Published in final edited form as:

Biochem Biophys Res Commun. 2017 November 18; 493(2): 1026–1029. doi:10.1016/j.bbrc.2017.09.098.

HIGH GLUCOSE DERIVED ENDOTHELIAL MICROPARTICLES INCREASE ACTIVE CASPASE-3 AND REDUCE MicroRNA-Let-7a EXPRESSION IN ENDOTHELIAL CELLS

Tyler D. Bammert, Jamie G. Hijmans, Whitney R. Reikvam, Ma'ayan V. Levy, Lillian M. Brewster, Zoe A. Goldthwaite, Jared J. Greiner, Kelly A. Stockelman, and Christopher A. DeSouza

Integrative Vascular Biology Laboratory, Department of Integrative Physiology, University of Colorado, Boulder, Colorado 80309

Abstract

The experimental aim of this study was to determine the effects of high glucose-induced endothelial microparticles (EMPs) on endothelial cell susceptibility to apoptosis. Human umbilical vein endothelial cells (HUVECs) were cultured (3rd passage) and plated in 6-well plates at a density of 5.0×10^5 cells/condition. Cells were incubated with media containing 25mM D-glucose (concentration representing a diabetic glycemic state) or 5mM D-glucose (normoglycemic condition) for 48 h to generate EMPs. EMP identification (CD144⁺ expression) and concentration was determined by flow cytometry. HUVECs (3×10^6 cells/condition) were treated with EMPs generated from either the normal or high glucose conditions for 24 h. Intracellular concentration of active caspase-3 was determined by enzyme immunoassay. Cellular expression of miR-Let7a, an anti-apoptotic microRNA, was determined by RT-PCR using the CT normalized to RNU6. High glucose-derived EMPs significantly increased both basal (1.5 ± 0.1 vs 1.0 ± 0.1 ng/mL) and staurosporine-stimulated (2.2 ± 0.2 vs 1.4 ± 0.1 ng/mL) active caspase-3 compared with normal glucose EMPs. Additionally, the expression of miR-Let-7a was markedly reduced (~140%) by high glucose EMPs (0.43 ± 0.17 fold vs control). These results demonstrate that hyperglycemic-induced EMPs increase endothelial cell active caspase-3. This apoptotic effect may be mediated, at least in part, by a reduction in miR-Let-7a expression.

Keywords

endothelial cells; glucose; apoptosis; caspase-3; microRNA; miR-Let-7a

Corresponding author: Christopher DeSouza, Ph.D., Integrative Vascular Biology Laboratory, Department of Integrative Physiology, 354 UCB, University of Colorado, Boulder, CO 80309, Ph: 303 492 2988, Fax: 303 492 6778, desouzac@colorado.edu.

Conflict of interest: The authors have no conflicts to disclose

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Glucose can have deleterious effects on the vasculature, particularly endothelial cells. Indeed, hyperglycemic conditions can damage endothelial cells, in turn, impairing endothelial vasomotor and fibrinolytic function, exacerbating inflammatory and oxidative processes and propagating a proapoptotic phenotype [1–4]. For example, in cultured endothelial cells high glucose concentrations induce endothelial cell apoptosis through a caspase-3-dependent mechanism [5]. Collectively, hyperglycemia induced endothelial cell damage, dysfunction and death are considered to be major factors underlying the increased risk of atherosclerotic vascular disease and acute thrombotic events associated with diabetes [1, 6].

Clinical interest in endothelial cell-derived microparticles (EMPs) has increased due to their role in the pathogenesis of vascular disease [7]. Although released by the endothelium, EMPs have autocrine properties that can significantly impact endothelial health and function [8]. Hyperglycemic conditions are known to stimulate EMP release both *in vitro* and *in vivo* [9–12]. For example, Jensen et al. [12] have demonstrated that high glucose concentrations stimulate EMP release in culture; whereas circulating EMPs have been shown to be markedly elevated in adults with diabetes and significantly associated with blood glucose concentrations [1]. However, the effects of EMPs derived from glucose stimulation on endothelial cell function are not well understood. It is possible, that glucose-stimulated EMPs may also confer deleterious effects on the endothelium.

Accordingly, the aim of this study was to determine the effects of glucose-stimulated EMPs on endothelial cell susceptibility to apoptosis. To address this aim, we assessed intracellular active caspase-3 concentration and expression changes in the anti-apoptotic microRNA, miR-Let-7a, in endothelial cells treated with EMPs derived from normal and high glucose conditions *in vitro*.

METHODS

Cell Culture and EMP Generation and Isolation

Human umbilical vein cells (HUVECs) were obtained from Life Technologies (ThermoFisher, Waltham MA), cultured in endothelial growth media (EBM-2 BulletKit, Clonetics LONZA, Walkersville MD), supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies™, Carlsbad, CA) under standard cell culture conditions (37°C and 5% CO₂). After reaching ~90% confluence on the 3rd passage, cells were seeded into tissue culture flasks (Falcon, Corning NY, USA) and incubated with RPMI 1640 media containing 25mM D-glucose (concentration representing a diabetic glycemic state) or 5mM D-glucose (control cells, normoglycemic condition) for 48 hours. All experimental conditions contained 25mM D-manitol (VWR Analytical, Radnor PA) to control for osmolality [13, 14]. After the 48-hour incubation period, cells and supernatant for both conditions were collected for analysis.

EMP Characterization and Enumeration

The supernatant collected from the treated cells was centrifuged at $13,000 \times g$ at room temperature for 2 minutes to pellet and discard cellular debris. 100 μL of the cell free supernatant was transferred to TruCount™ tubes (BD Biosciences, New Jersey, USA) and incubated with the fluorochrome labeled antibody CD144-phycoerythrin (VE-cadherin) to identify EMPs. Samples were then fixed with paraformaldehyde (ChemCruz Biochemicals, Santa Cruz, CA) and diluted with 500 μL of PBS [15]. All samples were analyzed using a FACS Aria I flow cytometer (BD Biosciences) at the University of Colorado Anschutz Medical Campus ACI/ID Flow Core. EMP size threshold was established using Megamix-Plus SSC calibrator beads (Biocytex, Marseille, France) [16]. EMP concentrations for the normal glucose EMPs (ngEMPs) and high glucose EMPs (hgEMPs) (expressed as MP/ μL) were determined using the formula: $([\text{number of events in region containing MPs}/\text{number of events in absolute count bead region}] \times [\text{total number of beads per test}/\text{total volume of sample}])$ [12].

EMP Treated Cells

HUVECs were cultured as described above and seeded into 6-well plates. Media containing EMPs harvested from either the normal glucose or high glucose conditions were centrifuged at $20,500 \times g$ for 30 minutes at 4°C to pellet EMPs [12]. The pelleted EMPs were then re-suspended in EBM-2 at a concentration of 1.0×10^7 MPs/mL. HUVECs were treated with an equal number of EMPs generated from either the normal glucose condition or EMPs from the high glucose condition for 24 hours.

Intracellular Active Caspase-3

After EMP treatment, harvested cells (3×10^6) for caspase-3 determination were treated with staurosporine (1 $\mu\text{mol/L}$) for 3 h at 37°C and biotin-ZVKD-fmk inhibitor for 1 h at 37°C . Intracellular concentration of active caspase-3 was determined by enzyme immunoassay [17].

Intracellular miR-Let-7a

After EMP treatment, 1.0×10^5 cells were harvested and total cellular RNA for each condition was isolated using the miRCURY RNA isolation kit (Exiqon, Vedbake, Denmark) and RNA concentration was determined using a Nanodrop Lite spectrophotometer (ThermoFisher, Waltham, MA) [18]. Thereafter, 150ng of RNA was reverse transcribed using the miScript II Reverse Transcription Kit (Qiagen, Hilden, Germany) RT-PCR was performed using the BioRad CFX96 RT-PCR platform with the miScript SYBR green PCR kit (Qiagen, Hilden, Germany) and specific primers for miR-Let-7a (Qiagen, Hilden, Germany). All samples were assayed in duplicate. miRNA expression was normalized to U6 and fold change of each transcript was calculated as the 2^{-C_t} where fold change (AU) $= 2^{-((C_t[\text{miR experimental}] - C_t[\text{RNU6experimental}] - C_t[\text{miR control}] - C_t[\text{RNU6control}]))}$ [18, 19].

Statistical Analysis

Differences in glucose-treatment derived EMP number, and EMP effects on caspase-3 and miR-Let-7a were determined by two-tailed, unpaired Student's t-test. Data are reported as

mean $\pm$ SEM for 5 independent HUVEC experiments. Statistical significance was set *a priori* at $P<0.05$.

RESULTS

The hyperglycemic condition resulted in markedly higher EMP release than the normoglycemic condition. The number of EMPs generated from the HUVECs treated with 25mM D-glucose was ~380% higher ($P<0.05$) compared with HUVECs treated with 5mM D-glucose (228 ± 70 vs 52 ± 14 EMP/uL). Basal (1.5 ± 0.1 vs 1.0 ± 0.1 ng/mL) and staurosporine-stimulated (2.2 ± 0.2 vs 1.4 ± 0.1 ng/mL) intracellular active caspase-3 concentrations were significant higher (~50%) in endothelial cells treated with hgEMPs compared with ngEMPs (Figure 1). Endothelial expression of miR-Let-7a was significantly affected by hgEMPs. Compared with cells treated with ngEMPs, relative expression of miR-Let-7a was ~140% lower in cells treated with hgEMPs (0.43 ± 0.17 fold; $P<0.05$) (Figure 2).

DISCUSSION

The primary new finding of the present study is that hyperglycemic-induced EMPs increase endothelial cell apoptotic susceptibility and this effect may be mediated, in part, by reduction in cellular miR-Let-7a expression. Although previous studies have demonstrated that high glucose induces both an increase in endothelial cell apoptosis and an increase in EMP release [5, 12], to our knowledge this is the first study to demonstrate that the EMPs produced in response to high glucose, in turn, also induce a pro-apoptotic endothelial phenotype. Thus, it appears that the adverse endothelial effects of high glucose are also conferred by the EMPs generated by the hyperglycemic state.

Apoptosis, or programmed cell death, is a highly-preserved, fundamental cellular process that is highly regulated to protect against aberrant cell death and/or proliferation [20]. Dysregulation of apoptotic programs can lead to blunted cell viability or unrestricted cell/tissue growth depending on the underlying physiologic or pathophysiologic state [21]. Initiation of apoptosis, either by extracellular or internal events, triggers a hierarchy of caspases, most notably caspase-3, leading to cell destabilization and destruction. Indeed, intracellular concentrations of active caspase-3, the so-called “executioner molecule”, provides specific biological insight into the apoptotic tendency of a cell [22]. The novel finding of the present study is that hgEMPs increased intracellular active caspase-3 concentrations under basal conditions and in response to staurosporine stimulation compared with ngEMPs. Although we did not assess the direct effect of high glucose on active caspase-3 in the parent endothelial cells, previous studies have demonstrated that the pro-apoptotic endothelial effect of high glucose exposure is mediated by caspase-3 [5, 23]. Thus, both high glucose and the EMPs it generates appear to induce an apoptotic endothelial phenotype via common mechanisms. It is interesting to note, that Jansen et al. [12] demonstrated that EMPs generated under apoptotic conditions (cell starvation) and exposed to, and incorporated by, endothelial cells reduced their susceptibility to apoptosis. Future studies are needed to characterize the high glucose-derived EMP phenotype in order to better understand their pro-apoptotic effect on endothelial cells.

MicroRNAs are endogenously expressed non-coding RNAs that regulate gene expression post-transcriptionally via translational repression or mRNA degradation [18, 24]. The microRNA Let-7a plays an important functional role in apoptosis through its regulatory effect on caspase-3. miR-Let-7a directly targets caspase 3 mRNA as the 3'UTR of the protein coding genes perfectly matches the seed sequence of miR-Let-7a facilitating mRNA translational repression and/or degradation. Indeed, miR-Let7a has been shown to suppress drug-induced apoptosis in cells via its regulatory effect on caspase-3 expression [25, 26]. A seminal finding of the present study was that relative expression of miR-Let-7a was markedly lower (~140%) in the endothelial cells exposed to hgEMPs compared with ngEMPs. Reduced expression of miR-Let-7a represents a viable contributing mechanism underlying the hgEMP-induced increase in caspase-3 activation [25]. Interestingly, in the parent endothelial cells exposure to high glucose, in addition to increasing EMP release, significantly reduced the expression of miR-Let-7a (0.75 ± 0.05 fold; data not shown). Thus, the effect of high glucose on miR-Let-7a expression (and caspase-3) in endothelial cells appears to be perpetuated by the subsequent glucose-generated EMPs and their endothelial interaction. These data provide further evidence that EMP phenotype is dictated by the stimulus underlying their release, epitomizing the complexity of microparticle biology [1].

In summary, both experimental and clinical studies have demonstrated that hyperglycemic conditions induce endothelial cell apoptosis and microparticle release. Herein, we demonstrate that EMPs generated from high glucose exposure induce a pro-apoptotic endothelial phenotype characterized by increased intracellular active caspase-3 and reduced miR-Let-7a expression. Increased endothelial cell apoptosis due to hyperglycemia is considered to be a key factor in diabetes-related vascular damage, dysfunction and disease [5, 27]. EMPs generated under high glucose conditions may significantly contribute to the profound vascular dysfunction and increased vascular risk associated with diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank all the staff at the University of Colorado Anschutz Medical Campus ACI/ID Flow Core for their technical assistance. This study was supported in part by National Institutes of Health (NIH) awards HL131458 and HL135598.

References

1. Santilli F, Marchisio M, Lanuti P, Boccatonda A, Miscia S, Davi G. Microparticles as new markers of cardiovascular risk in diabetes and beyond. *Thromb Haemost*. 2016; 116:220–234. [PubMed: 27173919]
2. Diehl KJ, Templeton DL, Ma J, Weil BR, Greiner JJ, Stauffer BL, DeSouza CA. Impaired fasting blood glucose is associated with increased endothelin-1 vasoconstrictor tone. *Atherosclerosis*. 2013; 229:130–133. [PubMed: 23643051]
3. Baumgartner-Parzer SM, Wagner L, Pettermann M, Grillari J, Gessl A, Waldhausl W. High-glucose--triggered apoptosis in cultured endothelial cells. *Diabetes*. 1995; 44:1323–1327. [PubMed: 7589831]

4. Du XL, Sui GZ, Stockklauser-Farber K, Weiss J, Zink S, Schwippert B, Wu QX, Tschöpe D, Rosen P. Introduction of apoptosis by high proinsulin and glucose in cultured human umbilical vein endothelial cells is mediated by reactive oxygen species. *Diabetologia*. 1998; 41:249–256. [PubMed: 9541163]
5. Ho FM, Liu SH, Liao CS, Huang PJ, Lin-Shiau SY. High glucose-induced apoptosis in human endothelial cells is mediated by sequential activations of c-Jun NH(2)-terminal kinase and caspase-3. *Circulation*. 2000; 101:2618–2624. [PubMed: 10840014]
6. Tabit CE, Chung WB, Hamburg NM, Vita JA. Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. *Reviews in endocrine & metabolic disorders*. 2010; 11:61–74. [PubMed: 20186491]
7. Koganti S, Eleftheriou D, Brogan PA, Kotecha T, Hong Y, Rakhit RD. Microparticles and their role in coronary artery disease. *Int J Cardiol*. 2017; 230:339–345. [PubMed: 28040277]
8. Dignat-George F, Boulanger CM. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol*. 2011; 31:27–33. [PubMed: 21160065]
9. Burger D, Turner M, Xiao F, Munkonda MN, Akbari S, Burns KD. High glucose increases the formation and pro-oxidative activity of endothelial microparticles. *Diabetologia*. 2017
10. Feng B, Chen Y, Luo Y, Chen M, Li X, Ni Y. Circulating level of microparticles and their correlation with arterial elasticity and endothelium-dependent dilation in patients with type 2 diabetes mellitus. *Atherosclerosis*. 2010; 208:264–269. [PubMed: 19674745]
11. Koga H, Sugiyama S, Kugiyama K, Watanabe K, Fukushima H, Tanaka T, Sakamoto T, Yoshimura M, Jinnouchi H, Ogawa H. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. *J Am Coll Cardiol*. 2005; 45:1622–1630. [PubMed: 15893178]
12. Jansen F, Yang X, Hoyer FF, Paul K, Heiermann N, Becher MU, Abu Hussein N, Kebschull M, Bedorf J, Franklin BS, Latz E, Nickenig G, Werner N. Endothelial microparticle uptake in target cells is annexin I/phosphatidylserine receptor dependent and prevents apoptosis. *Arterioscler Thromb Vasc Biol*. 2012; 32:1925–1935. [PubMed: 22701020]
13. Layton CJ. Diabetic levels of glucose increase cellular reducing equivalents but reduce survival in three models of 661W photoreceptor-like cell injury. *BMC ophthalmology*. 2015; 15:174. [PubMed: 26653778]
14. Risso A, Mercuri F, Quagliaro L, Damante G, Ceriello A. Intermittent high glucose enhances apoptosis in human umbilical vein endothelial cells in culture. *American journal of physiology Endocrinology and metabolism*. 2001; 281:E924–930. [PubMed: 11595647]
15. Jenkins NT, Padilla J, Boyle LJ, Credeur DP, Laughlin MH, Fadel PJ. Disturbed blood flow acutely induces activation and apoptosis of the human vascular endothelium. *Hypertension*. 2013; 61:615–621. [PubMed: 23319545]
16. Nielsen MH, Beck-Nielsen H, Andersen MN, Handberg A. A flow cytometric method for characterization of circulating cell-derived microparticles in plasma. *J Extracell Vesicles*. 2014; 3
17. Kushner EJ, MacEneaney OJ, Weil BR, Greiner JJ, Stauffer BL, DeSouza CA. Aging is associated with a proapoptotic endothelial progenitor cell phenotype. *J Vasc Res*. 2011; 48:408–414. [PubMed: 21625174]
18. Ye X, Hemida MG, Qiu Y, Hanson PJ, Zhang HM, Yang D. MiR-126 promotes coxsackievirus replication by mediating cross-talk of ERK1/2 and Wnt/beta-catenin signal pathways. *Cell Mol Life Sci*. 2013; 70:4631–4644. [PubMed: 23811937]
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(Delta Delta C(T))} Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
20. Elmore S. Apoptosis: a review of programmed cell death. *Toxicologic pathology*. 2007; 35:495–516. [PubMed: 17562483]
21. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science*. 1995; 267:1456–1462. [PubMed: 7878464]
22. Cohen GM. Caspases: the executioners of apoptosis. *Biochem J*. 1997; 326(Pt 1):1–16. [PubMed: 9337844]
23. Nakagami H, Morishita R, Yamamoto K, Yoshimura SI, Taniyama Y, Aoki M, Matsubara H, Kim S, Kaneda Y, Ogihara T. Phosphorylation of p38 mitogen-activated protein kinase downstream of

- bax-caspase-3 pathway leads to cell death induced by high D-glucose in human endothelial cells. *Diabetes*. 2001; 50:1472–1481. [PubMed: 11375350]
24. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009; 136:215–233. [PubMed: 19167326]
25. Tsang WP, Kwok TT. Let-7a microRNA suppresses therapeutics-induced cancer cell death by targeting caspase-3. *Apoptosis: an international journal on programmed cell death*. 2008; 13:1215–1222. [PubMed: 18758960]
26. Zhang Y, Chen N, Zhang J, Tong Y. Hsa-let-7g miRNA targets caspase-3 and inhibits the apoptosis induced by ox-LDL in endothelial cells. *International journal of molecular sciences*. 2013; 14:22708–22720. [PubMed: 24252910]
27. Ido Y, Carling D, Ruderman N. Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation. *Diabetes*. 2002; 51:159–167. [PubMed: 11756336]

Figure 1.

Effect of EMPs generated from normal (ngEMPs) and high glucose (hgEMPs) concentrations on endothelial cell basal and staurosporine-stimulated active caspase-3. Values are mean $\pm$ SEM (N=5). *P<0.05.

Figure 2.

Expression of miR-Let-7a in endothelial cells treated with EMPs generated from the high glucose (hgEMP) concentration relative to EMPs generated from the normal glucose concentration. Values are mean $\pm$ SEM (N=5). *P < 0.05.