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Nitrite-derived nitric oxide reduces hypoxia-inducible factor 1α-mediated extracellular vesicle production by endothelial cells

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ABSTRACT

Introduction: Extracellular vesicles (EVs) are small, spherical particles enclosed by a phospholipid bilayer (~30–1000 nm) released from multiple cell types, and have been shown to have pathophysiological roles in a plethora of disease states. The transcription factor hypoxia-inducible factor-1 (HIF-1) allows for adaptation of cellular physiology in hypoxia and may permit the enhanced release of EVs under such conditions. Nitric oxide (NO) plays a pivotal role in vascular homeostasis, and can modulate the cellular response to hypoxia by preventing HIF-1 accumulation. We aimed to selectively target HIF-1 via sodium nitrite ($NaNO_2$) addition, and examine the effect on endothelial EV, size, concentration and function, and delineate the role of HIF-1 in EV biogenesis.

Methods: Endothelial (HECV) cells were exposed to hypoxic conditions (1% O_2, 24 h) and compared to endothelial cells exposed to normoxia (21% O_2) with and without the presence of sodium nitrite ($NaNO_2$) (30 μM). Allopurinol (100 μM), an inhibitor of xanthine oxidoreductase, was added both alone and in combination with $NaNO_2$ to cells exposed to hypoxia. EV and cell preparations were quantified by nanoparticle tracking analysis and confirmed by electron microscopy. Western blotting and siRNA were used to confirm the role of HIF-1α and HIF-2α in EV biogenesis. Flow cytometry and time-resolved fluorescence were used to assess the surface and intravesicular protein content.

Results: Endothelial (HECV) cells exposed to hypoxia (1% O_2) produced higher levels of EVs compared to cells exposed to normoxia. This increase was confirmed using the hypoxia-mimetic agent desferrioxamine. Treatment of cells with sodium nitrite ($NaNO_2$) reduced the hypoxic enhancement of EV production. Treatment of cells with the xanthine oxidoreductase inhibitor allopurinol, in addition to $NaNO_2$ attenuated the $NaNO_2$-attributed suppression of hypoxia-mediated EV release. Transfection of cells with HIF-1α siRNA, but not HIF-2α siRNA, prior to hypoxic exposure prevented the enhancement of EV release.

Conclusion: These data provide evidence that hypoxia enhances the release of EVs in endothelial cells, and that this is mediated by HIF-1α, but not HIF-2α. Furthermore, the reduction of NO_2^- to NO via xanthine oxidoreductase during hypoxia appears to inhibit HIF-1α-mediated EV production.

1. Introduction

The production of extracellular vesicles (EVs) is a common feature of eukaryotic cells, including platelets, leukocytes, and endothelial cells [1]. EVs are spherical, submicron structures enclosed by a phospholipid bilayer, containing a variety of proteins, mRNAs and microRNAs [2]. Their application to modulate physiology is complex, with evidence for them both augmenting and

Abbreviations: Extracellular vesicles, (EVs); Hypoxia-inducible factor 1, (HIF-1); Nitrate, (NO_3^-); Nitrite, (NO_2^-); Nitric oxide, (NO); Nanoparticle tracking analysis, (NTA); Sodium nitrite, ($NaNO_2$); Time-resolved fluorescence, (TRF).

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alleviating disease, depending on their cellular origin and subsequent biophysical composition [3]. Elevated levels of EVs have been shown to have pathophysiological roles in a plethora of disease states, including cancer [4–6], neurodegenerative disorders [7–10], and cardiovascular disease [11–13]. Specifically, endothelial cell derived EVs have been shown to express tissue factor, suggesting a role in augmenting the coagulation cascade [14]. Additionally, EVs from patients with myocardial infarction have been shown to induce endothelial dysfunction *ex vivo* [15]. It has recently been shown that endothelial cells enhance EV secretion following temporary hypoxia exposure *in vivo* [16,17], a fundamental feature of the aforementioned diseases and resulting pathologies [18–20]. Indeed, EVs derived from endothelial cells exposed to hypoxia have been shown to produce a markedly altered RNA and protein composition, although the function of these EVs remains undetermined [21].

The adaptation of cellular physiology in response to hypoxia is largely mediated by the transcription factor hypoxia-inducible factor (HIF)-1, which promotes the transcription of genes involved in cell proliferation, metastasis, angiogenesis, and vascular remodelling [22,23]. HIF is comprised of an oxygen regulated HIF-α subunit (HIF-1α or HIF-2α) and the constitutively expressed HIF-1β. Whilst HIF-1α is ubiquitously expressed, HIF-2α is detected predominantly in vascular endothelial cells [24]. The HIF-α subunit is targeted for degradation under normoxic conditions by the O_2-dependent HIF-α prolyl hydroxylase enzymes. These enzymes hydroxylate two conserved prolyl residues (Pro 564 and Pro402) in the central oxygen-dependent degradation domain of the HIF-α subunit (both HIF-1α and HIF-2α), which promotes the binding of the Von Hippel-Lindau protein, allowing ubiquitination and subsequent degradation [25,26]. Inhibition of these enzymes in hypoxia prevents the degradation of HIF-α, allowing regulation of its transcriptional target genes [25]. HIF has been shown to increase expression of several proteins involved in cytoskeletal changes [27], a mechanism thought to be implicated in augmented EV release [28]. Thus, selective targeting and modulation of HIF-α could modulate endothelial cell EV release.

Endothelial-derived nitric oxide (NO) plays a pivotal role in vascular homeostasis, highlighted by the deficiency of NO prevalent in cardiovascular disease states [29]. NO can modulate the cellular response to hypoxia by preventing the stabilization of HIF-α via an increase in prolyl hydroxylase-mediated degradation [30,31]. Previously, impaired endogenous NO production in HUVECs has been shown to increase EV formation [32]. Recently, the inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-), once thought to be inert end products of NO metabolism, have been shown to be bioactive reservoirs for NO bioactivity, particularly during hypoxia [33,34]. NO_3^- is reduced to NO_2^- via commensal bacteria present in the oral cavity. NO_2^- can subsequently be reduced through reaction with various proteins that possess NO_2^- reductase activity, including Xanthine Oxidoreductase (XOR) [35,36], heme globins [37,38], and components of the mitochondrial electron transport chain [39,40].

Here, we aimed to elucidate the role of both HIF-1α and HIF-2α in endothelial EV release, and selectively target their expression in hypoxia via sodium nitrite ($NaNO_2$) addition, and investigate the effect on endothelial cell EV production.

2. Methods

2.1. Cell culture & viability

Human (HECV) endothelial cells were purchased from Interlab Cell Line Collection (ICLC, Naples, Italy). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, PAA Laboratories Ltd, UK) supplemented with 10% foetal calf serum (FCS, PAA Laboratories Ltd, UK), and 1% penicillin/streptomycin (P/S, Gibco®, Life Technologies, UK). Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords as previously described [41]. Human umbilical cords were obtained from the Antenatal Clinic, University Hospital Wales. Ethical approval was obtained from the Research Ethics Committee (REC) (REC reference: 14/NW/1459). HUVECs were maintained in M199 medium, supplemented with 10% foetal calf serum, 1% penicillin/streptomycin, human epidermal growth factor (1 ng/mL, Invitrogen, UK) and hydrocortisone (1 ng/mL, Sigma-Aldrich, UK). HUVECs were used at passage 0 and not sub-cultured. Cells were cultured using T25 cm^2 flasks (Cellstar®, Greiner Bio-One, Germany) and maintained in an incubator at 37 °C and 5% CO_2. Cell counts were undertaken using trypan blue exclusion (1:1 v/v) and a Cellometer Auto T4 (Nexcelom Biosciences, USA). Cell viability and apoptosis were determined using MTS and Caspase-Glo 3/7 assays (Promega, Southampton, UK), respectively, according to the manufacturers' instructions.

2.2. Hypoxia exposure

Hypoxic experiments were performed using an I-CO_2N_2 regulated InVivo 400 hypoxia workstation (Ruskinn, Bridgend, UK). Upon cells reaching ~80% confluency, culture medium was removed. HECVs were washed with phosphate-buffered saline (PBS) (Fisher Scientific, UK) and incubated with 10 mL EV-free serum free medium (SFM) for 24-hours. Cells were cultured at either normoxia (21% O_2, 5% CO_2, 37 °C) or hypoxia (1–20% O_2, 5% CO_2, 37 °C). The hypoxia mimetic agent desferrioxamine was added (100 μM) to HECVs incubated in normoxia to confirm the role of hypoxia in EV formation.

2.3. Extracellular vesicle isolation

EVs were isolated direct from cell culture as previously described [42]. Cells were cultured in serum-free medium (SFM) for 24 h prior to EV isolation to avoid contamination from foetal calf serum. Cell culture medium was extracted direct from the culture flask and subjected to differential ultra-centrifugation. Culture medium was spun at 500 × *g* for 10 min to remove any cells in suspension. The supernatant was then centrifuged at 15,000 × *g* for 15 min to remove any cell debris. Finally, supernatants were ultracentrifuged at 100,000 × *g* for 60 min to pellet EVs. This pellet was then resuspended in 1 x sterile PBS, stored at 4 °C and analysed within 1 week of isolation.

2.4. EV size and concentration analysis

Size and concentration distributions of EVs were determined using nanoparticle tracking analysis (NTA, NanoSight LM10 system, UK) as described previously [43]. NTA is a laser illuminated microscopic technique equipped with a 642 nm laser and a high sensitivity digital camera system (OrcaFlash2.8, Hamamatsu, NanoSight Ltd) that determines the Brownian motion of nanoparticles in real-time to assess size and concentration. Sixty-second videos were recorded and particle movement was analysed using NTA software (version 2.3). Camera shutter speed was fixed at 30.01 ms and camera gain to 500. Camera sensitivity and detection threshold were (11–14) and (4–6), respectively. A representative NTA trace can be seen in Appendix Figure A1. EV samples were diluted in EV-free sterile water (Fresenius Kabi, Runcorn, UK). Samples were run in quintuplicate, from which EV distribution, size and average concentration were calculated. EV concentrations were then normalised to cell count and expressed as EVs/cell.