Available online at www.sciencedirect.com

SCIENCE DIRECT

Free Radical Biology & Medicine 38 (2005) 1311–1322

www.elsevier.com/locate/freeradbiomed

Original Contribution

Hypoxia–reoxygenation-induced mitochondrial damage and apoptosis in human endothelial cells are inhibited by vitamin C

Manya Dhar-Mascareño[a,*], Juan M. Cárcamo[a,b], David W. Golde[a,c,⁂]

[a]*Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA*
[b]*Department of Clinical Laboratories, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA*
[c]*Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA*

Received 22 October 2004; revised 20 January 2005; accepted 20 January 2005
Available online 9 February 2005

Abstract

Hypoxia and hypoxia–reperfusion (H-R) play important roles in human pathophysiology because they occur in clinical conditions such as circulatory shock, myocardial ischemia, stroke, and organ transplantation. Reintroduction of oxygen to hypoxic cells during reperfusion causes an increase in generation of reactive oxygen species (ROS), which can alter cell signaling, and cause damage to lipids, proteins, and DNA leading to ischemia–reperfusion injury. Since vitamin C is a potent antioxidant and quenches ROS, we investigated the role of intracellular ascorbic acid (iAA) in endothelial cells undergoing hypoxia–reperfusion. Intracellular AA protected human endothelial cells from H-R-induced apoptosis. Intracellular AA also prevents loss of mitochondrial membrane potential and the release of cytochrome *C* and activation of caspase-9 and caspase-3 during H-R. Additionally, inhibition of caspase-9 activation prevented H-R-induced apoptosis, suggesting a mitochondrial site of initiation of apoptosis. We found that H-R induced an increase in ROS in endothelial cells that was abrogated in the presence of iAA. Our results indicate that vitamin C prevents hypoxia and H-R-induced damage to human endothelium.

Keywords: Dehydroascorbic acid; Hypoxia; Hypoxia–reoxygenation; Reactive oxygen species; Endothelium; Mitochondria; Free radicals

Introduction

Hypoxia and reoxygenation injury are common causes of mortality due to myocardial ischemia, circulatory shock, stroke, and transplantation of organs [1–3]. It has become increasingly evident that ROS play a significant role in reoxygenation injury. During hypoxia and reperfusion (H-R) vascular endothelium is a primary site of ROS generation and target of injury [4]. Endothelial cells function as a permeability barrier, regulating leukocyte migration and inhibiting thrombosis [5–8]. These cells are damaged by proinflammatory cytokines, bacterial endotoxins, and atherogenic factors such as homocysteine and oxidized lipoproteins [9]. Cellular models of H-R have provided useful tools for the study of ROS-mediated mechanisms of cellular dysfunction. The molecular mechanisms of reperfusion injury on vascular endothelium are not well understood [10].

Since ROS play an important role in H-R, antioxidants have been used to ameliorate consequent cellular injury [11–13]. Vitamin C, a strong antioxidant that quenches ROS, has also been used to reduce endothelial dysfunction in conditions such as diabetes, hyperhomocysteinemia, coronary artery disease, hypercholesterolemia, and renovascular

Abbreviations: H-R, hypoxia-reoxygenation; ROS, reactive oxygen species; DCFH-DA, 2,7′ dichlorodihydrofluorescein diacetate; Cyt C, cytochrome *C*; DAPI, (4,6-diamidino-2-phenylindole); HUVEC, human umbilical vein endothelial cells; HCAEC, human coronary artery endothelial cells; FCS, fetal calf serum; DTT, dithiothreitol; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; AA, ascorbic acid; DHA, dehydroascorbic acid; $DiOC_{(6)}$, 3,3-dihexyloxacarbocyanine iodide; ETC, electron transport chain; TUNEL, TdT-mediated dUTP nick end labeling; NAC, *N*-acetylcysteine; PDTC, pyrrolidinedithiocarbamate; ICAM-1, intercellular adhesion molecule-1; D-DOG, deoxy-D-glucose; L-DOG, 2-deoxy-L-glucose; MPT, mitochondrial permeability transition; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; pNA, *p*-nitroaniline.

* Corresponding author. Fax: (212) 772 8589.
E-mail address: mascarem@mskcc.org (M. Dhar-Mascareño)
⁂ Deceased.

0891-5849/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2005.01.017

hypertension [14–18]. However, experimental studies have shown that high physiological concentrations of vitamin C are required to prevent ROS-mediated vascular dysfunction [19].

The role of vitamin C in endothelial cells undergoing H-R has not been analyzed [20,21]. Certain specialized cells transport vitamin C directly as AA via sodium-dependent cotransporters localized on the cell membrane [22]. However, all cells can transport the oxidized form of vitamin C, dehydroascorbic acid (DHA), via facilititative glucose transporters (GLUTs) [23]. It has been shown that loading cells with vitamin C by treatment with DHA circumvents the prooxidant effects of AA in cell culture [24,25]. Loading cells with vitamin C by treatment with DHA protects against FAS-mediated apoptosis in monocytes, menadione-induced oxidant stress in endothelial cells, and hydrogen peroxide-induced cell death in HL-60 leukemic cells and protects against oxidant-induced DNA mutations [26–31]. In vivo, DHA administration has resulted in amelioration of ischemia-induced infarct size in experimental stroke models [32,17].

We have examined the role of vitamin C in reducing hypoxia and H-R-induced injury in human umbilical vein endothelial cells (HUVEC) and human coronary artery endothelial cells (HCAEC). Our results indicate that increased intracellular concentrations of vitamin C in endothelial cells substantially reduced hypoxia and H-R-induced apoptosis.

Materials and methods

Cell culture

HUVEC and HCAEC were obtained from Clonetics Corporation (Cambrex Inc., East Rutherford, NJ). HUVEC were grown in endothelial basal medium supplemented with 2% fetal calf serum (FCS), human epidermal growth factor, insulin growth factor-1, fibroblast growth factor, vascular growth factor, hydrocortisone (1.0 μg/ml), gentamicin (50 μg/ml), and heparin. HCAEC were maintained in EBM2-MV medium with 5% FCS and the growth factor supplements. Cells were used at passages 5 or less.

DHA uptake in HUVEC and HCAEC

Cells were grown in 6-well plates, washed twice with incubation buffer, pH 7.4 (15 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$), and then incubated in the same buffer with 0.2 μCi of L-[^{14}C] ascorbic acid (DuPont inc., Wilmington, DE), 1 mM DTT, and various concentrations of DHA. DHA was generated by converting AA to DHA using ascorbate oxidase (Sigma) [23]. Cells were incubated at 37°C with DHA for different times and the uptake was stopped by addition of cold PBS. After washing twice with cold PBS, cells were lysed in lysis buffer: 10 mM TRIS and 0.2% sodium dodecyl sulfate (SDS). The incorporated radioactivity was quantitated by scintillation spectrometry.

For inhibition and competition studies Cytochalasin B (Cyt B), Cytochalasin E (Cyt E), 2-deoxy-D-glucose (D-DOG), and 2-deoxy-L-glucose (L-DOG) were added 30 min prior to DHA treatment.

Vitamin C loading

HUVEC and HCAEC were loaded with controlled concentrations of AA by incubation with different concentrations of DHA for 30 min at 37°C before exposure to hypoxia. DHA was generated by incubating AA with ascorbate oxidase in incubation buffer at pH 5.5.

Estimation of cell volume

Intracellular volume was estimated as previously described [23] with 30% correction for trapped extracellular radioactivity [33]. Briefly, 2×10^6 cells were incubated in 200 μl incubation buffer containing 1 mM 3-oxy-methyl-D-glucose (OMG) and 5 μCi of ^{3}H-OMG for 1 h. During incubation, equilibrium (zero-trans) was established between intra- and extracellular concentrations of OMG. After incubation 2 μl of 2 mM Cyt B was added to prevent efflux of trapped OMG during washing. The cells were then washed with 20 μM Cyt B in cold PBS to remove unincorporated radioactivity. Cell incorporated radioactivity was determined by scintillation spectrometry for ^{3}H. The cell volume estimated for HUVEC was 1.2 μl/10^6 cells.

Hypoxia and H-R treatment in vitro

For H-R experiments, HUVEC and HCAEC were first incubated under hypoxia using a 95% N_2 and 5% CO_2 for 4 h at 37°C in a water-jacketed N_2/CO_2 incubator (NU-4950: NuAire, Inc.) and then in a standard incubator 5% CO_2 in normal air (normoxia) at 37°C for 18 h. Cells were preloaded with AA by incubating with different concentrations of DHA before subjecting to hypoxia. For hypoxia experiments HUVEC were rendered ischemic by incubating the cells in Hanks balanced salt solution (HBSS) without glucose and serum under hypoxic conditions for 1 h.

Determination of caspase-3 activity

Caspase-3 activity in cell extracts was measured using a colorimetric assay (Sigma). Briefly, cells loaded with AA were subjected to H-R and lysed in ice-cold lysis buffer (Sigma). Caspase-3 activity was measured by the amount of Ile Glu Thr Asp-*p*-nitroaniline (pNA) substrate cleaved after incubation and quantitated at 405 nm using a colorimetric plate reader.

Determination of apoptosis

In situ detection of apoptosis was performed by terminal deoxynucleotidyl transferase (TdT) labeling of DNA using a Fluorescence In Situ Cell Death Detection kit (Boehringer