FEBS Letters 583 (2009) 3448–3454

journal homepage: www.FEBSLetters.org

Cell sex determines anoikis resistance in vascular smooth muscle cells

Elisabetta Straface [a,*], Rosa Vona [a], Lucrezia Gambardella [a], Barbara Ascione [a], Maria Marino [b], Paola Bulzomi [b], Silvia Canu [c,d], Rita Coinu [c,d], Giuseppe Rosano [e], Walter Malorni [a,e,1], Flavia Franconi [c,d,1]

[a] Department of Drug Research and Evaluation, Section of Cell Aging and Degeneration, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
[b] Department of Biology, University Roma Tre, Viale G. Marconi 446, Rome, Italy
[c] University of Sassari-INBB Osilo-Sassari, Department of Pharmacology, Via Muroni 23a, Sassari, Italy
[d] Department of Pharmacology and Center for Biotechnology Development and Biodiversity Research, University of Sassari, Italy
[e] IRCCS San Raffaele, Rome, Italy

ARTICLE INFO

Article history:
Received 21 April 2009
Revised 28 August 2009
Accepted 28 September 2009
Available online 3 October 2009

Edited by Vladimir Skulachev

Keywords:
Reactive oxygen species
Vascular smooth muscle cells
Apoptosis
Gender
Anoikis
Cell adhesion
Autophagy
Senescence

ABSTRACT

Sexual dimorphism, detectable in vascular smooth muscle cells freshly isolated from aorta of male and female rats, is associated with a different susceptibility to radiation-induced apoptosis. In this work we investigated the mechanism underlying this difference and discovered that, in comparison with cells from male rats, cells from female rats show adhesion-associated resistance to apoptosis, the so called anoikis resistance. This is apparently due to a more adhering phenotype, characterized by a well organized actin microfilament cytoskeleton and to an increased phosphorylated focal adhesion kinase, and, more importantly, to a higher propensity to undergo survival by autophagy.

1. Introduction

Sexual dimorphism is a characteristic of cardiovascular system diseases [1]. Furthermore, some cardiovascular complications associated with hypertension, atherosclerosis and diabetes are largely gender associated [2] and sustained by redox alterations [3,4]. In a previous study performed in rat-derived vascular smooth muscle cells (VSMC) [3], of great importance in vascular diseases, we evidenced that, in basal conditions, male-derived VSMC (MVSMC) and female-derived VSMC (FVSMC) display marked gender differences in terms of redox balance. This sexual dimorphism is maintained after oxidative stress and reflects on cell fate, being cells from males more prone to apoptosis [3]. These observations offered a rationale for further investigations on the mechanisms underlying cellular gender differences. Here, we analyzed apoptotic resistance displayed by cells from female rats. In particular, we studied anoikis, a type of apoptosis induced by detachment of adhering cells from a substrate [5]. This has been investigated by studying cell adhesion and its determinants such as actin cytoskeleton assembly and focal adhesion kinase (FAK), whose phosphorylation occurs through signaling protein kinases activated by O_2^- and H_2O_2 [5,6]. Moreover, to better investigate the pathophysiological consequences of oxidative stress, 4-hydroxynonenal (4-HNE), an end-product of membrane lipid peroxidation, has been evaluated [7]. Finally, autophagy, an important mechanism of adaptation to stress, including the oxidative one, has also been evaluated [8]. We actually found that cells from female survive better to UVB-induced stress thanks to a more potent antioxidant system, to a better-adhering phenotype, to their anoikis resistance and, finally, to the development of a fruitful autophagic behavior.

Abbreviations: ROS, reactive oxygen species; VSMC, vascular smooth muscle cells; MVSMC, male-derived vascular smooth muscle cells; FVSMC, female-derived vascular smooth muscle cells; 4-HNE, 4-hydroxynonenal; FAK, focal adhesion kinase; p-FAK, phosphorylated focal adhesion kinase; SEM, scanning electron microscopy; UVB, ultraviolet B radiation; E2, estradiol; ERs, estrogen receptors; AR, androgen receptors

* Corresponding author. Department of Therapeutic Research and Medicines Evaluation, Section of Cell Aging and Degeneration, Istituto Superiore di Sanita', Viale Regina Elena 299, 00161 Rome, Italy. Fax: +39 06 49903691.
E-mail address: straface@iss.it (E. Straface).
[1] To be considered as senior authors.

0014-5793/$36.00
doi:10.1016/j.febslet.2009.09.052

2. Materials and methods

2.1. Cell cultures and treatments

Vascular smooth muscle cells (VSMC) were isolated from descending aorta of both female and male young rats. Primary cultures of VSMC were maintained in DMEM + GlutaMAX medium (GIBCO-Invitrogen, MI, Italy) containing 1 g/l D-glucose, supplemented with 10% fetal calf serum, 1% non-essential amino acids, 100 μ/ml penicillin and 100 ng/ml streptomycin. Cells were seeded at density of 2×10^5 cells in the Petri dishes and maintained at 37 °C. All the experiments, treatments and corresponding controls, were performed in phenol red-free DMEM culture medium (GIBCO-Invitrogen 11880-028). For fluorescence intensified charge-coupled device video microscopy (IVM) analysis, cells were seeded on 13 mm diameter glass cover-slips in separate wells. Cells at passage 2–4 were used in this study. However, gender differences shown below, e.g. redox imbalance or estrogen receptor expression, were detectable up to the 14–16th VSMC passage.

UVB exposure was performed as previously described [3]. FAK inhibitor (1,2,4,5-benzenetetramine tetrahydrochloride, Tocris Bioscience, Bristol, UK) has been added to the cells 1 h before UVB exposure at two different concentrations (0.5–1 μM). All analyses were carried out 24 h after UVB irradiation.

2.2. Protein extraction and Western blot analysis

Cells were lysed in RIPA buffer (100 mM Tris–HCL pH 8, 150 mM NaCl, 1% Triton X-100, 1 mM MgCl) in presence of complete protease-inhibitor mixture (Sigma Chemical Co., St. Louis, MO, USA). In total, 25 μg of total protein extracts were resolved on 12% SDS–PAGE (according to the different molecular weights) and electrically transferred onto nitrocellulose membranes PVDF. Membranes were probed using the following antibodies: polyclonal anti-LC3 (MBL, International Corporation, Woburn, USA), monoclonal anti-Beclin 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti α-tubulin (Sigma). Detection was achieved using HRP-conjugated secondary antibodies (monoclonal or polyclonal) and by ECL detection system (Pierce; Rockford, IL). Analysis of estrogen and androgen receptors has been performed as previously reported [3]. For densitometric analysis of the signals obtained by Western blot were using the ID-image analysis software Kodak digital system. These results were expressed as arbitrary units (a.u.).

2.3. Analytical cytology

For transmission electron microscopy (TEM) analyses, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide, dehydrated through graded ethanols, embedded in Agar 100 resin and dissected in ultrathin sections. Scanning electron microscopy (SEM) analyses have been performed as previously reported [5]. For cytometric analysis cells were fixed with 4% paraformaldehyde and permeabilized with 0.5 Triton X-100 (Sigma). For actin detection cells were stained with fluorescein-phalloidin (Sigma) at 37 °C for 30 min. Quantification of monomeric (G-actin) and polymeric (F-actin) actin was performed as previously reported [9]. For 4-HNE (R&D System, Inc., Minneapolis, USA), FAK (total and phosphorylated) (Chemicon, Tecumala, CA, USA) and LC3II (MBL) detection, cells were incubated with monoclonal antibodies for 30 min at 37 °C and, after washings in PBS, incubated with FITC-labeled anti-mouse antibodies (Sigma). Then the samples were observed with an Olympus Microphot fluorescence microscope (Olympus Corporation, Tokyo, Japan) or analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose', CA, USA) equipped with a 488 nm argon laser. Redox state and cell senescence have been evaluated as stated elsewhere [3]. The percentage of apoptotic cells was evaluated by using Annexin V kit (MBL).

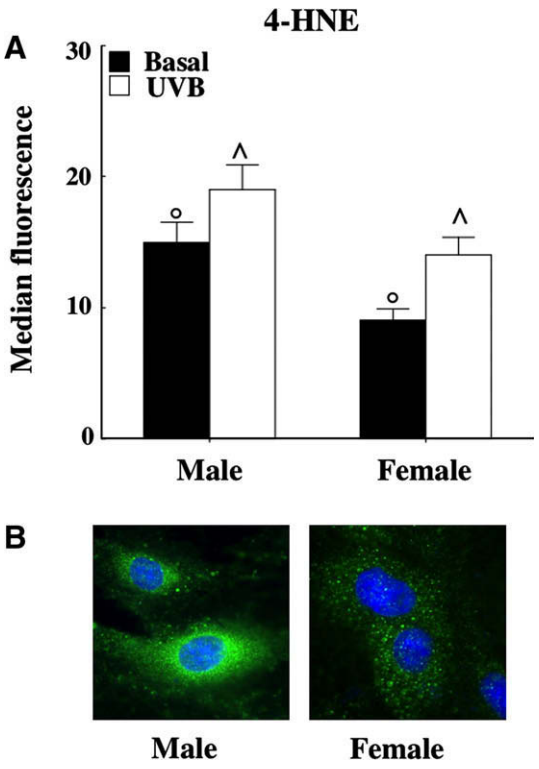

Fig. 1. (A) Quantitative evaluation of 4-HNE in basal and irradiated VSMC. Values are mean ± S.D. of four independent experiments. °$P < 0.01$ basal MVSMC versus basal FVSMC and ^$P < 0.05$ irradiated MVSMC versus irradiated FVSMC. (B) Representative micrographs showing the cytoplasmic distribution of 4-HNE in irradiated male and female VSMC.

2.4. Morphometric analyses

Quantitative evaluation of cells with actin "cross-links" was performed counting at least 300 cells at high magnification (500×) at the fluorescence microscopy equipped with a Zeiss CCD camera (Carl Zeiss, MI, Italy). Evaluation of apoptosis and senescence were performed as previously described [3].

2.5. Evaluation of the detached cells

Cells (density = 2×10^5) were seeded in the Petri dishes and maintained at 37 °C. Twenty-four hours after irradiation, detached and attached cells were collected separately and counted at the inverted microscope (Olympus) at 500×.

2.6. Statistical analysis

Cytofluorimetric results were statistically analyzed by using the parametric Kolmogorov–Smirnov test using Cell Quest Software. A least 20 000 events have been acquired. The median values of fluorescence intensity histograms were used to provide a semi-quantitative analysis. Results are presented as mean ± S.D. of at least three independent experiments. Statistical analyses were performed by using Student's *t*-test.