Digestive and Liver Disease xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Alimentary Tract

Myogenic oxidative imbalance interferes with antral motility in obese subjects

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ARTICLE INFO

Article history:
Received 3 November 2017
Received in revised form 3 March 2018
Accepted 6 March 2018
Available online xxx

Keywords:
Human gastric smooth muscle cells
Obesity
Oxidative stress
Vasoactive intestinal peptide

ABSTRACT

Background: Obesity is characterized by a systemic low-grade chronic inflammatory oxidative condition that affects vascular and cardiac smooth muscle relaxation. In human antrum, relaxation is mediated by vasoactive intestinal peptide (VIP) through cAMP and cGMP signaling pathways. A genome-wide association study has demonstrated an association between VIP and obesity.
Aim: To evaluate smooth muscle activity in human obese antrum, both in *in vitro* preparations as well as *in vivo*.
Methods: Antral muscle strips and cells were isolated from surgical gastric samples from obese and normal weight subjects. Muscle contraction and relaxation, myogenic oxidative stress and inflammatory status were analyzed *in vitro*. Distal antral motility was evaluated *in vivo* by magnetic resonance imaging.
Results: Obese antral muscle cells showed an oxidative-inflammatory imbalance with overexpression of NLRP3 inflammasome, increased IL-1β secretion and caspase1-activation, and reduced antioxidant capacity associated with a myogenic motor impairment of VIP-induced relaxation. The intracellular alterations were characterized by a decreased activation of the cAMP-signaling pathway and a decreased expression of eNOS. These *in vitro* alterations coincided with the hindering of antral motor activity observed *in vivo*. Apocynin treatment, counteracting oxidative stress, reverted alterations observed in obese antral muscle.
Conclusion: Antral myogenic activity of obese subjects can be impaired by alterations of signaling pathways induced by oxidative stress.

1. Introduction

Obesity is a condition closely associated with a systemic low-grade chronic inflammatory oxidative condition [1]. Inflammation occurs as a result of immune cell infiltration into the adipose tissue and increased production of pro-inflammatory cytokines intrinsically linked to oxidative stress and responsible of obesity-derived metabolic complications. Oxidative stress and inflammatory cytokines are also considered as potential factors in the induction of vascular dysfunction linked to hypertension, atherosclerosis, heart failure and diabetes [2].

Tissue inflammation occurring in obesity is apparently due to the nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) that, once activated by ROS, induces the release of the pro inflammatory cytokine IL-1β [3]. Generation of ROS, by reducing nitric oxide (NO) bioavailability, significantly affects vascular and cardiac smooth muscle relaxation [4,5]. The decreased NO production in obesity has also been ascribed to a reduced endothelial Nitric Oxide Synthase (eNOS) protein expression [6]. Furthermore, oxidative stress and inflammatory cytokines interfere in the regulation of G-protein coupled receptors (GPCR) that are essential molecules activating critical

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https://doi.org/10.1016/j.dld.2018.03.005
1590-8658/

intracellular signaling pathways such as the cAMP and the phosphatidylinositol signaling pathways [7]. In particular, human antral smooth muscle relaxation is mainly mediated by vasoactive intestinal peptide (VIP) through its interaction with two subtypes of GPCRs receptors, $VPAC_2$ and a single-transmembrane natriuretic peptide receptor C (NPR-C) [8]. $VPAC_2$ activates adenylyl cyclase and Protein Kinase A intracellular pathways whereas the NPR-C receptor activates Ca^{2+}/calmodulin-dependent eNOS, leading to NO formation and activation of soluble guanylyl cyclase and Protein Kinase G [9]. VIP binding to its receptors leads to the activation of both cGMP- and, more importantly, cAMP-dependent signaling pathway, which is essential for human antrum relaxation [8]. Of note, VIP pathway has been found to be significantly associated to obesity by a genome-wide association study [10].

The key hypothesis of the present study is that obesity-related inflammatory/oxidative imbalance may interfere with the VIP-pathway induced relaxation of human antrum. Hence, smooth muscle impairment in obese antrum was evaluated by using both *in vitro* preparations (strips and cells) as well as *in vivo* in obese subjects by using magnetic resonance imaging.

2. Materials and methods

2.1. Subjects

The 2 groups of subjects were studied consisting of 15 morbid obese subjects submitted to sleeve gastrectomy (OB) and 13 normal weight subjects submitted to gastrectomy for gastric cancer (NW), used as control group. Obesity was defined as BMI $\geq$30 kg/m^2. All subjects gave written informed consent and the study was approved by the Ethical Committee (University Sapienza, rif.2903-date: 26/09/2013).

2.2. Tissue preparations

Gastric antral muscle obtained from the 2 groups of subjects was used for *in vitro* muscle preparations. Smooth muscle cells (SMC) and strips were isolated separately from the muscle layer of surgical gastric samples of the terminal antrum [8] and primary cultures of human SMC were prepared as previously described [11]. Surgical specimen from gastrectomy for gastric cancer were removed at least 5–6 cm from the neoplastic area at the histologically disease-free margin of antral resection in NW patients [8].

2.3. Measurement of contractile and relaxant muscle response

2.3.1. Strips

Tone was raised on muscle strips, after the equilibration period, by a fixed dose of carbachol (10 μmol l^{-1}) that was repeated after 30 min and several washing steps. After, strips were exposed to increasing concentrations of carbachol (0.1–1000 μmol l^{-1}, cumulatively) in order to obtain concentration–response curves. Spontaneous phasic contraction of gastric antral strips was expressed as frequency (min^{-1}) and amplitude of contraction (mN cm^{-2}). The contraction to carbachol was expressed as mN cm^{-2}.

Relaxation was evaluated in response to cumulated increasing concentrations of VIP (0.01–3 μmol l^{-1}) added at a stable contraction plateau induced by KCl (80 mmol l^{-1}), causing a tonic contractile response of the strip that was stable for at least 20 min. After the contraction plateau was reached, VIP was added to the organ bath performing only one concentration response curve on each strip. The relaxation to VIP was expressed as a percentage decrease in the maximal contractile response to KCl. At the end of each experiment, strips were removed, blotted and weighted.

2.3.2. Cells and treatments

Contraction and relaxation studies were performed as previously described [8]. The length of 50 cells in sequential microscopic fields was measured by image-scanning micrometry both in the control state and upon addition of tested agents using a ProgRes® camera with CapturePro 2.6 application software (Jenoptik Laser Optik, Jena, Germany) installed on a phase-contrast microscope (Leica Microsystems, Wetzlar, Germany). Contraction was expressed as percentage decrease in cell length from control taken as 100 and relaxation as percentage inhibition of contraction.

2.3.2.1. Apocynin exposure. To evaluate the contribution of oxidative stress, OB gastric SMC were incubated for 24 h in the absence or presence of the NADPH oxidase inhibitor apocynin (APO) (60 μM) [12] and thereafter relaxation, antioxidant capacity, IL-1β secretion and eNOS transcript were re-evaluated. SMC without APO were considered as control.

2.3.2.2. Hydrogen peroxide treatment. To induce mild oxidative stress, cells were treated with 30 μM H_2O_2 for 30 min. After this time samples were prepared for contractile activity analyses.

2.4. Total RNA extraction and qPCR on SMC

Total RNA was harvested from NW and OB SMC, using Trizol (Invitrogen Life Technologies, Carlsbad, CA) and single-stranded cDNA was synthesized according to the manufacturer's protocol (Invitrogen Life Technologies). mRNAs were quantified using TaqMan Gene Expression Assays (Applied Biosystems), with specific human primers: nlpr3(Hs00918082_m1), pycard (Hs00203118_m1), caspase-1(Hs00354836_m1), IL-1β (Hs00174097_m1), eNOS (Hs01574659_m1). Data were analyzed and expressed as relative quantification (RQ) values obtained by determining ΔCt values followed by determining $\Delta\Delta$Ct values and then RQ values via the equation $2^{-\Delta\Delta Ct}$.

2.5. Antioxidant capacity and cytokine secretion measurement

NW and OB SMC antioxidant capacity was determined by evaluation of the combined non-enzymatic antioxidant capacity by TAC colorimetric assay Kit (Cell Biolabs Inc. San Diego, CA, USA), which measures the sample's capacity to convert Cu^{+2} to Cu^{+1}, following manufacturer's instructions. Results obtained by TAC assay are converted to trolox equivalents. IL-1β levels were evaluated by using a specific ELISA kit according to the manufacturer's protocol (Cayman; R&D) on the culture medium of an equal cells number.

2.6. Redox parameters

2.6.1. ROS

Cells isolated from NW and OB patients were incubated in Hank's balanced salt solution, pH 7.4, with dihydrorhodamine 123 (DHR123, Thermo Fisher) in polypropylene test tubes for 15 min at 37 °C (final concentration 10 μM). DHR123 dye freely diffuses into cells and is primarily oxidized by H_2O_2 producing green fluorescence.

2.6.2. GSH

Following the same protocol, GSH intracellular content was evaluated by using monochlorobimane (MBC, Thermo Fisher). Samples were washed twice in ice-cold PBS and immediately acquired by an LRS II cytometer (Becton and Dickinson, San Jose, CA, USA) equipped with a UVB laser.

Please cite this article in press as: Scirocco A, et al. Myogenic oxidative imbalance interferes with antral motility in obese subjects. Dig Liver Dis (2018), https://doi.org/10.1016/j.dld.2018.03.005