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Cell sex determines anoikis resistance in vascular smooth muscle cells

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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

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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).

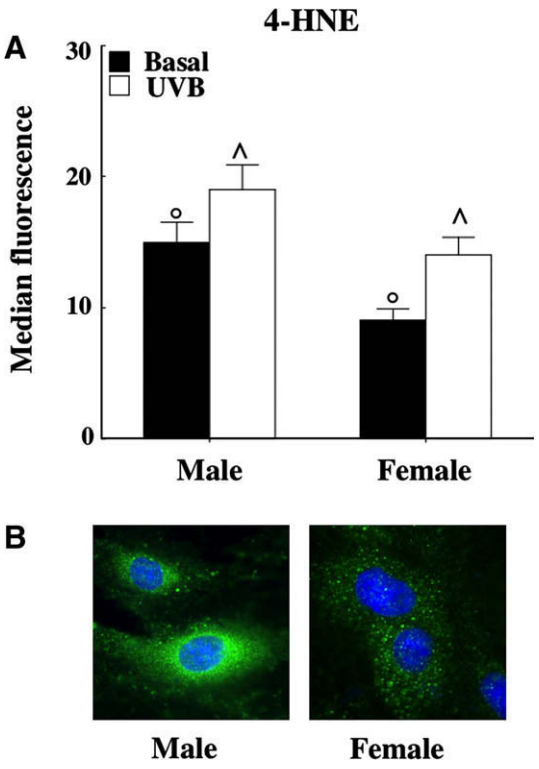

Fig. 1. (A) Quantitative evaluation of 4-HNE in basal and irradiated VSMC. Values are mean ± S.D. of four independent experiments. °$P < 0.01$ basal MVSMC versus basal FVSMC and ^$P < 0.05$ irradiated MVSMC versus irradiated FVSMC. (B) Representative micrographs showing the cytoplasmic distribution of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

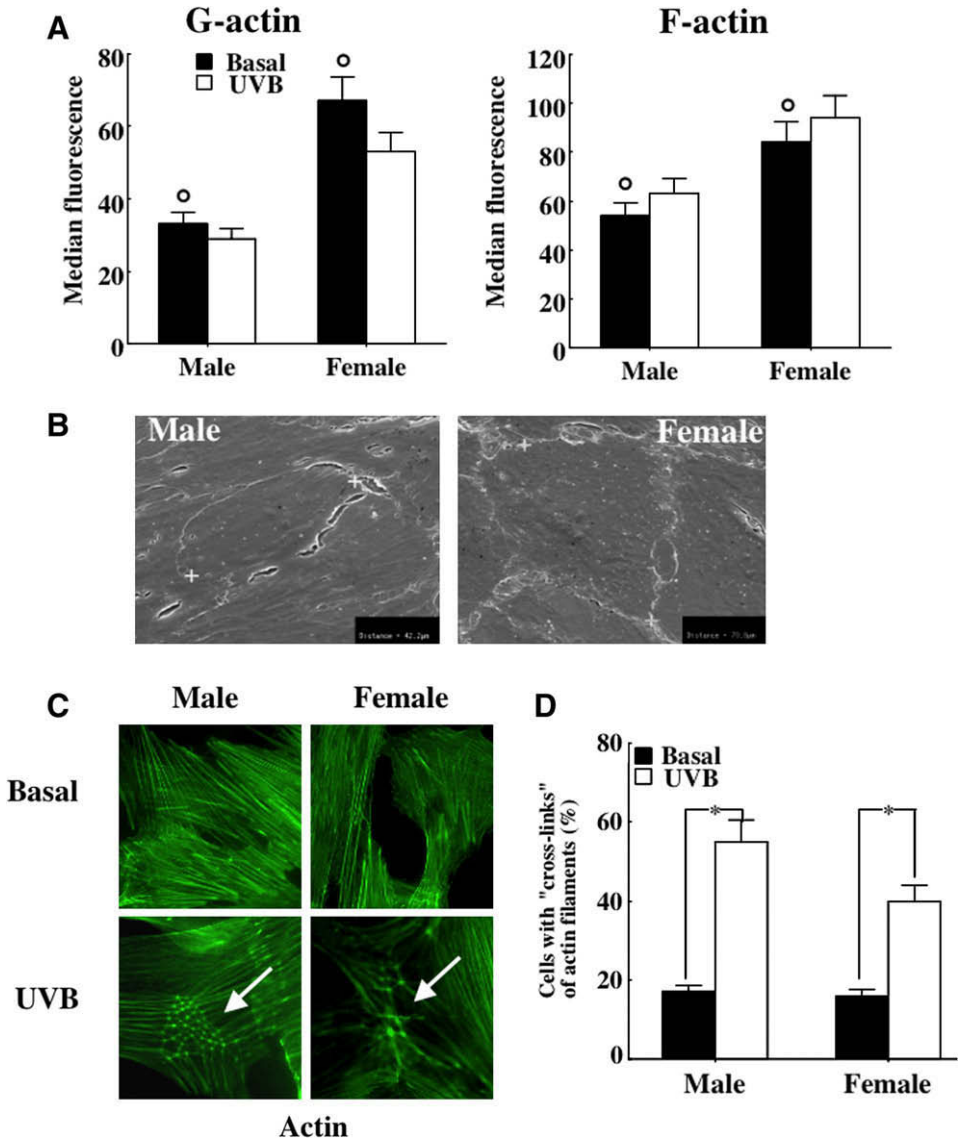

Fig. 2. (A) Flow cytometry evaluation of monomeric (G) and polymeric (F) actin obtained from four independent experiments ± S.D. (B) Representative micrographs obtained by scanning electron microscopy showing the typical polarized morphology of VSMC. The inset indicates a representative morphometric evaluation of cell diameter by SEM. (C) Fluorescence microscopy analysis of actin cytoskeleton. In basal cells (top panels) F-actin appears differently organized in FVSMC and MVSMC. After irradiation a marked reorganization of actin filaments occurs especially in cells from male rats. Arrows indicate actin cross-links. (D) Morphometric analysis of actin cross-links in cells from male and female before and after irradiation. Note the significant increase ($^{*}P < 0.01$) in irradiated versus basal VSMC of both sexes.

3. Results and discussion

3.1. Redox imbalance

As previously suggested [3], freshly isolated FVSMC (“basal FVSMC”): (i) produce less H_2O_2 and O_2^-; (ii) have higher levels of GSH; and (iii) have a major activity of SOD and catalase with respect to freshly isolated MVSMC (basal MVSMC). Thus, basal MVSMC display a sort of “physiological oxidative imbalance” when compared with basal FVSMC. Level of 4-HNE, a compound that provides a link between oxidant generation, lipid peroxidation, and cellular apoptosis [7] has also been measured 24 h after UVB irradiation. Basal MVSMC displayed a higher 4-HNE expression than FVSMC (Fig. 1A, black column), indicating that male cells have a higher level of lipid peroxidation products. UVB radiation significantly increased the 4-NHE levels in both male and female VSMC. The irradiated MVSMC produced more H_2O_2 and O_2^- and had a major decrement of antioxidant defenses in terms of GSH antioxidant enzyme with respect to FVSMC [3]. However, although the decrease of GSH is higher in irradiated FVSMC (49% in FVSMC versus 20% in irradiated MVSMC) the high level of this enzyme in basal FVSMC seems to provide these cells with a higher resistance to UVB-mediated oxidative stress [3]. Interestingly, the higher levels of 4-HNE and the lower levels of GSH in MVSMC could in part explain the major susceptibility of these cells to apoptosis induction (see below).

3.2. Cytoskeleton

Considering the importance of the actin filaments in cell shape maintenance, differentiation state and cell–substrate interaction, analyses have been conducted in order to evaluate the distribution and the organization of this protein. As a general rule, the assembly of actin cytoskeleton into parallel and elongated stress fibers [10] as well as FAK expression [11] have previously been considered as hallmarks of VMSC differentiated state, i.e. their

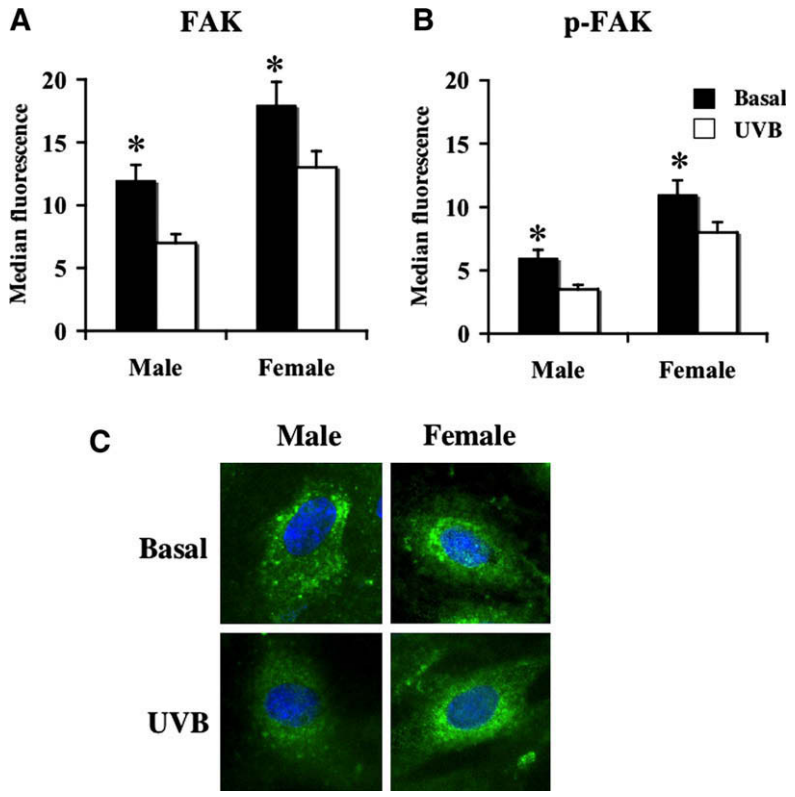

Fig. 3. Flow cytometry analyses of total FAK (A) and p-FAK (B). Data are means ± S.D. of four independent experiments. $^{*}P < 0.01$ basal MVSMC versus basal FVSMC. (C) Representative micrographs showing p-FAK distribution in basal (top panels) and irradiated cells (bottom panels).

contractile functional phenotype. In fact, these features were observable in our primary cultures (see below). Sex differences in VSMC cytoskeleton were already hypothesized by the pioneer study of Bacáková et al. [12]. Analyses carried out by static (morphometry) and flow cytometry 24 h after UVB exposure indicated that: (i) actin filaments clearly showed a different morphology in basal FVSMC in comparison with basal MVSMC (see below, Fig. 2B and C, upper panels) and (ii) basal FVSMC expressed higher amounts of actin (both monomeric, G-actin and polymeric, F-actin) than MVSMC (Fig. 2A). This is in line with previous works carried out in liver cells where a higher expression of F- and G-actin was found in cells from females than in those from males [9]. We can thus hypothesize that microfilament "sexual dimorphism" could concern more than one cell type. After UVB exposure, not significant variations of both G-actin and F-actin amounts were detected (Fig. 2A). Conversely, marked actin filaments reorganization was observed (Fig. 2C, bottom panels). In particular, a redistribution and thickening of the actin stress fibers with numerous "cross-links" of cytoskeleton filaments, visible as fluorescent punctuate spots, were especially evident in MVSMC (Fig. 2D). The presence of actin cross-links seems to indicate the occurrence of an oxidative remodeling of cytoskeleton with formation of side-by-side thiol group oxidative cross-linking [3]. These cytoskeleton sex-associated differences, probably due to radiation-induced oxidative stress, could have salient consequences in view of the importance of cytoskeleton in several cell processes including cell proliferation, adhesion and contractility as well as in cell death [13]. In fact, MVSMC and FVSMC proliferate differently and show different adhesion features and apoptotic susceptibility (see below). Accordingly, scanning electron microscopy (S.E.M.) analyses also suggest different morphological features of freshly isolated smooth muscle cells from males and females (Fig. 2B).

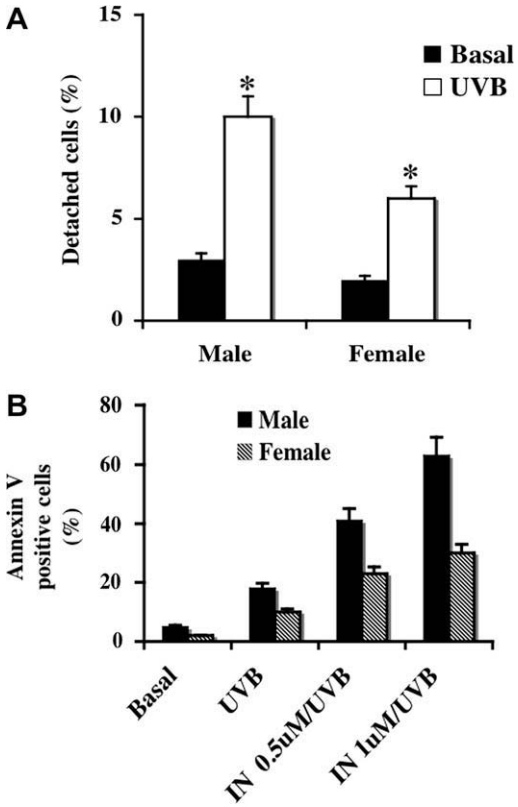

Fig. 4. (A) Percentage of detached cells 24 h after irradiation. Data are reported as mean values ± S.D. of four independent experiments. $^{*}P < 0.01$ irradiated MVSMC versus irradiated FVSMC. (B) Percentage of Annexin V positive cells treated with two different concentrations of FAK inhibitor 1 h before UVB exposure. Note the significant increase ($P < 0.01$) in treated versus basal VSMC of both sexes.

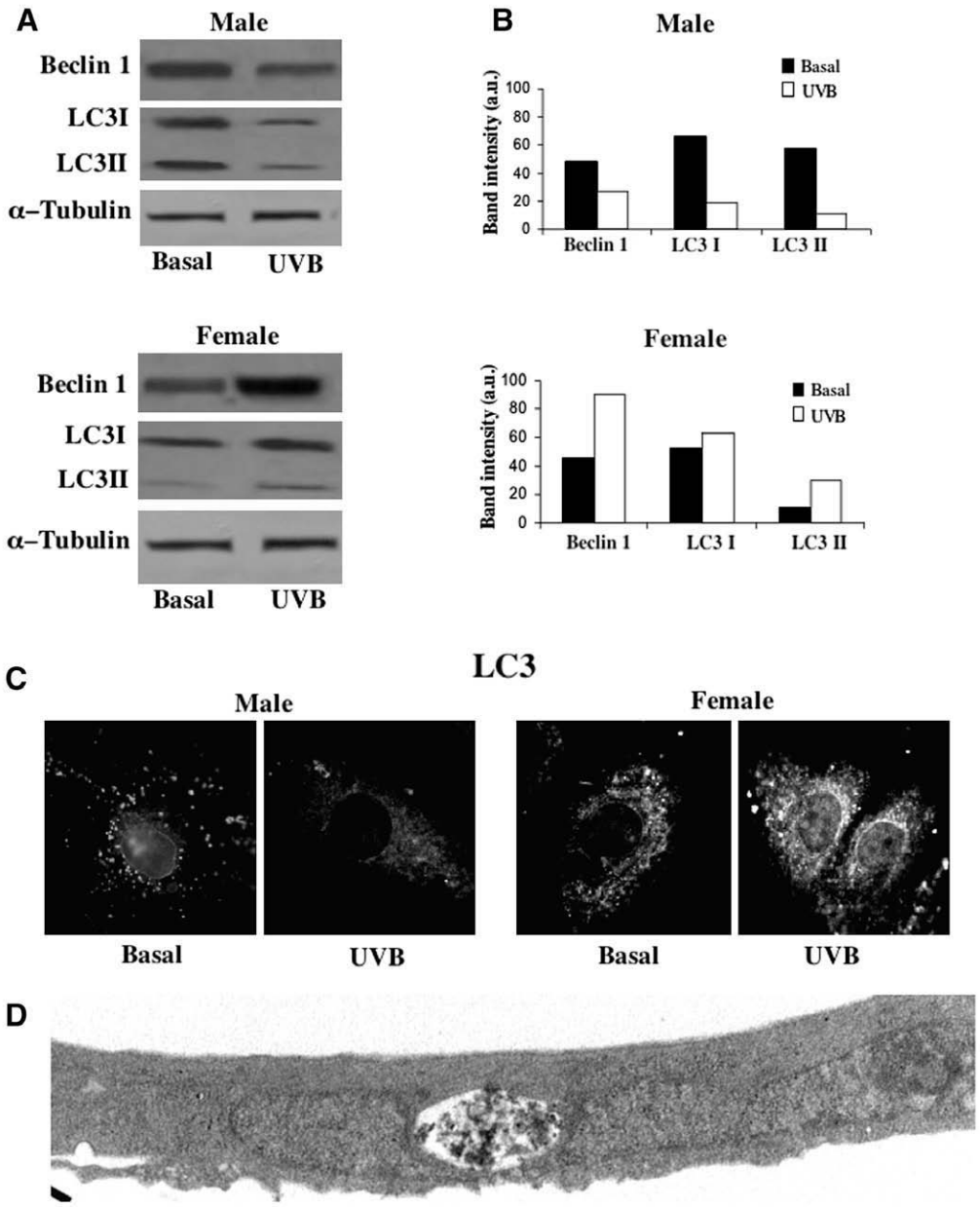

Fig. 5. Representative Western blot analysis (A) and relative densitometric analysis (B) of Beclin 1, LC3I and LC3II in both basal and irradiated VSMC. (C) Representative micrographs showing LC3 distribution in basal and irradiated cells of both sexes. Note the marked presence of autophagic vacuoles in irradiated FVSMC. (D) Representative micrograph obtained by TEM of FVSMC after radiation. The arrows indicate an autophagic vacuole. Magnification 12 000×.

3.3. Cell fate

On the basis of sex-associated differences (adhesion feature and apoptosis susceptibility) we decided to investigate the anoikis, a form of cell adhesion-dependent apoptosis. We first analyzed both total and phosphorylated FAK, an adhesion kinase that acts by promoting cell adhesion turnover and actin cytoskeleton remodeling. Here, we showed that both FAK and p-FAK is more expressed in basal FVSMC than in MVSMC (Fig. 3A and B). This is not surprising since the "physiological oxidative imbalance" that occurs in MVSMC could activate metalloproteinases, block some inhibitors of such enzymes and enhance proteolytic susceptibility of adhering cells [14]. Furthermore, after irradiation-induced oxidative stress (to which cells from male were highly susceptible), FAK and p-FAK levels decreased significantly in MVSMC with respect to FVSMC. Thus, FVSMC could have a survival advantage because the maintenance of cell anchorage to the substrate, one of the fundamental stimuli able to induce anoikis resistance [15] together with their cytoskeletal network features, which appeared less influenced by stress (Fig. 2). Our data seem also to suggest for the first time the existence of sex-based differences in FAK levels of VSMC either in basal conditions or after irradiation. Since cell–substrate adhesion represents a key factor in anoikis resistance, a specific analysis of this parameter has been carried out. In fact, the detachment test, i.e. the evaluation of the number of cells flowing freely in the culture medium 24 h after oxidative stress (Fig. 4A), further suggested that FVSMC were more resistant to oxidative stress-induced detachment. It is known in fact that cell

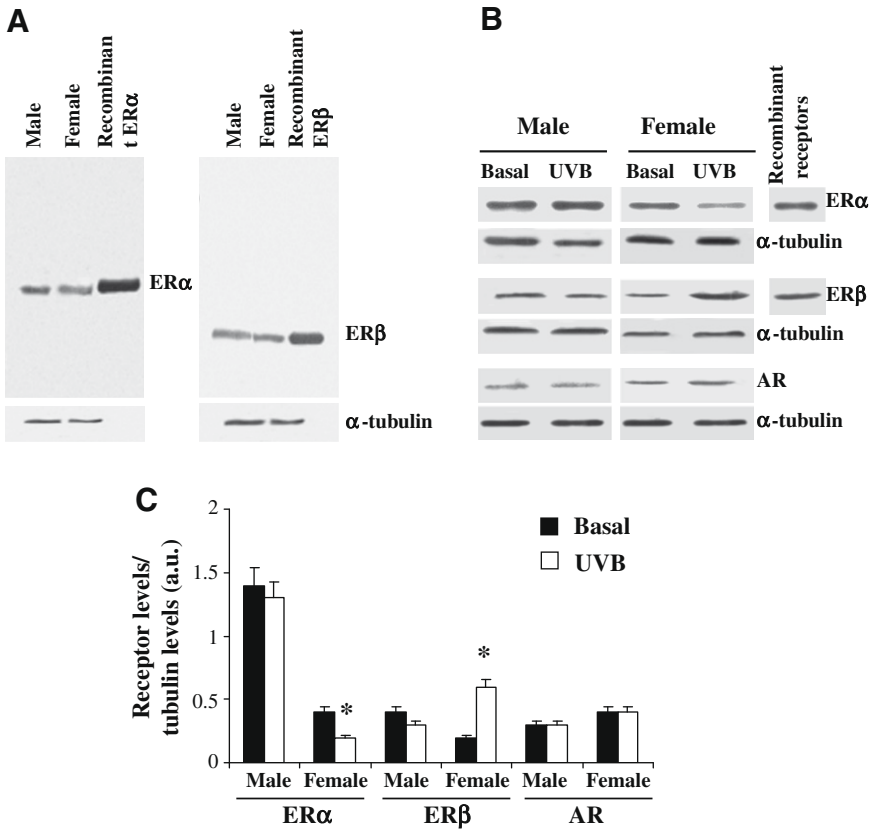

Fig. 6. Representative Western blot analysis of estrogen receptors (ER). ERα, ERβ and androgen receptor (AR) levels in VSMC cells from male and female rats. (A and B) Typical Western blot and (C) densitometric analysis are shown. $^{*}P < 0.01$ irradiated versus basal FVSMC.

detachment after oxidative stress, e.g. induced by H_2O_2, plays a key role in anoikis [5] whereas the overexpression of manganese SOD delays it [16].

To better investigate the role of FAK in the anoikis resistance, experiments were carried out in both male and female cells by using two different concentrations of FAK inhibitor (added at the cells 1 h before UVB exposure). In particular, the percentage of apoptotic cells have been evaluated 24 h after UVB exposure. As shown in Fig. 4B, the percentage of apoptotic cells (evaluated in terms of positivity to Annexin V) increased significantly in VSMC. A particular sensibility to apoptosis was detected in female cells. This datum confirms a key role of FAK in anoikis resistance.

A further point to be considered is represented by the intracellular levels of GSH, which are known to be able to reduce apoptotic susceptibility [17]. As previously suggested [3], basal FVSMC are rich in GSH and this could provide a further survival advantage.

3.4. Autophagy

Autophagy, previously named type II cell death, is instead nowadays considered as a survival pathway that the cells bring into play in unfavorable microenvironmental conditions [18]. Oxidative stress alters proteins and organelles and the proper cytoplasmic protein turnover influencing autophagic process [15,19]. Autophagy was thus analyzed in our system by evaluating the expression of two specific biomarkers: (i) the soluble form of LC3 (LC3I) and its converted form (LC3II/ATG8) localized in autophagosomal membranes and (ii) Beclin 1 (ATG5), part of a phosphoinositide 3-kinase complex that seems to play an important role during the initial steps of autophagosome formation. Strikingly, the expression of LC3II protein was found higher in basal MVSMC than in FVSMC (Fig. 5A). Conversely, after irradiation, was found increased in FVSMC and decreased in MVSMC (Fig. 5A and C). As concerns the levels of Beclin 1, these are quite similar in basal MVSMC and FVSMC whereas an increased expression of Beclin 1 was detected in FVSMC after irradiation (Fig. 5A and B). This increased autophagic activity detectable 24 h after irradiation (also shown by means of TEM observation in Fig. 5D) could confer to FVSMC a survival advantage. In fact, it has been suggested that cells undergoing autophagy "recycle" damaged organelles and proteins to survive [20] but, also, that when autophagy increases, apoptosis is inhibited and that Beclin 1 could play a key role in such a shift [21]. Moreover, as previously affirmed, in female cells (but no in male cells) the expression of β-galactosidase, considered as a senescence marker, increases after UVB exposure. Hence, senescent FVSMC probably use autophagy as an adaptive mechanism to counteract an unfavorable environmental condition, e.g. oxidative stress [22]. The literature on this argument is still lacking. However, it has been hypothesized that 17β-estradiol could influence autophagy [23] whereas Beclin 1 could down-regulate estrogenic signaling and growth response suggesting an important interaction between the two systems [24]. Similar results have also been observed in a recent work dealing with a different cell type, i.e. neuronal cells [25], exposed to a different stress type (starvation): neurons from males more readily undergo autophagy and die, whereas neurons from females mobilize fatty acids, accumulate triglycerides, form lipid droplets, and survive longer.

3.5. Sex hormone receptors

Estradiol (E2) via estrogen receptors (ERs) (both ERα and ERβ) regulates the expression of numerous genes involved in the redox control and in E2-induced effects on cardiovascular system [26]. ERs mediate diverse and opposite E2 effects. ERα is considered

the master mediator of the E2-induced VSMC growth inhibition; whereas, in acute ischemia/reperfusion injury characterized by oxidative stress, ERβ mediates cardioprotection [27]. We found that basal FVSMC express less ERα and ERβ than MVSMC whereas the androgen receptor (AR) level did not differ between sexes (Fig. 6). More importantly, irradiation did not modify the level of both AR and ERs in MVSMC, whereas in female-derived cells both ERs were found altered: ERβ significantly increased whilst ERα decreased (Fig. 6C). On the basis of literature that suggests a general "protective" role of ERβ [27] we cannot rule out the possibility that the increased ERβ levels induced by irradiation in FVSMC could represent and adaptive mechanism resulting in the anoikis resistance of these cells.

In conclusion, the results reported here suggest that differentiated VSMC obtained from male and female rats show intrinsic differences with respect to their redox state and their capability of reacting to oxidative stress and survive, being FVSMC more resistant. This can result in a higher anoikis resistance of FVSMC with respect to MVSMC that can be due to their cytoskeleton-dependent adhesion features and to a higher propensity to undergo autophagy and survival in unfavorable environmental conditions. Altogether these data, obtained with contractile smooth muscle cells, could provide useful clues in the comprehension of the gender/sex differences actually detected in the pathogenesis and outcome of some diseases, including cardiovascular diseases.

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