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Repetitive hypoxia increases lipid loading in human macrophages—a potentially atherogenic effect

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Abstract

Obstructive sleep apnea (OSA) is characterized by repetitive episodes of hypoxia and is associated with an increase in cardiovascular disease. We, therefore, investigated the effect of repetitive hypoxia on two key early events in atherogenesis; lipid loading in foam cells and monocyte adhesion to endothelial cells. Human macrophages were loaded with acetylated low-density lipoproteins. During lipid loading, the cells were exposed to 30 min cycles of 2%/21% oxygen or control (room air, 5% CO_2 incubator). Human umbilical vein endothelial cells (HUVECs) were also exposed to 30 min cycles of repetitive hypoxia or control conditions and monocyte adhesion measured. Cell adhesion molecules E-selectin, ICAM-1 and VCAM-1 were measured by ELISA. Repetitive hypoxia increased cholesteryl ester uptake by macrophages ($127 \pm 5\%$ compared to controls; $p = 0.003$). By contrast, monocyte adhesion to HUVECs and cell adhesion molecule expression were unchanged by exposure to repetitive hypoxia, compared to controls ($p > 0.1$). Repetitive hypoxia, at levels relevant to tissues such as the arterial wall, enhances lipid uptake into human macrophages. This may contribute to accelerated atherosclerosis in OSA patients.

Keywords: Sleep apnea; Atherosclerosis; Foam cell

1. Introduction

Obstructive sleep apnea (OSA) is a common condition in adults [1] and is associated with an increased risk of cardiovascular events [2,3]. Recent studies have demonstrated a direct causal relationship between OSA and systemic hypertension [4]. In addition, OSA is common in the metabolic syndrome of obesity, increased hip to waist ratio, dyslipidaemia and insulin resistance, which also predisposes to vascular disease [5].

Moderate to severe obstructive sleep apnea is characterized by repetitive episodes of hypoxia and surges of sympathetic activity. These cycles can occur hundreds of times a night [6].

An independently increased risk of coronary events has been found in a number of studies of patients with OSA [7,8]. Mechanisms to explain this observation have included sympathetic nervous system activation [9] and systemic endothelial dysfunction [10]. The effects of prolonged repetitive hypoxia on atherogenic processes, however, have never been studied [11]. We, therefore, designed the first tissue culture system to allow prolonged exposure to varying degrees

Abbreviations: HMDM, human monocyte-derived macrophage; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; LDL, low-density lipoprotein; OSA, obstructive sleep apnea; VCAM, vascular cellular adhesion molecule

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of repetitive hypoxia. Using this novel system we investigated the effects of repetitive hypoxia on two key events in atherosclerosis; macrophage lipid loading, a process that occurs within the arterial wall, and the adhesion of monocytes to vascular endothelial cells [12].

2. Methods

2.1. Induction of repetitive intermittent hypoxia in vitro

Primary human cells were grown in conditions of repetitive hypoxia in a customized gas flow chamber, or in normoxic conditions (room air, 5% CO_2). Two premixed gases, run via two-stage regulators through flow meters at 6 l/min, were humidified and warmed to 37 °C (Fischer and Paykel 730 humidifier). Repetitive hypoxic cycles were controlled by a customized gas alternator. Gassing regimes chosen were prolonged repetitive hypoxia, 48 h; short repetitive hypoxia, 4 h; and short repetitive hypoxia, 4 h, followed by 20 h of normoxia, to assess for any delayed effects of repetitive hypoxia. Five percent oxygen, 5% CO_2 and nitrogen balance was the premixed gas used to replicate intravascular repetitive hypoxia; 2% oxygen was used for repetitive hypoxia relevant to extravascular tissue and atherosclerotic plaque [13,14].

2.2. Isolation of human monocytes

Human monocytes were isolated from white cell concentrates or whole blood donated by healthy volunteers, as previously described by us [15]. Only aliquots with a monocyte purity of >90% [16] were plated out for cell culture.

2.3. Lipid loading of human monocyte-derived macrophages (HMDMs)

2.3.1. Culture and acLDL loading of HMDMs

Isolated monocytes were resuspended onto 24 mm diameter tissue culture wells. After 1.5 h of adherence, the media was changed to RPMI supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mmol/l) (ICN Biomedicals) and 10% (v/v) filtered and heat inactivated human serum.

Low-density lipoproteins (LDL), isolated from whole blood, was acetylated at 4 °C, as previously described [15]. On days 8–10, the HMDMs were lipid loaded with acLDL 50 μg/ml in RPMI supplemented with lipoprotein deficient serum ($d > 1.25$) and Hepes (50 mmol/l) (Sigma). During lipid loading the cells were exposed to repetitive hypoxia gas mixtures, as above.

2.3.2. Analysis of cholesterol and cholesteryl esters

Cell extracts were prepared and free cholesterol and cholesteryl esters were separated by reverse-phase high-performance liquid chromatography at room temperature on a C-18 column (Supelco), as described previously [17]. Cholesteryl esters were analyzed with acetonitrile/isopropanol (30:70, v/v), whereas free cholesterol values were assessed with acetonitrile/isopropanol/water (45:54:2, v/v/v) with detection at 205 nm for both parameters (Activon UV-200 absorbance detector). In our laboratory, the coefficient of variation for measuring macrophage cholesteryl ester formation is 11%.

2.4. Monocyte adhesion to endothelial cells and expression of cell adhesion molecules

2.4.1. Monocyte–endothelial cell adhesion studies

Human umbilical vein endothelial cells (HUVECs) were harvested and grown to confluence, as previously described [18,19], Confluent endothelial cell monolayers were incubated under repetitive hypoxia or normoxic conditions. Monocyte adhesion assays were performed as previously described [19]. The percentage of adherent monocytes was then calculated in the basal state and stimulated by pre-incubation with IL-1β (25 U/ml). Cell viability >90% was confirmed for all experiments with LDH assays and light microscopy for trypan blue inclusions.

2.4.2. Cell adhesion molecule detection on endothelial monolayers

Confluent endothelial monolayers were pre-incubated in 96-well plates under the same conditions as the adhesion studies. Endothelial cell surface expression of adhesion molecules was assessed by ELISA (as described previously [19]); the coefficient of variation for such measurements in our laboratory is 10%.

2.5. Statistical analysis

Descriptive data are expressed as mean ± S.E.M. Groups were compared by a one-way ANOVA with Scheffe's test for post hoc pairwise comparisons. Statistical significance was inferred at a two-sided value of $p < 0.05$.

3. Results

3.1. Effects of repetitive on macrophage lipid loading

In the absence of lipid loading, HMDM's usually contain 50–100 nmol free cholesterol/mg cell protein, and little or no cholesteryl esters. Incubation with acLDL leads to additional cholesteryl ester accumulation, consistent with foam cell formation. Prolonged repetitive hypoxia, with an oxygen tension relevant to atherosclerotic plaques (2% alternating with 21%), was associated with a significant increase in macrophage cholesteryl ester accumulation (Fig. 1) after 48 h of exposure, compared to macrophages cultured in control conditions (127 ± 5%, $p = 0.003$). The increase was demonstrated in all cholesteryl esters measured including docosahexaenoate, arachidonate, linoleate, palmitate and oleate (Fig. 2). The greatest proportional increase was in cholesteryl