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Sex-specific pharmacological modulation of autophagic process in human umbilical artery smooth muscle cells

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ABSTRACT

Sex has largely been neglected in cell studies. Therefore, we investigated the occurrence of sexual dimorphism in human umbilical artery smooth muscle cells (HUASMCs). In particular, we investigated the existence of sex differences in basal and in drug-induced autophagy, a process involved in cardiovascular diseases.

HUASMCs were isolated from healthy and normal weight male and female newborns (MHUASMCs and FHUASMCs, respectively). Expression of the primary molecules involved in the autophagic process [beclin-1 and microtubule-associated protein 1 light chain 3 (LC3)], and PmTOR were detected using western blotting in basal conditions, after serum starvation, rapamycin and verapamil treatments.

The level of constitutive autophagy, measured as the LC3II/I ratio, was similar in male and female HUASMCs in the basal condition. Serum starvation promoted autophagy in both cell types, but the increase was more pronounced in FHUASMCs, while 250 nM rapamycin induced autophagy only in female cells. Moreover, the level of verapamil-induced autophagy was not different between the two sexes. Notably, in the basal condition, Beclin-1 was more elevated in MHUASMCs than in FHUASMCs, and the difference disappeared after serum starvation and exposure to rapamycin. After exposure to verapamil, the differences in Beclin-1 increased, with more elevated expression levels in female cells. PmTor did not differ in basal conditions, but it was significantly down-regulated by starvation only in FHUASMCs and by rapamycin both in male and female cells. Finally, a strong negative correlation was observed between the newborn's weight and basal autophagy in female cells and between the newborn's weight and the LC3II/I ratio in male verapamil-treated cells.

These results indicate that sex-differences begin in utero, are parameter-specific and drug specific suggesting that HUASMCs are a suitable model for the screening of drugs and to study the influence of sex. The sex differences in the autophagy suggest sexually different pharmacodynamics effects of verapamil and rapamycin.

1. Introduction

Sex bias is an on-going problem in medicine, and sex differences are observed in physiological or pathological conditions as well as in drug responses [1–3]. Cardiovascular system is highly affected by sex differences both physiologically and pathologically, and in response to drug treatments [1,2,4–6]. As examples, women suffer from endothelial and smooth muscle dysfunction to a greater extent than men [7]. Although sex and gender can affect outcomes and the interpretation and applicability of the data, the sex of cells in culture is not consistently reported in many studies [8,9].

Abbreviations: HUASMC, human umbilical artery smooth muscle cells; HRP, horseradish peroxidase; LC3, microtubule-associated protein 1 light chain; PmTOR, phosphorylated mammalian target of rapamycin; PBS, phosphate-buffered saline.

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Vascular smooth muscle cells (VSMCs) have a pivotal role in maintaining the physiology of blood vessel [10]. Changes in autophagic function has been described in pulmonary hypertension [11], vascular aging [12], and atherosclerosis [13]. Autophagy represents an important homeostatic mechanism, which occurs through lysosomial enzyme [14], for the maintenance of normal cardiovascular function and morphology [15]. Recently, it has been shown that sex may influence autophagy either in vitro or in vivo, in studies performed in animal cells and organs [16–23], or in human cells, such as HUVECs [24].

Autophagy can be stimulated by a number of events including nutrient deprivation, exposure to pathogens, oxidative stress [25] and drugs such as rapamicyn, lithium, sodium valproate, clonidine, calcium channel blockers [26–29]. We analysed constitutive and starvation, rapamycin and verapamil induced autophagy in human VSMCs through the analysis of the main molecules involved: beclin-1, LC3 and PmTOR, a critical regulator of autophagy (29). These three different stimuli induce autophagy through different mechanisms. In particular, serum starvation acts by reducing mTOR activation [30], acting through the mTOR complex 1 (mTORC1), which senses the changes in the metabolism of the organism [32], whereas rapamycin acts inhibiting mTOR [29]. Finally, verapamil, a calcium channel blocker with anti-atherosclerotic properties [32], induces autophagy through a calcium-dependent [26] or independent pathway [27]. Indeed, sex-specific effects of these selected stimuli have been reported. Some authors, as example, show sexually specific effects of rapamycin on the proteasome-chaperone network [33], and a decrease in maladaptive cardiac hypertrophy and a promotion of the detrimental dilative cardiomyopathy, in male and female rapamycin-treated rodents, respectively [34]. Finally a sex specificity in the effects of enteric rapamycin treatment is described by Fischer et al. [35]. According to DrugCite, adverse effects due to verapamil are more frequent in women than in men [36]. Hepatocytes obtained from women are more sensitive to hepatotoxicant damage being verapamil more toxic in the post-menopausal female group [37]. Also serum starvation effects may be sex different, as female HUVECs show a higher cell viability after serum starvation and an increased tube formation capacity compared to male cells [38] and neurons from males rodents during starvation more readily undergo autophagy and die than female ones [20].

2. Materials and methods

2.1. Donors

To overcome the limited availability of human VSMCs, we used human cord umbilical arteries as a source of VSMCs (i.e., HUASMCs). The successful culture of HUASMCs depends not only on the culture conditions but also on factors preceding the cell's harvest, such as birth weight and maternal smoking status [39]. Therefore, we selected umbilical cords from healthy and normal weight human male and female newborns (total males = 21; total females = 23) that were vaginally delivered at term (37–42 weeks) at the Obstetrics and Gynaecology Clinic, University of Sassari, from healthy non-obese and non-smoking mothers who were also drug-free, with the exception of folic acid and iron supplementation. The neonate's normal weight range was established in accordance to the Ines charts as described by Bertino et al. [40](2550–4190 g for males and 2430–4050 g for females, representing the 10th and 90th centiles in Ines Charts). Informed consent was obtained from all mothers donating their neonate's umbilical cord, in accordance with the Declaration of Helsinki. The local ethics board of University of Sassari approved the experiments.

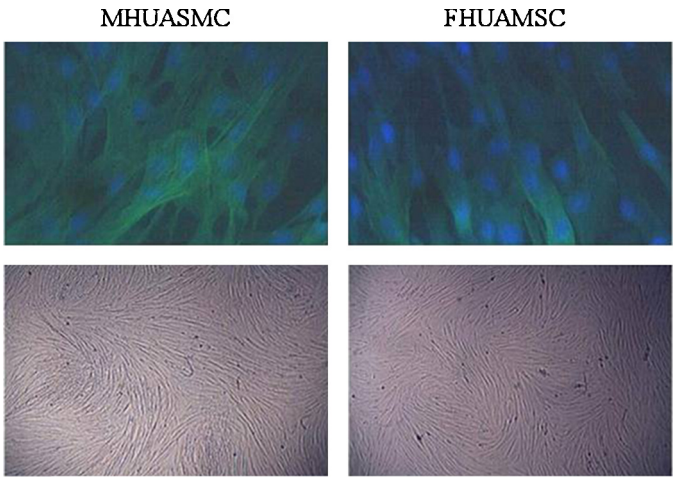

Fig. 1. Morphology of male and female HUASMCs and typical immunofluorescence images for α-smooth muscle actin. The green filaments represent α-smooth muscle actin. Nuclei are shown in blue. Images were acquired by microscopy at 20× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

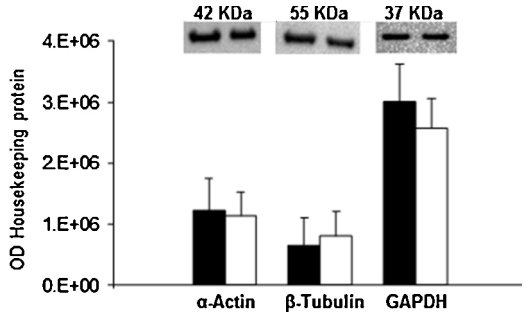

Fig. 2. Representative Western blot images and densitometric analysis is shown for α-actin, β-tubulin and GAPDH expression.
Data are expressed as the mean of 12 samples for each sex ± SD. black is male, white is female.

2.2. Isolation, culture and characterisation of HUASMCs

Cells were isolated within the next 12 h after delivery. Under sterile conditions, cord arteries were identified and excised along their entire length, and all the surrounding connective tissue was carefully removed. Then, HUASMCs were isolated as previously described [41], with some modifications. Briefly, the arteries were incubated in DMEM (Gibco, Carlsbad, CA, USA) containing 80 U/ml of collagenase from *Clostridium histolyticum* (Sigma-Aldrich, Saint Louis, MI, USA) for 1 h. Then, arteries were placed in a Petri dish with sterile PBS, and the outer layer composed of muscle cells was gently scrubbed in PBS until the arteries reached a translucent aspect. The cell-containing PBS was centrifuged for 15 min at 2000 rpm. The pellet was collected and suspended in DMEM with 20% FBS and 1% antibiotic/antimycotic (Sigma-Aldrich, Saint Louis, MI, USA), and the cells were seeded in gelatin-coated flasks (Sigma-Aldrich, Saint Louis, MI, USA). The flasks were kept at 37 °C in a humidified atmosphere with 5% CO2 for 5 days; after this period, the attached cells were washed with PBS, and the flasks were replenished with fresh medium. As previously described [42,43], the cells were characterized by immunofluorescence using an anti-α-smooth muscle actin antibody, a marker of muscular cells. Briefly, cells were washed and fixed with freshly prepared 4% paraformaldehyde (Sigma-Aldrich, Saint Louis, MI, USA). Next, the cells were incubated with the specific antibody, which was then detected using a FITC-conjugated secondary antibody (Sigma-Aldrich, Saint Louis, MI, USA). Cells