View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 582 (2008) 635–642

Redox state and gender differences in vascular smooth muscle cells

Walter Malorni[a,*,1], Elisabetta Straface[a,1], Paola Matarrese[a], Barbara Ascione[a], Rita Coinu[b], Silvia Canu[b], Paola Galluzzo[c], Maria Marino[c], Flavia Franconi[b]

[a] Department of Drug Research and Evaluation, Section of Cell Aging and Degeneration, Istituto Superiore di Sanita', Viale Regina Elena 299, 00161 Rome, Italy
[b] University of Sassari-INBB Osilo-Sassari and Gender Pharmacology Section, School of Biomedical Sciences, Department of Pharmacology, Via Muroni 23a, Sassari, Italy
[c] Department of Biology, University Roma Tre, Viale G. Marconi 446, Rome, Italy

Received 19 December 2007; revised 23 January 2008; accepted 23 January 2008

Available online 31 January 2008

Edited by David Lambeth

Abstract **Vascular smooth muscle cells (VSMC) have been isolated from male and female rat aorta and studied to assess their susceptibility to ultraviolet radiation-induced oxidative stress. Interestingly, a gender difference, in terms of reactive oxygen species production, was detected in both basal and irradiated VSMC. Namely, VSMC from male rats were more susceptible to radiation-induced stress and easier underwent apoptosis in comparison to cells from female rats. Conversely, the latter, in the same experimental conditions, clearly displayed signs of premature senescence. These results indicate that a sort of "gender memory" can be conserved in VMSC in primary culture.**
© 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Reactive oxygen species; Vascular smooth muscle cells; Gender; Senescence; Apoptosis

1. Introduction

Changes in the arterial structure and function are associated with aging and may constitute an early cardiovascular risk marker [1]. In fact, after middle age, dilation and stiffening of large arteries, caused by alterations of extracellular matrix and of vascular smooth muscle cells (VSMC), clearly occur [2]. Furthermore, many experimental, clinical and epidemiological studies emphasize that gender differences are detectable in the manifestations of cardiovascular diseases [3,4]. In particular, hypertension, diastolic dysfunction, diabetes, obesity and inactivity are more important factors in women, whereas ischemic heart disease and systolic dysfunction are more important factors in men [5]. Thus, since many years, several preclinical studies on this matter have been performed using vascular smooth muscle cells (VSMC) in primary culture with the aim to reveal a sexual dimorphism in these cells [6]. On these bases, considering that reactive oxygen species (ROS) play a pivotal role in the pathogenesis of vascular disease [7], a specific study was undertaken to evaluate whether male and female VSMC freshly isolated from descending aorta of female and male rats were basically different and whether they respond differently to ultraviolet B (UVB) radiation, here considered as a prototypic inducer of oxidative stress.

2. Materials and methods

2.1. Cell cultures

Vascular smooth muscle cells (VSMC) were isolated from descending aorta of both female and male rats as previously reported [8]. Primary cultures of VSMC were maintained in DMEM medium (GIBCO-Invitrogen, MI, Italy) supplemented with 10% fetal calf serum, 1% non-essential amino acids, 100 u/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. 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.

2.2. UVB exposure

Exposure to a single dose of UVB (200 mJ/cm^2) was obtained as follows: 24 h after plating, the cells were exposed to UVB radiation in a thin layer of PBS (pH 7.4) using one Philips TL 20 W/12 lamp (Philips, Netherlands) which was placed 10 cm above the Petri dishes. In order to eliminate UV radiations not in the UVB range, a Kodak filter (type Kodacell TL 401) was used. In these conditions the UVB radiant flux density was measured with an Osram Centra radiometer. The radiometric measurements were performed for each experiment. This procedure has been described elsewhere [9]. Control samples were submitted to the same conditions without UVB radiation.

2.3. Evaluation of the redox state

Cells (5×10^5) were incubated with 1 μmol/l of dihydroethidium (DHE, Molecular Probes) or 10 μmol/l dihydrorhodamine 123 (DHR 123, Molecular Probes) for 15 min at 37 °C [10]. Intracellular GSH was detected by monochlorobimane (MBC, Molecular Probes, Leiden, The Netherlands) staining as previously described [11]. After washing, samples were immediately analyzed with an LRS II cytometer (Becton & Dickinson, San Jose', CA, USA) equipped with a 488-Argon laser and a UVB laser. Data obtained were analyzed by DIVA software (B&D). The median values of fluorescence intensity histograms were used to provide semi-quantitative assessment of GSH content and

*Corresponding author. Fax: +39 06 49903691.
E-mail addresses: malorni@iss.it, malorni@mclink.it (W. Malorni)

[1] Both these authors are first authors.

Abbreviations: BSO, L-buthionine-[*S*,*R*]-sulfoximine; DHE, dhydroethidium; DHR123, dihydrorhodamine 123; MBC, monochlorobimane; IVM, intensified charge-coupled device video microscopy; ROS, reactive oxygen species; SE, standard error; SOD, superoxide dismutase; UVB, ultraviolet B; VSMC, vascular smooth muscle cells

0014-5793/$34.00 © 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2008.01.034

reactive oxygen species (ROS) production. To deplete GSH, VSMC were exposed to 2.5 mM L-buthionine-[*S*,*R*]-sulfoximine (BSO), an inhibitor of γ-glutamylcysteine, six hours prior to UVB irradiation. BSO was always removed from the medium before UVB exposure.

2.4. Enzymatic activity assays

Superoxide dismutase (SOD) and catalase activities were evaluated by using spectrophotometric assay kits (Cayman Chemical Company, MI, USA) following manufacturer instruction. SOD assay kit

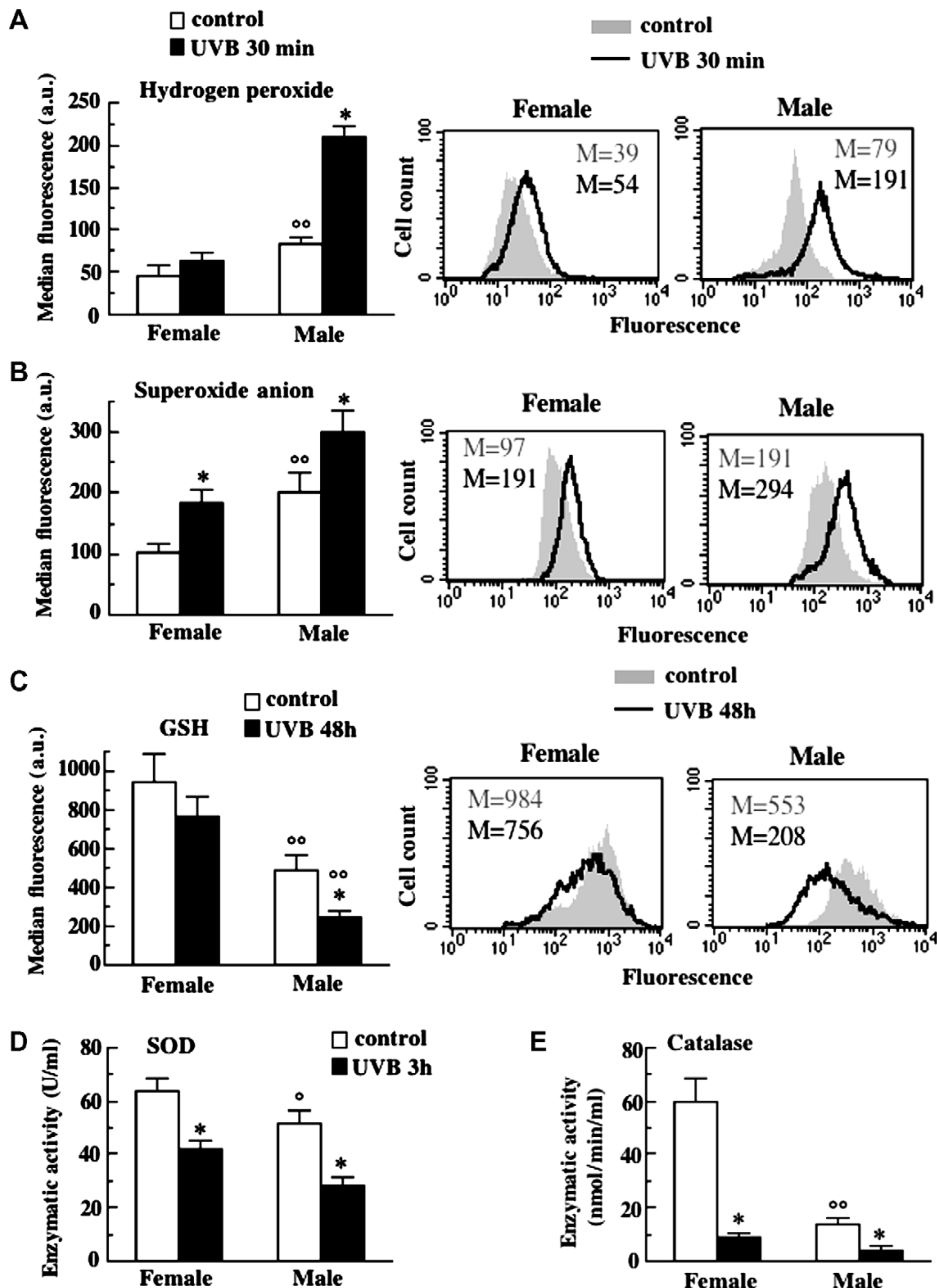

Fig. 1. Cytofluorimetric analysis of (A) hydrogen peroxide production, (B) superoxide anion production and (C) intracellular GSH 30 min (A and B) or 48 h (C) after UVB irradiation. Values reported represent the median fluorescence. In the left column the mean of the results obtained from four independent experiments ± S.E. are reported. In the right column, results obtained in a representative experiment are shown. Spectrophotometric analysis of SOD activity (D) and catalase activity (E) are also shown. (°) $P < 0.05$ between VSMC from males vs females; (°°) $P < 0.01$ between VSMC from males vs females; (∗) $P < 0.01$ between UVB-treated VSMC vs the respective control cells.

measures different types of SOD (Cu/Zn, Mn-SOD and Fe-SOD) and utilizes tetrazolium salt for detection of superoxide radicals generated by xantine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Absorbance of the samples (performed in triplicate for each experimental condition) was read at 450 nm using a plate reader. To obtain SOD activity (expressed as U/ml) experimental samples were compared with standard samples.

Catalase assay kit utilizes the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as chromogen. Absorbance of the samples (performed in triplicate for each experimental condition) was read at 540 nm using a plate reader. To obtain catalase activity (expressed as nmol/min/ml) experimental samples were compared with standard samples.

2.5. Analytical cytology

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 Chemical Co., St. Louis, MO, USA) at 37 °C for 30 min. For β-galactosidase detection, cells were incubated with monoclonal antibody (Immunological Sciences) for 30 min at 37 °C. For p53 and phosphorylated H2AX detection, cells were fixed in acetone/methanol 1/1 (v/v) and incubated at room temperature with monoclonal anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal anti H2AX (Abcam, Cambridge, UK) for 30 min. After washings in PBS, cells were incubated with FITC-labeled anti-mouse or anti-rabbit antibodies (Sigma). To analyze the nuclei and to evaluate apoptosis, cells were counterstained with Hoechst 33258 (Sigma) and observed with a Nikon Microphot fluorescence microscope as previously described [12]. For flow cytometry analysis, samples were recorded with a FACScan flow cytometer (Becton Dickinson) equipped with a 488 nm argon laser. A least 20000 events have been acquired. The median values of fluorescence intensity histograms were used to provide a semi-quantitative analysis. To ensure staining specificity, negative controls using monoclonal FITC-labeled anti-mouse or anti-rabbit antibodies (Sigma), were performed.

2.6. Morphometric analyses

For qualitative analyses, samples were mounted on glass cover-slips with glycerol-PBS (2:1) and analyzed by intensified video microscopy (IVM) with an Olympus fluorescence microscope equipped with a Zeiss CCD camera. Quantitative evaluation of apoptotic cells, cells with actin cross-links, heterochromatic foci, and nuclear p53 translocation was performed counting at least 300 cells at high magnification (500×) at the fluorescence microscope.

2.7. Analyses of sex steroid hormone receptors by electrophoresis and immunoblotting

Cells were lysed and solubilized [in 0.125 M Tris, pH 6.8, containing 10% (w/v) SDS, 1.0 mM phenylmethylsulfonyl fluoride, and 5.0 μg/ml leupeptin] and finally boiled for 2 min. Total proteins were quantified using the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Solubilized proteins (20 μg) were electrophoretically resolved by 10% SDS–PAGE (100 V, 1 h, 24 °C) and then transferred to nitrocellulose (100 V, 45 min, 4 °C). The nitrocellulose membrane was treated with 3% (w/v) BSA in 138.0 mM NaCl, 25.0 mM Tris, pH 8.0, at 24 °C for 1 h and then probed overnight at 4 °C with either anti-ERα MC20 (*C*-terminus, Santa Cruz, CA, USA) or anti-ERβ L20 (*C*-terminus, Santa Cruz, CA, USA) or anti-AR (Abcam, Cambridge, UK) antibodies. The nitrocellulose membrane was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical Company, Rockford, IL, USA) for 10 min at room temperature and then probed with anti-tubulin antibody (MP Biomedical, LLC, Solon, OH, USA). Antibody reactions were visualized by chemiluminescence with the Western blotting detection reagent (Amersham Biosciences, Little Chalfont, UK).

2.8. Statistical analyses

Cytofluorimetric results were statistically analyzed by using the parametric Kolmogorov–Smirnov test using Cell Quest Software. A least 20000 events have been acquired. The median values of fluorescence intensity histograms were used to provide a semi-quantitative analysis.

Morphometric data (reported as mean ± standard error (S.E.), from at least four separate experiments) were analyzed by using Student's *t*-test. Only $P < 0.05$ was considered as significant.

3. Results and discussion

ROS are reactive derivatives of O_2 metabolism, including superoxide anion, hydrogen peroxide, hydroxyl radical and nitric oxide. Cardiovascular diseases, such as hypertension, are associated with increased ROS formation. Oxidative stress in the vasculature reduces levels of the vasodilator nitric oxide, causes tissue injury, promotes protein oxidation and DNA damage, and induces proinflammatory responses [13]. ROS are also important intracellular signaling molecules that regulate vascular function by modulating vascular cell contraction/dilation, migration, growth/apoptosis, and extracellular matrix protein turnover, which contribute to vascular remodeling. In order to study gender differences in terms of response to oxidative stress, we exposed VSMC from female and male rats in primary culture at the same and early passages (up to 4th subseeding) to a sub-cytotoxic dose of UVB radiation. UVB is a well-known prototypic stimulus capable of inducing oxidative stress, apoptosis or premature senescence [14–16] and the different ability of the cells to respond to this insult seems to depend from differences in basal antioxidant defenses [17].

3.1. Redox imbalance and gender differences in VSMC

Cellular redox status, monitored by quantitative flow cytometry and spectrophotometric analyses, clearly indicated the presence of "basal" differences between VSMC from male and female rats in terms of redox balance (Fig. 1A–E). In fact, in control cells, either H_2O_2 (Fig. 1A) or $O_2^{\cdot}$ (Fig. 1B) production was significantly ($P < 0.01$) higher in VSMC from male then in VSMC from female rats. Cytofluorimetric analysis, performed at different time points after UVB exposure

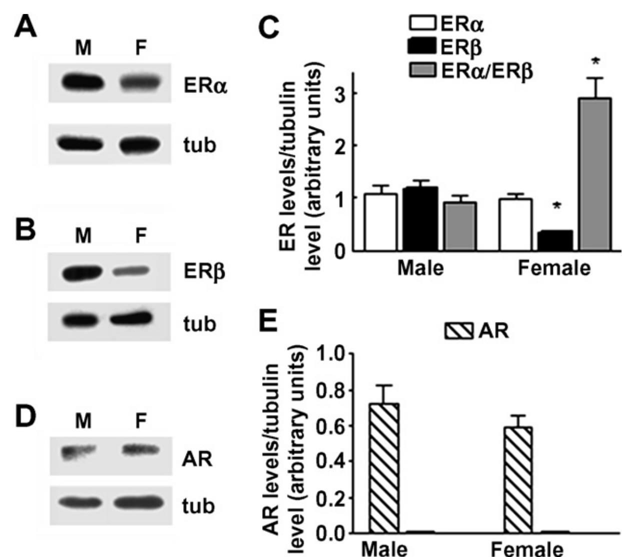

Fig. 2. Western blot analysis of estrogen receptor (ER) α, ERβ and androgen receptor (AR) levels were performed in VSMC cells from male (M) and female (F) rats. Typical Western blot (panels A, B, and D) and relative densitometric analysis (panels C and E) of three different experiments are shown. Data are the means ± S.D. (*) $P < 0.001$, calculated with student's *t*-test, compared with male values.

(15 min, 30 min 3 h and 48 h), demonstrated that irradiation-induced ROS production rapidly increased after 15 min (not shown), peaked at 30 min (Fig. 1A and B) and diminished after 48 h (not shown). Fittingly, a time course analysis revealed that (i) in VSMC from male GSH intracellular level was significantly lower than in VSMC from female, also in absence of any stress (Fig. 1C) and that (ii) UVB reduced GSH content both in cells from female or male, although to a different extent. This reduction of GSH content was more marked 48 h after UVB exposure (Fig. 1C).

The presence of GSH is essential, but not sufficient per se, to prevent the cytotoxicity of ROS. In fact, a series of antioxidant enzymes contribute to cellular antioxidant defenses. Thus, a time-dependent analysis of SOD (Fig. 1D) and catalase (Fig. 1E) activity was also carried out. We found that both SOD and catalase activity: (i) was significantly higher in VSMC from female than in VSMC from male, independently of UV-induced stress; (ii) was significantly impaired 3 h after UVB exposure, and (iii) was recovered 48 h after irradiation (data not shown).

3.2. Gender differences and levels of sex steroid hormone receptors

Differences in the levels of both estrogen receptors (ERα and ERβ) and androgen receptor (AR) were assessed in VSMC from male and female rats. As reported in Fig. 2A, B, and D

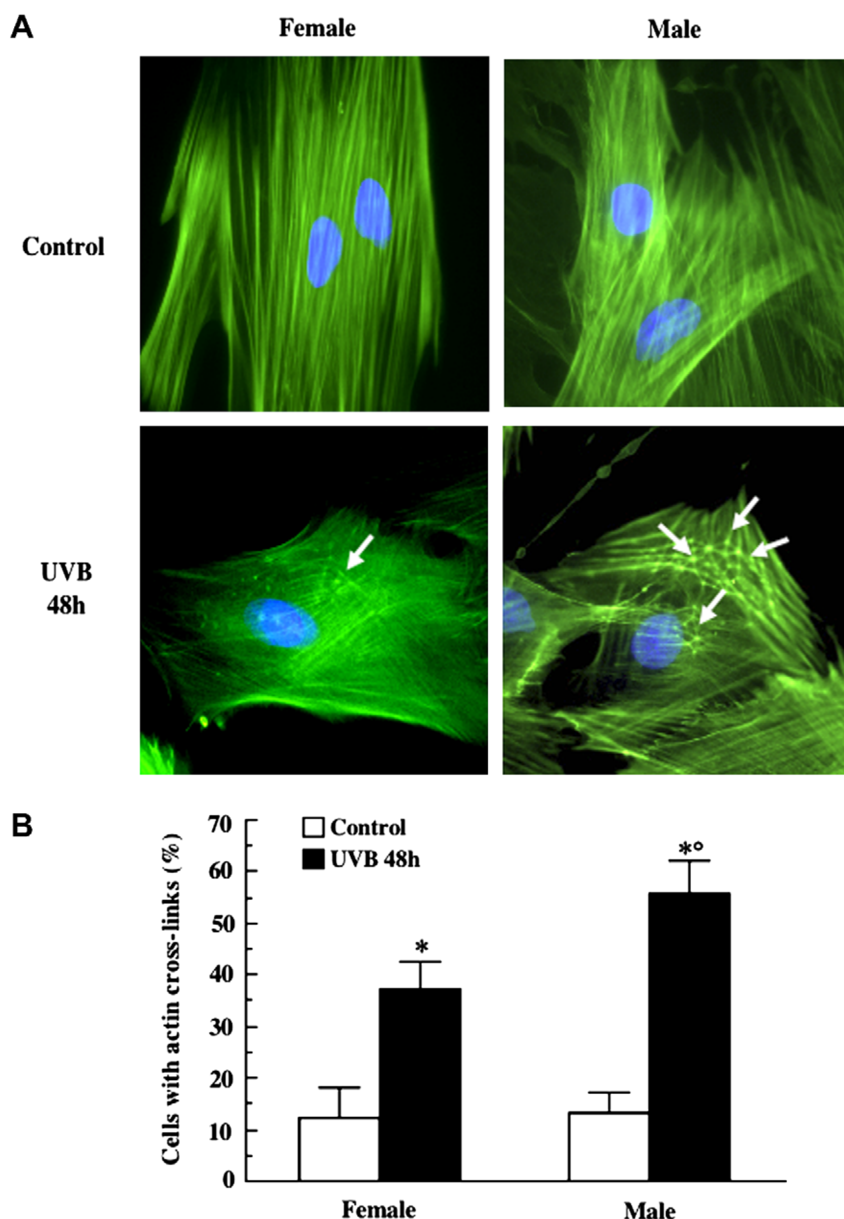

Fig. 3. (A) Fluorescence microscopy analysis of actin cytoskeleton in cells stained with FITC-phalloidin. In untreated VSMC (top panels) F-actin appears organized in thin and oriented stress fibers through the cytoplasm. After UVB exposure (48 h) a marked actin filament reorganization occurs, (bottom panels) especially in cells from male rats (bottom right panel). Arrows indicate actin cross-links. (B) Morphometric analysis of actin cross-links in VSMC from female and male before and after UVB exposure. (*) represents $P < 0.01$ of UVB-treated vs untreated cells; (°) represents a significant difference ($P < 0.01$) between UVB-treated VSMC from males vs females (black columns).

only the ERβ isoform level showed a clear gender difference, being lower in VSMC from female than in cells derived from male. This result reveals a significant gender difference in the ERα/ERβ ratio (Fig. 2C) which, was completely abolished in VSMC cells at 14th passage (data not shown). The existence of gender differences in the incidence and outcome of cardiovascular disease is well known and it has been associated to the presence of sex steroid hormone receptors [18]. In particular, ERα and ERβ display distinct physiological roles in estrogen-induced vascular functions. Knockout mouse models revealed a role for ERα in mediating the vascular protective effect of estrogen; whereas, ERβ has a distinct role in controlling systemic blood pressure, in part by modulating endothelial-independent vasodilation and potassium ion channel function in vascular smooth muscle cells [18]. Therefore, the final estrogen effects could be dictated by the balance of ERα vs. ERβ activities, which in turn would be dependent on the relative levels of the two ERs. The high $ER\alpha/ER\beta$ ratio in cells derived from female (≃2.8) could be related to the differences reported in ROS production as suggested above [see also [19]]. However, the mechanism(s) underlying these differences are under active evaluation in this laboratory.

3.3. Gender differences and cytoskeleton damage

Considering that cytoskeleton, responsible for cell shape and polarity maintenance [20], is one of the targets of ROS production [21], studies were carried out in order to investigate if cytoskeleton actin filaments were affected by exposure to UVB. As shown in Fig. 3A, a marked actin filament reorganization was observed in VSMC from male rats. In fact, 48 h after UVB exposure a dramatic redistribution and "thickening" of the actin stress fibers with numerous "cross-links" of cytoskeletal filaments were detectable in these cells (Fig. 3A, right column, bottom panel). By contrast, minor alterations were detected in VSMC from female rats (Fig. 3A, left column, bottom panel). A morphometric evaluation of cells with actin cross-links was then performed. As shown in Fig. 3B, the percentage of cells with actin cross-links was found significantly increased in both cell types but was higher in VSMC from male rats. This major cytoskeleton damage detectable in cells from males is in line with the higher production of ROS induced by UVB exposure in these cells in comparison to VMSC from females (see Fig. 1). In fact, according to literature, the presence of actin cross-links seem to indicate the occurrence of an oxidative remodeling of cytoskeleton with formation of side-by-side thiol group oxidative cross-linking [22].

3.4. Gender differences and cell fate: apoptosis versus senescence

In order to investigate if a gender difference, in terms of redox imbalance, could influence cell fate, we first evaluated the percentage of apoptotic cells in UVB-exposed VSMC. As

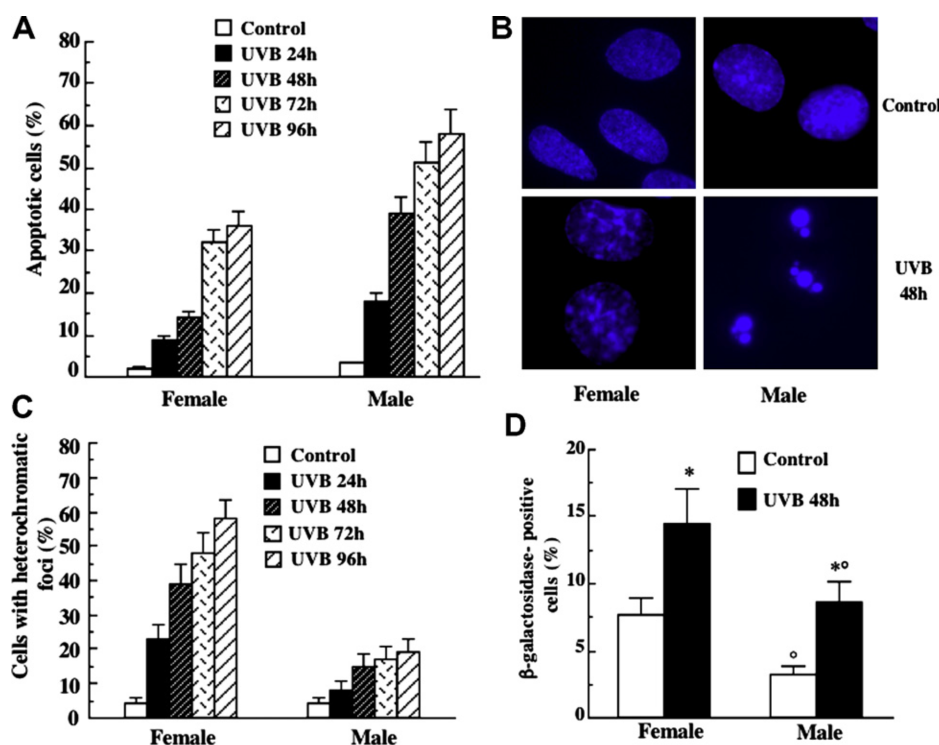

Fig. 4. (A) Flow cytometry analysis of apoptosis after double staining with annexin V/propidium iodide at different time points after UVB irradiation. Note that UVB induces apoptosis in a time dependent manner in both experimental settings. However, the percentage of apoptotic cells was significantly higher ($P < 0.01$) in VSMC from male rats compared to VSMC from female rats. (B) Fluorescence micrographs of cells stained with Hoechst dye to reveal nuclei. Representative micrographs showing: (i) heterochromatic foci in UVB-exposed VSMC from female (compare with untreated cells in the upper panel) and (ii) chromatin condensation in UVB-exposed VSMC from male rats (compare with untreated cells in the upper panel). (C) Quantitative time-course evaluation, performed by morphometric analysis as reported in Section 2, of heterochromatic foci as observed by fluorescence microscopy. Note that the percentage of cells with heterochromatic foci was significantly higher ($P < 0.01$) in VSMC from female rats compared to VSMC from male rats. (D) Cytofluorimetric analysis of the expression of β-galactosidase in VSMC from female and male 48 h after UVB exposure. (∗) represents $P < 0.01$ of UVB-treated vs untreated cells; (°) represents a significant difference ($P < 0.01$) between VSMC from males vs females (white vs white and black vs black columns).

shown in Fig. 4A, evaluation of apoptosis performed by annexin V staining (not shown) or by Hoechst staining clearly indicated that UVB induced apoptosis in a time dependent manner especially in cells from male rats (representative micrographs are shown in Fig. 4B, right). Strikingly, distinct heterochromatic foci were observed in the nuclei of cells from females (representative micrographs in Fig. 4B, left). These morphological features are generally referred as typical of replicative senescence [16,23]. A quantitative evaluation of cells with heterochromatic foci was then carried out at different time points (Fig. 4C). The data obtained indicated that the percentage of cells with heterochromatic foci increased significantly with time, especially in radiation-exposed cells from female rats. We confirmed these data by quantitatively analyzing β-galactosidase, a further marker of replicative senescence [16]. The analysis of this enzyme revealed that 48 h after UVB exposure the percentage of β-galactosidase-positive cells was significantly higher in VSMC from female rats than in VSMC from males (Fig. 4D).

3.5. Gender-associated redox state determines cell fate

Senescence induced by oxidative stress is named premature senescence and might be triggered by DNA damage (i.e. double-strand breaks) at sites that are distinct from telomeres [16,24]. In the recent years, the early events of DNA damage have been well characterized [25]. Signaling kinases are recruited to the site of damage and are activated, leading to phosphorylation of Ser-139 of histone H2AX molecules adjacent to the site of DNA damage [26]. In order to verify if histone H2AX phosphorylation was involved in senescence occurring in UVB-exposed VSMC, specific immunocytochemistry experiments were carried out. As shown in Fig. 5A, 3 h

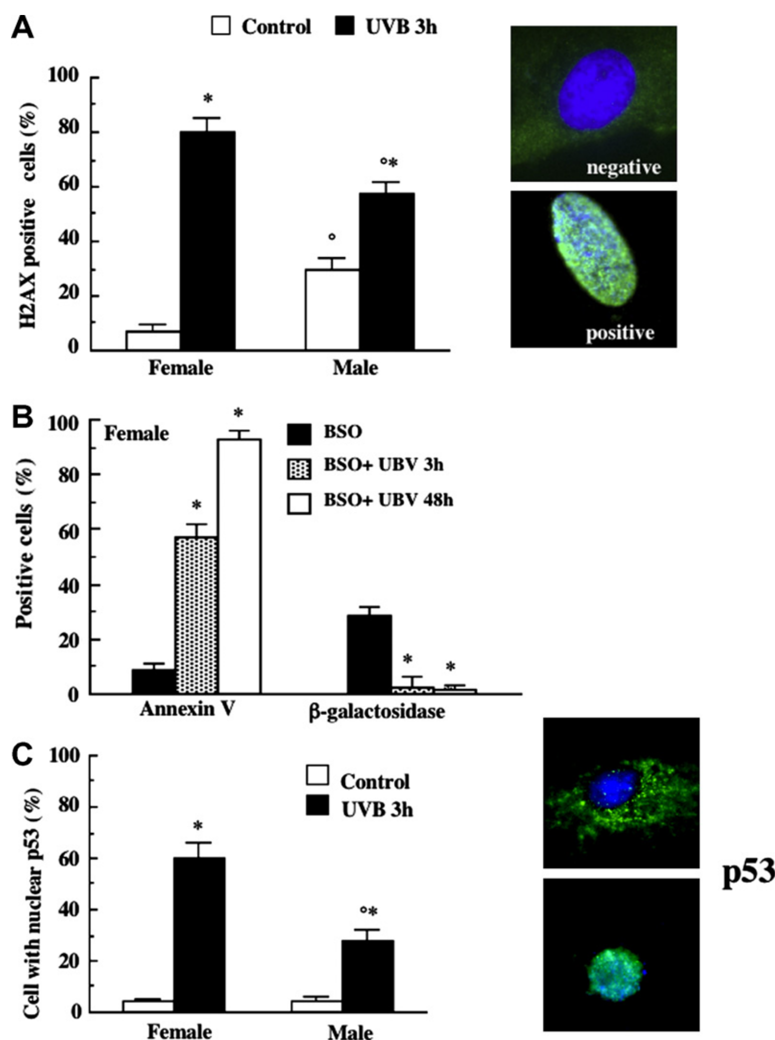

Fig. 5. (A) Quantitative evaluation of phospho-H2AX-positive cells by fluorescence microscopy after double staining with Hoechst dye and a specific polyclonal antibody against phospho-H2AX. Representative micrographs (right panels) show a nucleus negative (upper panel) or positive (bottom panel) to phospho-H2AX. (B) Flow cytometry analysis of apoptosis after staining with annexin V/propidium iodide and β-galactosidase expression at two different time points after UVB irradiation performed in VSMC from female rats pre-treated with BSO to deplete GSH before irradiation. (*) Represents $P < 0.01$ vs the same time points in Fig. 4A (apoptosis) and 4D (β-galactosidase) by Student's *t*-test. (C) Quantitative analysis of translocation of p53 from the cytoplasm into the nucleus. Representative micrographs (right), after double staining with Hoechst dye (blue) and a specific antibody to p53 (green), showing a cell with cytoplasmic (upper panel) or nuclear (bottom panel) localization of p53 are shown. In (A,C): (*) represents $P < 0.01$ of UVB-treated vs untreated cells; (°) represents a significant difference ($P < 0.01$) between VSMC from males vs females (white vs white or black vs black columns).

after UVB exposure, a high percentage of nuclei positive to phosphorylated H2AX was observed, especially in VSMC from female rats. Moreover, 48 h after UVB exposure, virtually all cells from both female and male rats were positive to H2AX (data not shown). In fact, as previously suggested [16], cells undergoing death clearly detached from the substrate so that only surviving cells undergoing premature senescence were still detectable on the coverslips. Finally, according to the results on ROS production reported above, Fig. 5A (white columns) also points out a gender difference in terms of H2AX phosphorylation in ''basal'' conditions.

Why the fate of cells from males and females appeared quite different in terms of susceptibility to apoptosis and senescence? First, in order better elucidate this point, the role of redox balance was evaluated by using BSO to deplete intracellular GSH content. We thus evaluated either apoptosis or senescence at two different time points. We found that BSO-induced GSH reduction deeply influenced the fate of VSMC from female rats. In fact, a high percentage of BSO pre-treated VSMC from females underwent apoptosis after UVB irradiation (Fig. 5B, compare with Fig. 4A). At the same time, in BSO pre-treated cells, the percentage of ''female'' senescent cells was significantly lower (Fig. 5B, compare with Fig. 4D). By contrast, as expected, the effects of BSO treatment in VSMC from male rats, probably due to their apoptosis-proneness, were negligible (not shown). These results seem to indicate a key role for the redox state in determining gender differences in terms of apoptotic susceptibility.

Further studies were then carried out on p53, a signaling molecule that is known to be activated by ROS [27] and was suggested to play a critical role in determining cell fate in terms of senescence or apoptosis [28]. Interestingly, 3 h after UVB exposure, p53 molecule translocated from the cytoplasm into the nucleus in a high percentage of VMSC from female rats (Fig. 5C). By contrast, this translocation was significantly lower in VSMC from male rats (Fig. 5C). No signs of translocation were detected in unexposed cells. These data emphasized once again the ability of cells from female rats to counteract the effects of ROS. These data might also suggest that p53 could play a role in the survival mechanism of VSMC from female rats in the radiation-induced senescence.

These results seem to suggest that freshly isolated VSMC at early passages could still maintain a sort of ''memory'' of sexual dimorphism. In particular they display: (i) a different basal redox state and (ii) a different susceptibility to oxidative stress, being cells from males more susceptible. This was associated, or could also lead, to a different cell fate, being cells from male rats more apoptotic-prone and cells from female rats more senescence-prone. These results are in line with some recent views [29] and could suggest a careful reappraisal of the use of freshly isolated non-tumor cultured cells as a reliable model system per se, independently from their ''gender source''. Furthermore, our findings also suggest a different role of VSMC in the pathogenetic mechanisms leading to cardiovascular diseases. Gender differences of VSMC could in fact contribute to the onset of hypertension or atherosclerotic plaque formation, known to develop differently in males and females [19,30,31]. Hence, the differences detected in our experimental system could provide some useful clue in the assessment of novel pathogenetic mechanisms of vascular morbidity states and the development of new pharmacological strategies aimed at the maintenance of vascular integrity and function.

Acknowledgements: We thank Roberta Terlizzi for her precious help. This work has been supported by Grants from Ministero della Sanita' to W.M. and FF and Telethon to W.M.

References

[1] Jani, B. and Rajkumar, C. (2006) Ageing and vascular ageing. Postgrad. Med. J. 82, 357–362.

[2] Izzo Jr., J.L. and Mitchell, G.F. (2007) Aging and arterial structure–function relations. Adv. Cardiol. 44, 19–34.

[3] Legato, M.J. (2004) Gender-specific medicine: the view from Salzburg. Gend. Med. 1, 61–63.

[4] Hirakawa, Y., Masuda, Y., Kuzuya, M., Iguchi, A., Kimata, T. and Uemura, K. (2007) Impact of gender on in-hospital mortality of patients with acute myocardial infarction undergoing percutaneous coronary intervention: an evaluation of the TAMIS-II data. Int. Med. 46, 363–366.

[5] Lund, L.H. and Mancini, D. (2004) Heart failure in women. Med. Clin. North Am. 88, 1321–1345.

[6] Loukotova, J., Kunes, J. and an Zicha, J. (1998) Cytosolic free calcium response to angiotensin II in aortic VSMC isolated from male and female SHR. Physiol. Res. 47, 507–510.

[7] Harrison, D., Griendling, K.K., Landmesser, U., Hornig, B. and Drexler, H. (2003) Role of oxidative stress in atherosclerosis. Am. J. Cardiol. 91, 7A–11A.

[8] Coinu, R., Chiavello, A., Galleri, G., Franconi, F., Crescenzi, E. and Palumbo, G. (2006) Exposure to modeled microgravity induces metabolic idleness in malignant human MCF-7 and normal murine VSMC cells. FEBS Lett. 580, 2465–2470.

[9] Straface, E., Giacomoni, P.U. and Malorni, W. (2001) Cultured cells as a model system for the study of UV-induced cytotoxicity. J. Photochem. Photobiol. 63, 52–60.

[10] Frey, T. (1997) Correlated flow cytometric analysis of terminal events in apoptosis reveals the absence of some changes in some model systems. Cytometry 28, 253–263.

[11] Sebastia, J., Cristofol, R., Martin, M., Rodriguez-Farre, E. and Sanfeliu, C. (2003) Evaluation of fluorescent dyes for measuring intracellular glutathione content in primary cultures of human neurons and neuroblastoma SH-SY5Y. Cytometry 51, 16–25.

[12] Straface, E., Santini, M.T., Donelli, G., Giacomoni, P.L. and Malorni, W. (1995) Vitamin E prevents UVB-induced cell blebbing and cell death in A431 epidermoid cells. Int. J. Radiat. Biol. 68, 579–587.

[13] Antoniades, C., Tousoulis, D., Marinou, K., Vasiliadou, C., Tentolouris, C., Bouras, G., Pitsavos, C. and Stefanadis, C. (2006) Asymmetrical dimethylarginine regulates endothelial function in methionine-induced but not in chronic homocystinemia in humans: effect of oxidative stress and proinflammatory cytokines. Am. J. Clin. Nutr. 84, 781–788.

[14] Wan, Y.S., Wang, Z.Q., Shao, Y., Voorhees, J.J. and Fisher, G.J. (2001) Ultraviolet irradiation activates PI 3-kinase/AKT survival pathway via EGF receptors in human skin in vivo. Int. J. Oncol. 18, 461–466.

[15] Debacq-Chainiaux, F., Borlon, C., Pascal, T., Royer, V., Eliaers, F., Ninane, N., Carrard, G., Friguet, B., de Longueville, F., Boffe, S., Remacle, J. and Toussaint, O. (2005) Repeated exposure of human skin fibroblasts to UVB at subcytotoxic level triggers premature senescence through the TGF-beta1 signaling pathway. J. Cell Sci. 118, 743–758.

[16] Straface, E., Vona, R., Ascione, B., Matarrese, P., Strudthoff, T., Franconi, F. and Malorni, W. (2007) Single exposure of human fibroblasts (WI-38) to a sub-cytotoxic dose of UVB induces premature senescence. FEBS Lett. 581, 4342–4348.

[17] Saquet, T., Robin, S., Nicod, L., Binda, D., Viennet, C., Aubin, F., Coulomb, B. and Humbert, P. (2006) Comparison of ultraviolet B-induced imbalance of antioxidant status in foreskin- and abdominal skin derived human fibroblast. Eur. J. Dermatol. 16, 368–374.

[18] O'Lone, R., Knorr, K., Jaffe, I.Z., Schaffer, M.E., Martini, P.G., Karas, R.H., Bienkowska, J., Mendelsohn, M.E. and Hansen, U. (2007) Estrogen receptors α and β mediate distinct pathways of vascular gene expression, including genes involved in mitochondrial electron transport and generation of reactive oxygen species. Mol. Endocrinol. 21, 1281–1296.

[19] Malorni, W., Campesi, I., Straface, E., Vella, S. and Franconi, F. (2007) Redox features of the cell: a gender perspective. Antioxid. Redox Signal. 9, 1779–1801.

[20] Louvet-Vallee, S. (2000) ERM proteins: from cellular architecture to cell signaling. Biol. Cell 92, 305–316.

[21] Nimnual, A.S., Taylor, L.J. and Bar-Sagi, D. (2003) Redox-dependent downregulation of Rho by Rac. Nat. Cell Biol. 5, 236–241.

[22] Masella, R., Straface, E., Giovannini, C., Di Benedetto, R., Scazzocchio, B., Viora, M., Cantafora, A. and Malorni, W. (2000) Subcellular alterations induced by UV-oxidized low-density lipoproteins in epithelial cells can be counteracted by alpha-tocopherol. Photochem. Photobiol. 71, 97–102.

[23] Zhang, R. and Adams, P.D. (2007) Heterochromatin and its relationship to cell senescence and cancer therapy. Cell Cycle 6, 784–789.

[24] Sedelnikova, O.A., Horikawa, I., Zimonjic, D.B., Propescu, N.C., Bonner, W.M. and Barrett, J.C. (2004) Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. Nat. Cell Biol. 6, 168–170.

[25] Shilo, Y. (2003) ATM and related protein kinases: safeguarding genomic integrity. Nat. Rev. Cancer 3, 155–168.

[26] VonZglincki, T., Saretzki, G., Ladhoff, J., d'Adda di Fagagna, F. and Jackson, S.P. (2005) Human cell senescence as DNA damage response. Mech. Ageing Dev. 126, 111–117.

[27] Macip, S., Igarashi, M., Berggren, P., Yu, J., Lee, S.W. and Aaronson, S.A. (2003) Influence of induced reactive oxygen species in p53-mediated cell fate decisions. Mol. Cell Biol. 23, 8576–8585.

[28] Cotton, J. and Spandau, D.F. (1997) Ultraviolet B-radiation dose influences the induction of apoptosis and p53 in human keratinocytes. Radiat. Res. 147, 148–155.

[29] Bacakova, L., Pellicciari, C., Bottone, M.G., Lisa, V. and Mares, V. (2001) A sex-related difference in the hypertrophic versus hyperplastic response of vascular smooth muscle cells to repeated passaging in culture. Histol. Histopathol. 16, 675–684.

[30] Ling, S., Komesaroff, P. and Sudhir, K. (2006) Cellular mechanisms underlying the cardiovascular actions of oestrogens. Clin. Sci. (Lond). 111, 107–118.

[31] Watanabe, T., Yasunari, K., Nakamura, M. and Maeda, K. (2006) Carotid artery intima-media thickness and reactive oxygen species formation by monocytes in hypertensive patients. J. Hum. Hypertens. 20, 336–340.