs) were seeded in T75 flasks with 15 mL endothelial growth medium 2 (EGM2), then passed into separate 6-well plates at 80% confluence.
- HUVECs were then passed and cultured in 6-well plates to 80% confluence with EGM2

Generation of Conditioned Media:
- At 80% confluence, conditions were applied to the cells (1 µg/mL LPS for 24h in EGM2, 400 µM H_2O_2 for 1 hour in EGM-2, or EGM-2 for 24h to serve as control).
- Each condition was performed in each cell lot and processed in duplicate.
- Following 1h of H_2O_2 incubation, original medium was removed from the cells and new EGM2 was added. This was done because of the negative effect of H_2O_2 on EMPs after long periods of incubation.
- Following the 24h, medium from all 3 conditions (LPS, H_2O_2, and control) was collected.

EMP isolation and Flow Cytometry:
- Endothelial microparticles were isolated from conditioned medium via sequential centrifugation.
- Isolated samples were stained with fluorescent conjugated antibodies for $CD31^+42b^-$ and $CD62E^+$ EMPs, then quantified using flow cytometry.

Statistical Analysis: Analysis of variance was used to test for differences among H_2O_2, LPS, and control conditions. A paired two-sample t-test was then used to determine significant differences among conditions. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

Table 1. Endothelial microparticle counts and comparisons among conditions. * = significant difference with $P < 0.01$, † = significant difference with $P < 0.05$

	$CD31^+42b^-$	$CD42b^-$
LPS	1.31 ± 0.64*	1.96 ± 0.92
H_2O_2	5.00 ± 1.84*	4.29 ± 3.26
Control	3.03 ± 2.04	2.51 ± 1.69
ANOVA P-value	0.0211†	0.3293

Figure 1. Flow cytometric analysis gating strategy

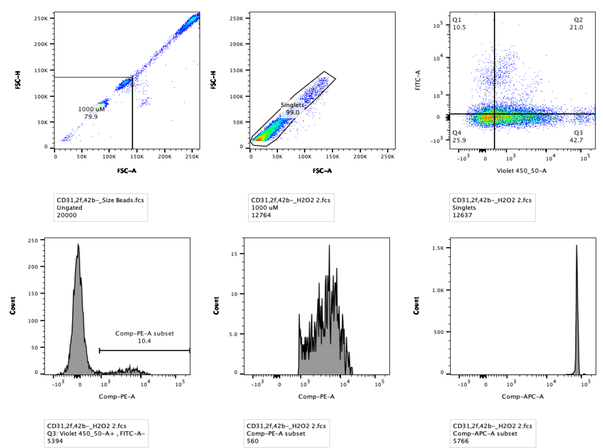


Figure 2. Human umbilical vein endothelial cells following 1 hour of exposure to each inflammatory condition. A = H_2O_2, B = LPS, C = Control

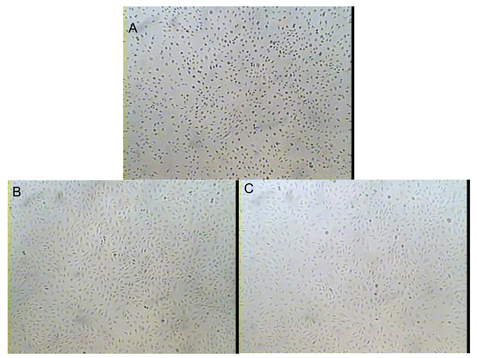
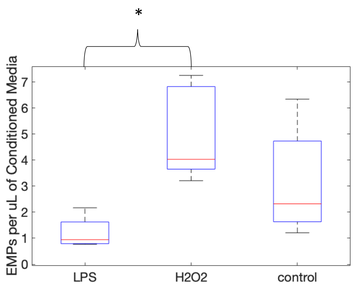


Figure 3. Total endothelial microparticle release in response to H_2O_2 and LPS. * = significant difference, $P < 0.05$.



Summary

- Following the 24-h incubation period for each condition, there was a significantly higher number of $CD31^+42b^-$ EMPs released per µL of conditioned media in the H_2O_2-stimulated condition compared with the LPS-stimulated condition (5.00 ± 1.84 vs. 1.31 ± 0.64, $P = 0.03$).
- There was no significant difference between $CD31^+42b^-$ EMPs brought on by the LPS condition compared to the control condition ($P = 0.19$), nor was there a significant difference between H_2O_2 and control ($P = 0.17$) among those EMPs.
- There was no significant effect of LPS or H_2O_2 on $CD62E^+$ EMPs compared to control ($P = 0.69$ and $P = 0.49$). There was also no significant difference between LPS and H_2O_2 conditions ($P = 0.14$).

CONCLUSIONS

- Endothelial cells regularly release EMPs into circulation in response to both acute and chronic inflammation. In non-pathological situations, EMPs are taken back up into the cells at the same rate at which they are released. In pathological conditions, a greater number of EMPs is released and therefore they cannot all be taken back up into the cells, resulting in increased EMP counts. This has been associated with endothelial dysfunction and the development of atherosclerosis.
- In the present study, H_2O_2-induced oxidative stress appears to increase the release of apoptotic $CD31^+42b^-$ EMPs compared to LPS-induced cytokine-mediated inflammation.
- In contrast, $CD62E^+$ EMPS, which typically signal endothelial cell activation, were not significantly affected by either stimuli.
- As a result, oxidative stress appears to be a strong stimulus for the release of apoptotic EMPs at the onset of endothelial dysfunction compared to cytokine-mediated inflammation.
- Future research in this area would benefit from focusing on intracellular EMP counts versus extracellular counts under similar inflammatory conditions.

ACKNOWLEDGEMENTS

The authors would like to thank all members of the research team for their contributions to the study.

Funding: Department of Veterans Affairs