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Original Article

Anti-oxidative effects of 17 β-estradiol and genistein in human skin fibroblasts and keratinocytes

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ABSTRACT

Background: Estrogens and phytoestrogens can hinder the aging process through mechanisms related to estrogen receptors (ERs), guanine nucleotide-binding protein-coupled receptor (GPER30), mitochondria function and nitric oxide (NO) release. Up to date, however, the above issues are a matter of debate.
Objective: To examine the effects elicited by 17 β-estradiol and genistein against peroxidation in human keratinocytes/fibroblasts and evaluate the role played by ERs, GPER30, mitochondria and NO.
Methods: Human fibroblasts/keratinocytes, either subjected to peroxidation or not, were exposed to 17 β-estradiol/genistein in the absence or presence of the NO synthase (NOS) inhibitor, the ERs and GPER30 blockers, fulvestrant and G15, the phosphatidyl-inositol-3-kinase (PI3K-Akt), the p38 mitogen-activated protein (MAP) kinase and the extracellular signal–regulated kinases (ERK) 1/2 inhibitors. Specific kits were used for cell viability, NO, ROS and glutathione (GSH) detection and mitochondrial membrane potential measurement. Western Blot analysis was performed for kinases expression/activation detection.
Results: In physiological and peroxidative conditions, 17 β-estradiol/genistein respectively increased and reduced NO release by fibroblasts/keratinocytes. Moreover, both agents prevented the ROS release and the fall of cell viability and mitochondrial membrane potential, while increasing GSH levels and the proliferation rate. Fulvestrant and G15 counteracted all above responses. Also, the NOS, and the kinases blockers reduced the protection exerted by 17 β-estradiol/genistein on cell viability/mitochondria function. The involvement of PI3K-Akt and p38-MAPK was confirmed by Western blot.
Conclusion: 17 β-estradiol/genistein protected fibroblasts/keratinocytes against peroxidation by modulating oxidant/antioxidant system and mitochondria membrane potential, through mechanisms related to ERs and GPER30 and kinases activation.

1. Introduction

Estrogens have been widely shown to exert many important beneficial effects in skin physiology [1] targeting, among others, keratinocytes and fibroblasts [2]; their importance in the maintenance of human skin homeostasis is highlighted by the acceleration of skin aging that can be observed in postmenopausal women [3]. Overall, estrogens not only improve collagen content and quality, but they also increase skin thickness and vascularization [4] and enhance keratinocytes migrations, as demonstrated also in the wound healing process [5,6]. The protective effects elicited by estrogens in skin cells could be related to the modulation of peroxidation, as shown in dermal fibroblasts taken from patients suffering from Friedreich's ataxia [7]. Also phytoestrogens (i.e. non-steroidal plant compounds with estrogen-like biological activity) represent promising alternatives for skin ageing treatment. Among the others, and in particular, the phytoestrogens genistein could exert antiphotocarcinogenetic and antiphotoaging properties by modulation of oxidant/antioxidant balance [8,9]. Hence, genistein was shown to protect human dermal fibroblasts against UV-induced senescence by upregulating intracellular superoxide dismutase (SOD) activity [10], suggesting a potential

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role in skin rejuvenation [11]. Genistein exerts protective effects against reactive oxygen species (ROS)-induced apoptosis in the human keratinocyte, HaCaT cell line [12]. In addition, it reduces free radicals both in normal and in hyperproliferative fibroblasts and counteracts the inhibition of collagen biosynthesis and the antiproliferative effects of peroxidative stimuli more effectively than ascorbate [13,14].

The effects of both estrogens and genistein on peroxidation could be of particular relevance for clinical perspectives, since increased inflammation and oxidative stress play a crucial role in skin photoaging. ROS are generated from both the ultraviolet (UV) sun light and endogenous oxidative metabolism by mitochondria [15–17]. In physiological conditions, antioxidant enzymes, such as SOD or glutathione (GSH), keep normal levels of ROS homeostasis and counteract cellular stress; however, when the oxidant/antioxidant ratio is shifted in favor of peroxidation, increased ROS and oxidative stress can damage DNA, proteins, and lipids leading to premature aging [17].

Another molecule that can be involved in photoaging phenomena is represented by nitric oxide (NO), that may constitute a target for estrogens and phytoestrogens [18,19]. NO is synthesized from L-arginine by three isoforms of NOS, which are the inducible and calcium-independent NOS (iNOS), and the constitutive and calcium-dependent neuronal NOS (nNOS) and the eNOS [20]. While the constitutive NOS may act as a regulator of short-term and physiological phenomena and is involved in a small amount of NO release, the inducible NOS could be involved in longer-lasting cytotoxic and inflammatory immunological functions, by producing NO at higher extent [21,22]. NO has been reported to be associated with common forms of inflammatory skin diseases, thus representing a possible therapeutic target [23,24]. Moreover, it was also found to be over-secreted in response to UV irradiation by iNOS isoform [25]. Hence, the reduction of NO release by human keratinocytes was reported to be a mechanism of protection against UV-induced senescence by garlic [26]. Similar effects were found in the mouse skin, where the use of polyphenols and anthocyanin was able to counteract iNOS activation by UV irradiation [27].

Finally, although not clearly examined to date, changes of mitochondrial function could also play a role in the mechanisms of action of estrogens and phytoestrogens as antioxidant and anti-aging factors in skin [7,28]

Both estrogen receptors (ERs), ERα and ERβ, which belong to the superfamily of nuclear hormone receptors, and guanine nucleotide-binding protein-coupled receptor, GPER30, could be involved in eliciting the protective effects of estrogens, even if explicit data regarding the mechanisms through which estrogens and phytoestrogens would exert their protection in the skin are still lacking [29,30]. In respect of genistein, it would exert its effects on skin mainly through ERβ [31], whereas information about the involvement of GPER30 has not yet been reported.

Thus, in this study, we aimed to examine the protective effects elicited by 17-β-estradiol and genistein against peroxidation in human keratinocytes and fibroblasts and to analyze the involvement of ERs, GPER30, NO, ROS and mitochondria.

2. Materials and methods

2.1. Isolation of human keratinocytes and fibroblasts from skin biopsy and cell culture

Primary human keratinocytes were obtained from healthy skin specimens surrounding benign cutaneous lesions excised at the Dermatologic Unit of the Department of Health of Sciences, University Eastern Piedmont "A. Avogadro". To avoid variations due to the age of the donor or prior photo exposure, skin from subjects under the age of 50 and from non-photo-damaged areas was used only. Informed consent was previously obtained from all donors. The study was approved by the local ethical committee. For at least three times, the skin was immersed alternatively in 70% ethanol and subsequently in saline solution to reduce contamination. Then 0.5–1 cm^2 skins were incubated at 4 °C overnight in 5 mg/ml Dispase II (Sigma, Milan, Italy), 100 u/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). The following day, separation of dermis/epidermis was performed. The epidermis was immersed in 2.5 ml 0.25% trypsin/0.02% EDTA (Euroclone, Pero, Milan, Italy) and incubated at 37 °C for 15–20 min. Then, 5 ml Dulbecco's Modified Eagle Medium (DMEM, Sigma) with 10% Foetal Bovine Serum (FBS, Sigma) was added; the solution was pipetted vigorously up and down 20–30 times and passed through a 100 μm Nylon filter (Corning, Fisher Scientific Italia, Rodano, Italy). Extracted cells were centrifuged at 970 rpm for 10 min. The cell pellet was suspended in a 1 ml Epilife medium (Life Technologies Italia, Monza, Italy), added with 1% Human Keratinocytes Growth Supplement (HKGS, Life Technologies), 100 u/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma), 1% Gentamycin (Sigma) and 1% Amphotericin B (Sigma). Keratinocytes were transferred to a Collagen IV-coated dish (100 μg/ml, Collagen from human placenta; Sigma) containing 5 ml complete Epilife medium and incubated at 37 °C, 5% CO_2 and 95% humidity. The medium was changed after 24 h [32]. Following the same previously described protocol, the dermis was immersed in 3 mg/ml Dispase II, 100 u/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) and left for 4–5 h at 37 °C, 5% CO_2 and 95% humidity. Then, 6 ml DMEM with 10% FBS (Sigma) was added; the solution was pipetted vigorously and passed through a 100 μm Nylon filter (Corning). Extracted cells were centrifuged at 970 rpm for 10 min. The cell pellet was suspended in a 1 ml of DMEM with 20% FBS (Euroclone), 2 mM L-glutamine (Sigma) and 100 u/ml penicillin and 100 μg/ml streptomycin (Sigma). Fibroblasts were transferred into a Collagen IV-coated dish (Euroclone) containing 5 ml complete DMEM (Sigma) and incubated at 37 °C, 5% CO_2 and 95% humidity. The medium was changed after 24 h. For glutathione (GSH) quantification and Western Blot, $3-4 \times 10^5$ cells were plated in 6 wells (Euroclone) in a complete culture medium, and at the confluence, they were incubated with starvation medium (DMEM without FBS) overnight. For ROS quantification, 25×10^3 cells/well were plated in 96-well (Euroclone) in a complete culture medium. For proliferation rate, 5×10^3 cells/well were plated in xCELLigence 16-well plates (Prodotti Gianni, Milan, Italy). Each experimental protocol was repeated in five different cell samples.

2.2. NO production

The NO production was measured in keratinocytes and fibroblasts culture supernatants using the Griess method (Promega, Milan, Italy), as previously performed in the same or similar cellular models [33–36]. Fibroblasts and keratinocytes plated in 96-well plates in starvation medium were treated for 30 min with genistein (10 nM- 1 μM- 100 μM; Sigma) and 17 β estradiol (100 pM-10 nM-100 nM; Sigma). In different cell samples, the effects of 30 min hydrogen peroxide (200 μM; Sigma) on NO release alone or in presence of genistein and 17 β estradiol given 30 min before hydrogen peroxide, were also examined. The dose of hydrogen peroxide was similar to the one used in similar studies performed in both fibroblasts and keratinocytes [37–39]. In addition, in some experiments, 30 min genistein (100 μM; Sigma) and 17 β estradiol (100 nM; Sigma) were given after the pre-stimulation with the ERs inhibitor, fulvestrant (equimolar; 15 min; Sigma), the GPER30 antagonist, G15 (equimolar; 15 min; Santa Cruz Biotechnologies, Inc., CA, USA), the NOS inhibitor, L-NAME (equimolar; 15 min; Sigma), the phosphatidyl inositol 3 kinase

inhibitor (PI3K), wortmannin (equimolar; 15 min; Sigma), the p38 mitogen-activated protein (MAP) kinase inhibitor, SB203580 (equimolar; 15 min; Sigma), and the extracellular signal–regulated kinases (ERK) 1/2 inhibitor, UO126 (equimolar; 15 min; Sigma). Acetylcholine was used for positive control (10 μM; 15 min; Sigma). At the end of the stimulations, NO production in the sample's supernatants was examined by adding an equal volume of Griess reagent following the manufacturer's instruction. At the end of incubation, the absorbance at 570 nm was measured by a spectrometer (BS1000 Spectra Count, San Jose, CA, USA) and the NO production was quantified in respect to nitrite standard curve and expressed as a percentage. The values obtained correspond to the NO (μmol) produced, after each stimulation, by samples containing 1.5 μg of proteins each.

2.3. Cell viability

As described for NO release, dose-response experiments were performed to examine the effects of genistein and 17 β-estradiol on cell viability of fibroblasts and keratinocytes cultured in the starvation medium. Control cells were treated with DMEM without FBS and phenol red only. Cell viability was examined by using the 1% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Life Technologies Italia, Monza, Italy) dye, as previously described [26–28]. Keratinocytes and fibroblasts were also treated with hydrogen peroxide (200 μM; Sigma) alone or in the presence of genistein (100 μM; Sigma) and 17 β estradiol (100 nM; Sigma) given alone or 30 min before hydrogen peroxide. In some experiments, genistein and 17 β-estradiol were given in keratinocytes and fibroblasts pretreated with the same inhibitors used for NO measurement. After each treatment, the medium was removed, and fresh culture medium without red phenol and FBS and with MTT dye was added in 96-well plates containing the cells and incubated for 2 h at 37 °C in an incubator. Thereafter, the medium was removed, and an MTT solubilization solution in equal volume to the original culture medium was added and mixed in a gyratory shaker until the complete dissolution of formazan crystals. Cell viability was determined by measuring the absorbance at 620 nm through a spectrometer (BS1000 Spectra Count, San Jose, CA, USA), and cell viability was calculated by comparing results with the control.

2.4. XCELLigence

For examining the proliferation rate, the xCELLigence™ MP Instrument (Roche, Basel, Switzerland) was used. Fibroblasts and keratinocytes were harvested using trypsin-EDTA and counted under light microscope. xCELLigence 16-well plates (Prodotti Gianni) were prepared by the addition of complete media to every well. After equilibration to 37 °C, plates were inserted into the xCELLigence station and the baseline impedance was measured to ensure that all wells and connections were working within acceptable limits. The software automatically informs the researchers if any connection problem arises. Following harvesting and counting, cells were diluted to the correct seeding density and added to the wells in 100 μl. In different cell samples, keratinocytes and fibroblasts were treated with H_2O_2 for 30 min (200 μM; Sigma) alone or in the presence of genistein (100 μM and 10 nM; Sigma) and 17β-estradiol (100 nM and 100 pM; Sigma). The experiment was stopped after 70 h and results analyzed after 12 h, 24 h, 48 h and 70 h. Cell-sensor impedance was expressed as an arbitrary unit called the Cell Index. The Cell Index at each time point is defined as (Rn-Rb)/15, where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the media alone.

2.5. Mitochondrial membrane potential measurement

Mitochondrial membrane potential measurement in fibroblasts and keratinocytes was performed with 5,51,6,61-tetrachloro-1,11,3,31 tetraethyl-benzimiazolyl carbocyanine iodide assay. Fibroblasts and keratinocytes were stimulated as for cell viability examination. After stimulations, the medium of cells was removed and incubated with 5,51,6,61-tetrachloro-1,11,3,31 tetraethyl-benzimidazolyl carbocyanine iodide 1X diluted in Assay Buffer 1× for 15 min at 37 °C in an incubator following the manufacturer's instruction (Invitrogen, Life Technologies Europe BV, Monza, Italy) [34,36]. The dyes were dissolved in dimethylsulfoxide (Sigma), and the percentage of the organic solvent in the samples never exceeded 1% vol/vol. After incubation, the cells were washed twice with Assay Buffer 1× and then the suspensions were transferred in triplicates to a black 96-well plate. The red (excitation 550 nm/emission 600 nm) and green (excitation 485 nm/emission 535 nm) fluorescence were measured using a fluorescence plate reader (BS1000 Spectra Count). To establish the cells undergoing apoptosis, the ratio of red to green fluorescence was determined and expressed as a percentage.

2.6. ROS quantification

The oxidation of 2,7-dichlorodihydrofluorescein diacetate into 2,7- dichlorodihydrofluorescein was used to assess ROS generation, following the manufacturer's instructions (Abcam, Cambridge, United Kingdom), and as previously performed [34,35]. Briefly, fibroblasts and keratinocytes in 96-well plates were stimulated with hydrogen peroxide (200 μM, 30 min) in the absence or presence of genistein (10 nM, 1 μM, 100 μM; Sigma) and 17 β-estradiol (100 pM, 10 nM, 100 nM; Sigma). After treatments, the reactions were stopped by removing the medium and washing with phosphate buffer saline followed by staining with 10 μM 2,7-dichlorodihydrofluorescein diacetate for 20 min at 37 °C. The fluorescence intensity of 2,7-dichlorodihydrofluorescein diacetate was measured at excitation/emission wavelengths of 485 nm and 530 nm by using a spectrometer (BS1000 Spectra Count).

2.7. Glutathione (GSH) quantification

In keratinocytes and fibroblasts treated as described for ROS quantification, the content of GSH was determined by using a commercial kit according to the manufacturer's instructions (Cayman, Ann Arbor, Michigan, USA) [34–36]. Briefly, fibroblasts and keratinocytes were lysed on ice with 2 ml ice-cold Glutathione Assay Buffer and a rubber policeman. Thereafter, cells were collected by centrifugation (2000*g* for 10 min at 4 °C). After centrifugation, the cell pellet was homogenized in 2 ml cold Glutathione Assay Buffer [50 mM 2-(*N*-morpholino) ethanesulfonic acid, containing 1 mM ethylene diamine tetra-acetic acid] and vortexed for several seconds to achieve a uniform emulsion. After samples were centrifuged at 10,000*g* for 15 min, at 4 °C, the supernatants were removed and stored on ice. The supernatants were deproteinized before assaying to remove as much protein as possible from the samples and avoid any interferences due to particulates and sulfhydryl groups on proteins, as described in Glutathione assay kit booklet. Briefly, an equal volume of metaphosphoric acid (Sigma) was added to the samples which were vortexed. After centrifugation at 2000*g* for 2 min, the supernatants were collected and mixed with 4 M solution of triethanolamine (TEAM; Sigma; 50 μl TEAM per ml of the supernatants). Then, 50 μL of the samples were transferred to a 96-well plate where GSH was detected following the manufacturer's instructions through a spectrometer (BS1000 Spectra Count) at an excitation

and emission wavelength of 340 and 420 nM. GSH content was expressed as nmol/10^5 cells.

2.8. Cell lysates

When keratinocytes and fibroblasts reached confluence, the complete medium was replaced with starvation medium (DMEM without FBS) and incubated overnight at 37 °C with 5% CO_2.

For Western Blot analysis of p38MAPK, Akt and NOS, fibroblasts and keratinocytes were stimulated with 30 min hydrogen peroxide (200 μM) in the absence or presence of 30 min genistein (10 nM, 100 μM; Sigma) and 30 min 17 β-estradiol (100 pM, 100 nM; Sigma). For Western Blot analysis of metalloproteases 1 (MMP-1) and 9 (MMP-9), fibroblasts and keratinocytes were stimulated with 30 min hydrogen peroxide (200 μM) in the absence or presence of 24 h genistein (10 nM, 100 μM; Sigma) and 24 h 17 β-estradiol (100 pM, 100 nM; Sigma). After the end of stimulations, keratinocytes and fibroblasts were lysed in iced-Ripa-buffer supplemented with 1:200 sodium orthovanadate and 1:100 protease inhibitors cocktail and phenylmethanesulfonyl fluoride (PMSF; 1:100; Sigma). The extract proteins were quantified through bicinchoninic acid protein (BCA; Pierce, Rockford, IL, USA) and used for electrophoresis and immunoblotting studies [34–36].

2.9. Western blotting

Cell lysates (30 μg protein each sample) dissolved in Laemmli buffer 5× and boiled for 5 min, were resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-Rad Laboratories, Hercules, CA, USA) and after electrophoresis, were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories), which were incubated overnight at 4 °C with specific primary antibodies: anti phospho-Akt (p-Akt, 1:1000; Ser473, Santa Cruz Biotechnology, Dallas, USA), anti Akt (1:1000; Santa Cruz Biotechnology), anti phospho-p38 MAPK (pp-38 MAPK, 1:1000; Thr180/Thr182, Cell Signalling Technologies, Danvers, USA), anti p38 MAPK (1:1000; Cell Signalling Technologies), anti phospho-endothelial NOS (eNOS; 1:1000; Ser1177, Cell Signalling Technologies), anti-endothelial NOS (eNOS: 1:500; Santa Cruz Biotechnology), iNOS (1:500; Santa Cruz Biotechnology), anti MMP-1 (2 μg/ml; R&D Systems, Minneapolis, USA) and anti MMP-9 (0.1 μg/ml; R&D Systems). The membranes were washed and then incubated with horseradish peroxidase-coupled goat anti-rabbit IgG (Sigma), peroxidase-coupled rabbit anti-goat IgG and horseradish peroxidase-coupled goat anti-mouse IgG (Sigma) for 45 min and were developed through a nonradioactive method using Western Lightning Chemiluminescence (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). Phosphorylated protein expression was calculated as a ratio towards specific total protein expression or β-actin (1:5000; Sigma) detection.

2.10. Statistical analysis

All data were recorded using the Institution's database. Statistical analysis was performed by using STATVIEW version 5.0.1 for Microsoft Windows (SAS Institute Inc., Cary NC, USA). Data were checked for normality before statistical analysis. All the results obtained were examined through one-way ANOVA followed by Bonferroni post hoc tests. The non-parametric Mann Whitney *U* test for unpaired data was used to compare percentage responses. Pearson coefficient was calculated for linear correlation analysis in dose-response studies. All data are presented as means ± SD of five independent experiments for each experimental protocol. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of genistein and 17 β-estradiol on NO release

In fibroblasts and keratinocytes cultured in physiological conditions, genistein, 10 nM, 1 μM and 100 μM, increased NO release to 1.8%, 2.9%, 4.5% and 2.2%, 3.5% and 4.4% from respective control values (Fig. 1A and B). In the same cells, the NO release caused by 17 β-estradiol, 100 pM, 10 nM and 100 nM, amounted to 2.8%, 3.8%, 6.1% and 3.7%, 4.6%, 4.9% from respective control values (Fig. 1). In cells pretreated with hydrogen peroxide, NO release was increased to about 55% and 61% of control values, an effect which was counteracted by both agents (Fig. 1). Moreover, genistein and 17 β-estradiol were able to dose-dependently reduce the eNOS and iNOS activation/expression caused by the peroxidative stimuli (Fig. 2). The dose of hydrogen peroxide chosen, amounting to 200 μM, was similar to the one previously used for studies about skin aging induced by irradiation [37] and about protective effects elicited by isoflavones or peptides in both fibroblasts and keratinocytes [38–40].

In addition, on the ground of the above results, we decided to use 17 β-estradiol and genistein for all next experiments at

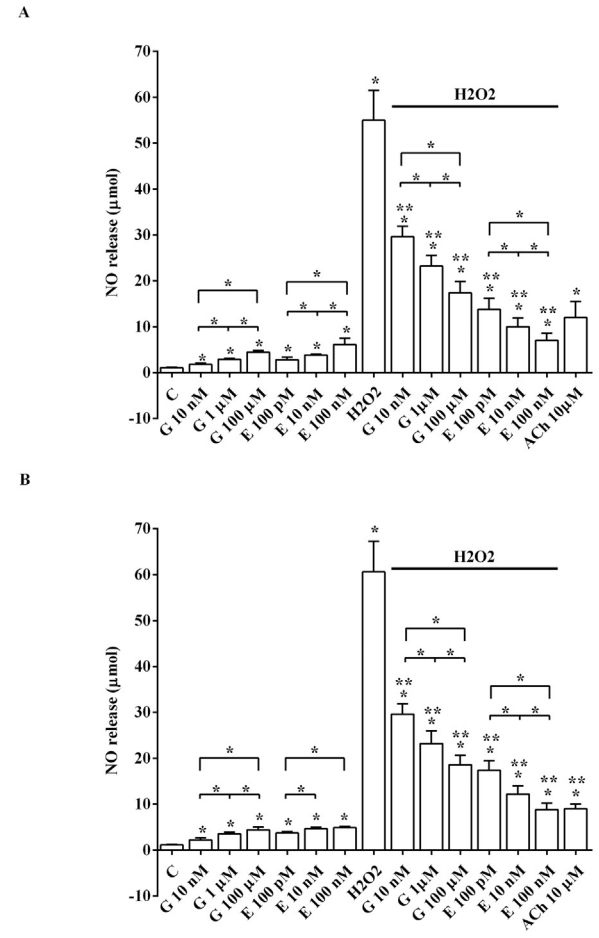

Fig. 1. Dose-response effects of genistein and 17 β-estradiol on NO release in human fibroblasts (A) and keratinocytes (B). The values obtained correspond to the NO (μmol) produced after each stimulation, by samples containing 1.5 μg of proteins each. Reported data are means ± SD of five independent experiments. C: control; G: genistein; E: 17 β-estradiol. Square brackets indicate significance between groups. *P < 0.05. *P < 0.05 vs C. **P < 0.05 vs H_2O_2.

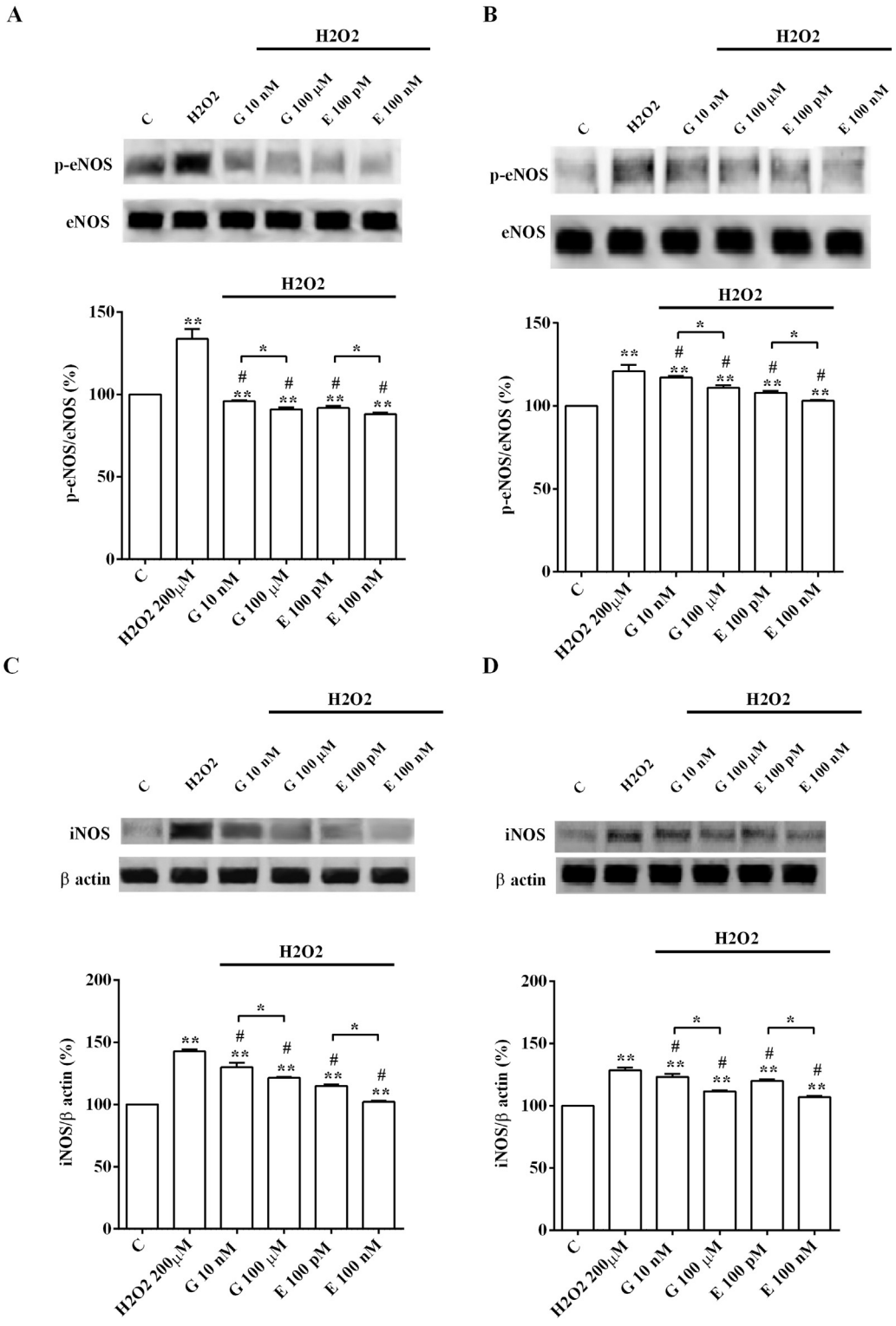

Fig. 2. Effects of genistein and 17 β-estradiol on activation/expression of eNOS and iNOS in human fibroblasts (A, C) and keratinocytes (B, D) cultured in peroxidative conditions. In A–D, densitometric analysis and an example of Western Blot taken from 5 different experiments of p-eNOS and iNOS are shown. In each sample, densitometric analysis was performed by taking the percentage change of phosphorylated eNOS vs eNOS or iNOS vs β actin. Abbreviations are as in Fig. 1. Reported data are means ± SD of five independent experiments. Square brackets indicate significance between groups. $^{*}P < 0.05$. $^{**}P < 0.05$ vs control. $^{\#}P < 0.05$ vs H_2O_2.

100 nM and 100 μM, respectively, since similar concentrations were used in previous studies aimed to examine the protective effects elicited by 17 β-estradiol and genistein in fibroblasts and keratinocytes against aging or UV-mediated damages [6,13,41].

3.2. Role of ERs, GPER30, NOS, PI3K, ERK1/2 and p38MAPK in the effects of genistein and 17 β-estradiol on NO release

The effects of genistein and 17 β-estradiol in fibroblasts (Fig. 3A and B) and keratinocytes (Fig. 3C and D) cultured in

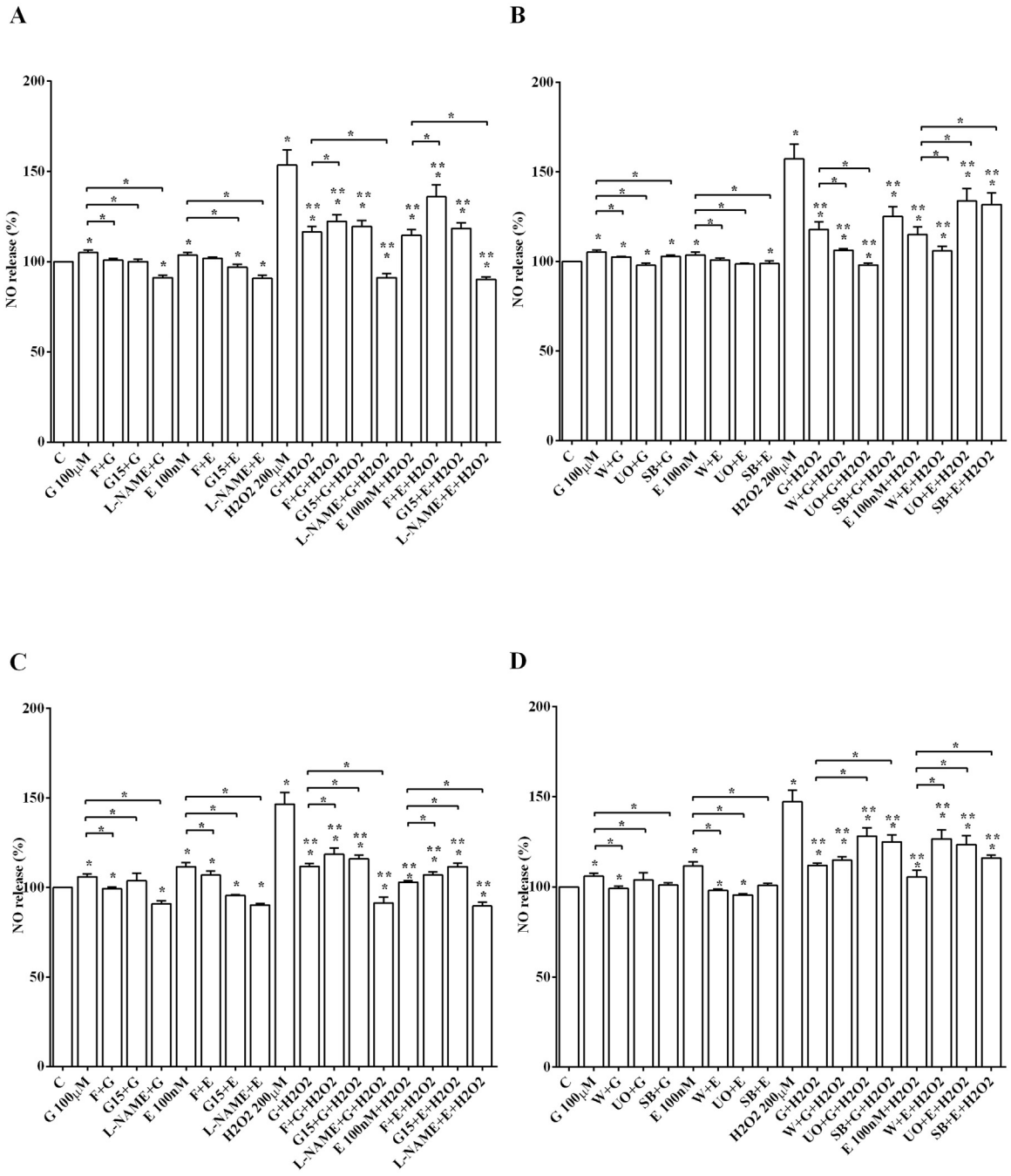

Fig. 3. Effects of genistein and 17 β-estradiol on NO release in human fibroblasts (A, B) and keratinocytes (C, D) in the presence or absence of various inhibitors. The values obtained correspond to the NO (μmol) produced after each stimulation, by samples containing 1.5 μg of proteins each. F: fulvestrant, ERs inhibitor; G15: GPER30 inhibitor; L-NAME = Nω-nitro-L-arginine methylester, NOS inhibitor; W: wortmannin, the phosphatidyl inositol 3 kinase inhibitor; UO: UO126, the extracellular signal–regulated kinases inhibitor; SB: SB203580, the p38 mitogen activated protein kinases inhibitor. Other abbreviations are as in previous Figures. Reported data are means ± SD of five independent experiments. Square brackets indicate significance between groups. *P < 0.05. *P < 0.05 vs C. **P < 0.05 vs H_2O_2.

physiological conditions were reduced or abolished by the ERs inhibitor, fulvestrant, the GPER30 blocker, G15, the NOS inhibitor, L-NAME, the PI3K blocker, wortmannin, the ERK1/2 blocker, UO126, and the p38MAPK blocker, SB203580. In contrast, in fibroblasts in peroxidative condition the protective effects of genistein and 17 β-estradiol were reduced by fulvestrant and G15 (Fig. 3A), as NO release was higher than NO release under genistein and 17 β-estradiol given without the blockers. Nevertheless, wortmannin improved the effects of both estradiol and genistein as the NO releases decreased (Fig. 3B). L-NAME improved as well both protective effects (Fig. 3A). UO126 improved the protective effects of genistein as NO release decreased, but it contributed to the NO release in the presence of estradiol. SB203580 counteracted the protective effects of genistein and 17 β-estradiol as in both cases NO release increased (Fig. 3B). In keratinocytes treated with hydrogen peroxide all the above inhibitors except L-NAME hindered the response of cells to both the phytoestrogens and estrogens. Hence, the release of NO caused by hydrogen peroxide was reduced less by genistein and 17 β-estradiol (Fig. 3C and D).

3.3. Effects of genistein and 17 β-estradiol on cell viability and mitochondrial membrane potential

Regarding cell viability, genistein and 17 β-estradiol elicited opposite effects in fibroblasts and keratinocytes cultured in physiological conditions. In fibroblasts, we observed a dose-related increase of cell viability after treatment with estrogens (Fig. 4A) and a reduction after treatment with phytoestrogens (Fig. 4B). Opposite results were obtained in keratinocytes (Fig. 4C and D). In any case, the mitochondrial membrane potential was improved by both agents (Fig. 5). In peroxidative conditions, 17 β-estradiol enhanced the mitochondrial membrane potential in a dose-dependent manner in both cell types (Fig. 5), while genistein in fibroblasts decreased mitochondrial membrane potential with increasing doses (Fig. 5A) and increased it in a dose-dependent way in keratinocytes (Fig. 5B). Moreover, in keratinocytes and

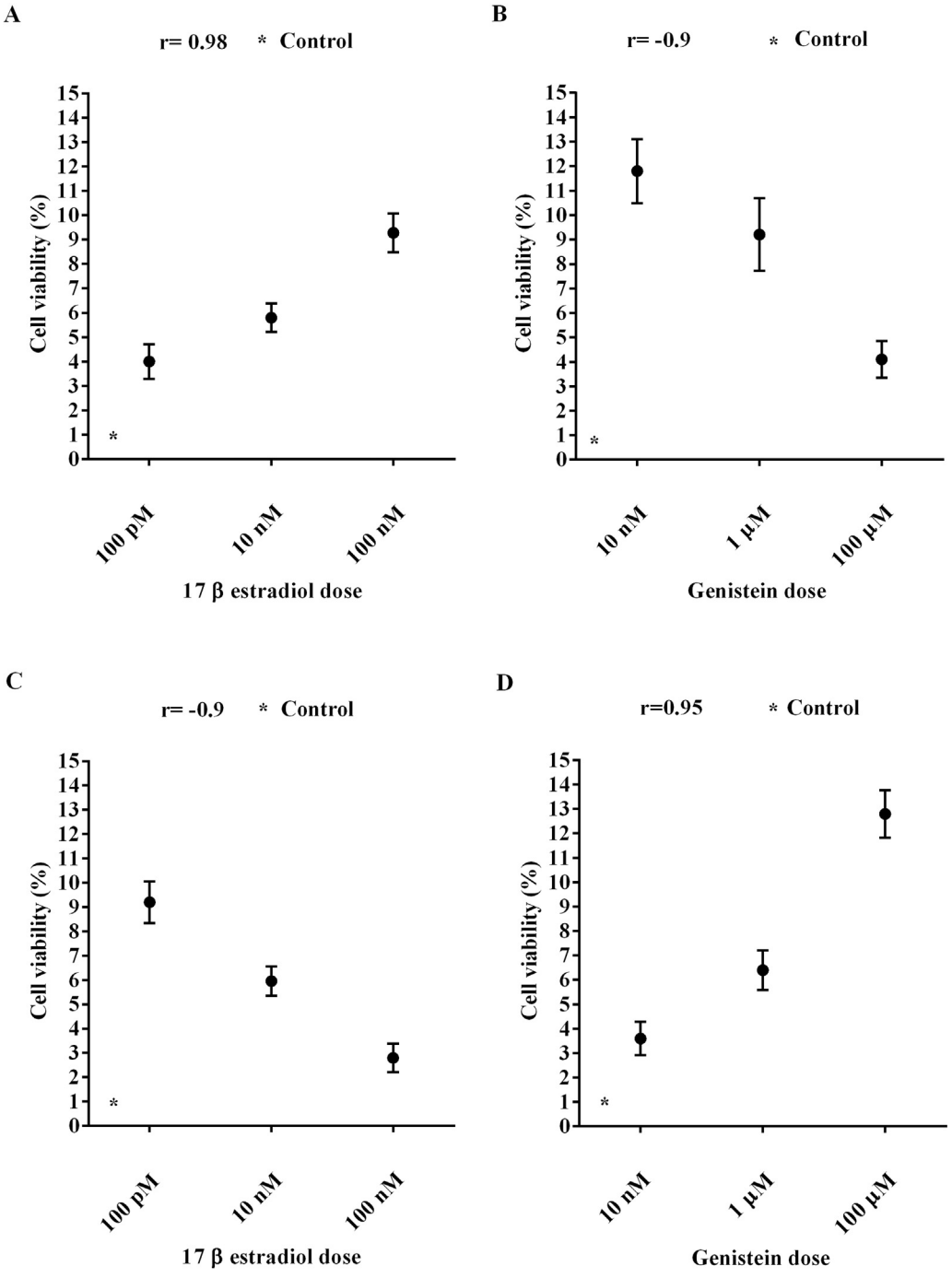

Fig. 4. Dose-response effects of 17 β-estradiol and genistein on cell viability of human fibroblasts (A, B) and keratinocytes (C, D) cultured in physiological conditions. Reported data are means ± SD of five independent experiments. R: Pearson's relation coefficient. Abbreviations are as in previous Figures.

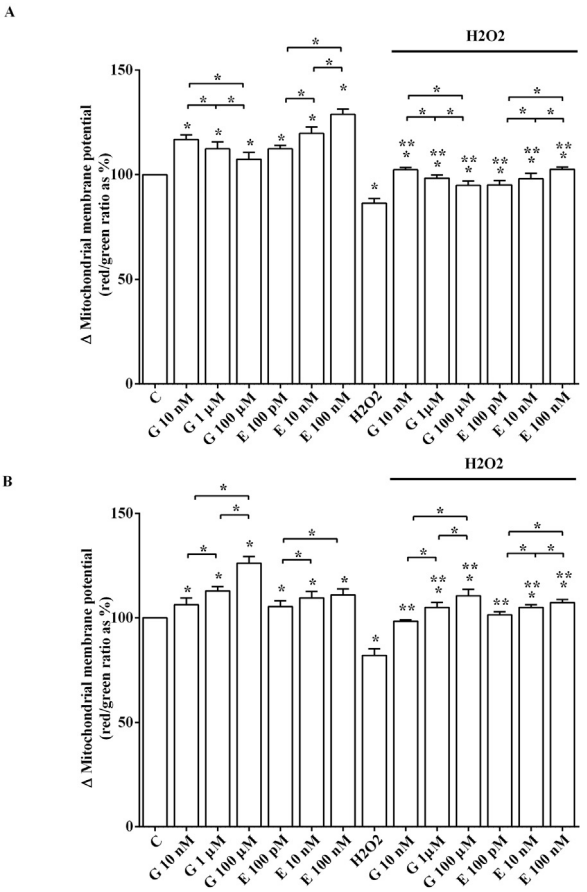

Fig. 5. Dose-response effects of genistein and 17 β-estradiol on mitochondrial membrane potential of human fibroblasts (A) and keratinocytes (B) cultured in physiological and peroxidative conditions. Reported data are means ± SD of five independent experiments. Abbreviations are as in previous Figures. Reported data are means ± SD of five independent experiments. Square brackets indicate significance between groups. *P < 0.05. *P < 0.05 vs C. **P < 0.05 vs H_2O_2.

fibroblasts pretreated with hydrogen peroxide, both genistein and 17 β-estradiol prevented the fall of cell viability and of mitochondrial membrane potential (Figs. 5–7).

3.4. Role of ERs, GPER30, NOS, PI3K, ERK1/2 and p38MAPK in the effects of genistein and 17 β-estradiol on cell viability and mitochondrial membrane potential

In addition, and as shown in Figs. 6 and 7, A and C, in keratinocytes and fibroblasts treated with fulvestrant and G15, the effects of those agents on cell viability and mitochondrial membrane potential were reduced, both in physiological and peroxidative conditions. Hence, in the presence of those blockers, the effects of genistein and estradiol were lower than those found in the absence of blockers. Similar responses were observed in cells pretreated with L-NAME, wortmannin, UO126, and SB203580 (Figs. 6 and 7).

3.5. Role of PI3K and p38MAPK in the effects of genistein and 17 β-estradiol against peroxidation

As shown in Fig. 8, also Western Blot analysis confirmed the involvement of PI3K-Akt (A and B) and p38MAPK (C and D) in the effects of both genistein and 17 β-estradiol against the peroxidative damage in fibroblasts (A, C) and keratinocytes (B, D). In particular, and regarding Akt, both genistein and 17 β-estradiol were able to hinder the reduction of its activation caused by hydrogen peroxide (Fig. 8A and B); regarding p38MAPK, both agents enhanced the effects of the oxidative stimuli (Fig. 8C and D).

3.6. Antioxidant effects elicited by genistein and 17 β-estradiol

Of more relevance is that the protective effects elicited by genistein and 17 β-estradiol were accompanied by a dose-related reduction of ROS release (Fig. 9A and B) and by an increase of GSH content in both cell types (Fig. 9C and D). Since GSH content is one of the main mechanisms, among others, to cope with ROS, it could be argued that the protective effects elicited by both genistein and estradiol were related to the dose-dependent modulation of the oxidant/antioxidant system in fibroblasts and keratinocytes.

3.7. Modulation of proliferation and MMPs expression by genistein and 17 β-estradiol

In addition, genistein and 17 β-estradiol increased proliferation of cells cultured in physiological medium (Fig. 10A and B) and prevented the effects of hydrogen peroxide (Fig. 10C and D). Those effects were similar to those concerning cell viability and also in relation to the doses administrated (Fig. 6). Hence, in fibroblasts, we observed a dose-related increase of cell proliferation after treatment with 17 β-estradiol and a reduction in the presence of genistein (Fig. 10A). The opposite results were found in keratinocytes (Fig. 10C). Finally, both the estrogen and phytoestrogen were able to dose-dependently counteract the effects of hydrogen peroxide on MMP-1 and MMP-9 expression (Fig. 11). Since, MMPs play an important role in photoaging, our results showing inhibitory effects on MMPs activation caused by peroxidation would highlight a role for estrogens and phytoestrogens as potential antiaging tools.

4. Discussion

In this study we demonstrate a protective effect against peroxidative injuries of genistein and 17 β-estradiol on fibroblasts and keratinocytes, by modulating NO and ROS release, GSH content and mitochondria function. The involvement of ERs, GPER30 and of signaling related to PI3K-Akt, p38 MAPK, and ERK1/2 has also been highlighted.

Oxidative stress induced by ROS is widely accepted as the major driving force of the aging process. Hence, the age-related increase in ROS generation and oxidative injuries have been reported in a variety of tissues including the skin [42]. Human skin is exposed to ROS generated from both environmental sources as well as the endogenous oxidative metabolism [43] and, for this reason, photoaging (i.e. extrinsic aging mainly caused by UV) accelerates the physiological aging process of the skin (i.e. intrinsic aging) [44,45].

Physiological skin aging is a process which can be modulated by multiple factors, including hormonal fluctuations. Many studies demonstrated that estrogens can exert significant beneficial and protective roles in the skin: variations in skin thickness are observed during the menstrual cycle [3], whereas in the postmenopausal age estrogens deficiency accelerates structural and functional changes (i.e. fragmentation of elastin fibers, damage of dermal vessels and reduction of collagen production) [46]. Many of these effects can be reversed by estrogen replacement, which increases epidermal hydration, elasticity, and thickness, and enhances the content and quality of collagen [13]. Consequently, estrogens can also offer some protection against skin photoaging [47,48].

Genistein, the main isoflavone contained in soybeans and in certain fermented soy foods, has been shown to be a promising anti-aging and anti-carcinogenic agent for skin care, due to its

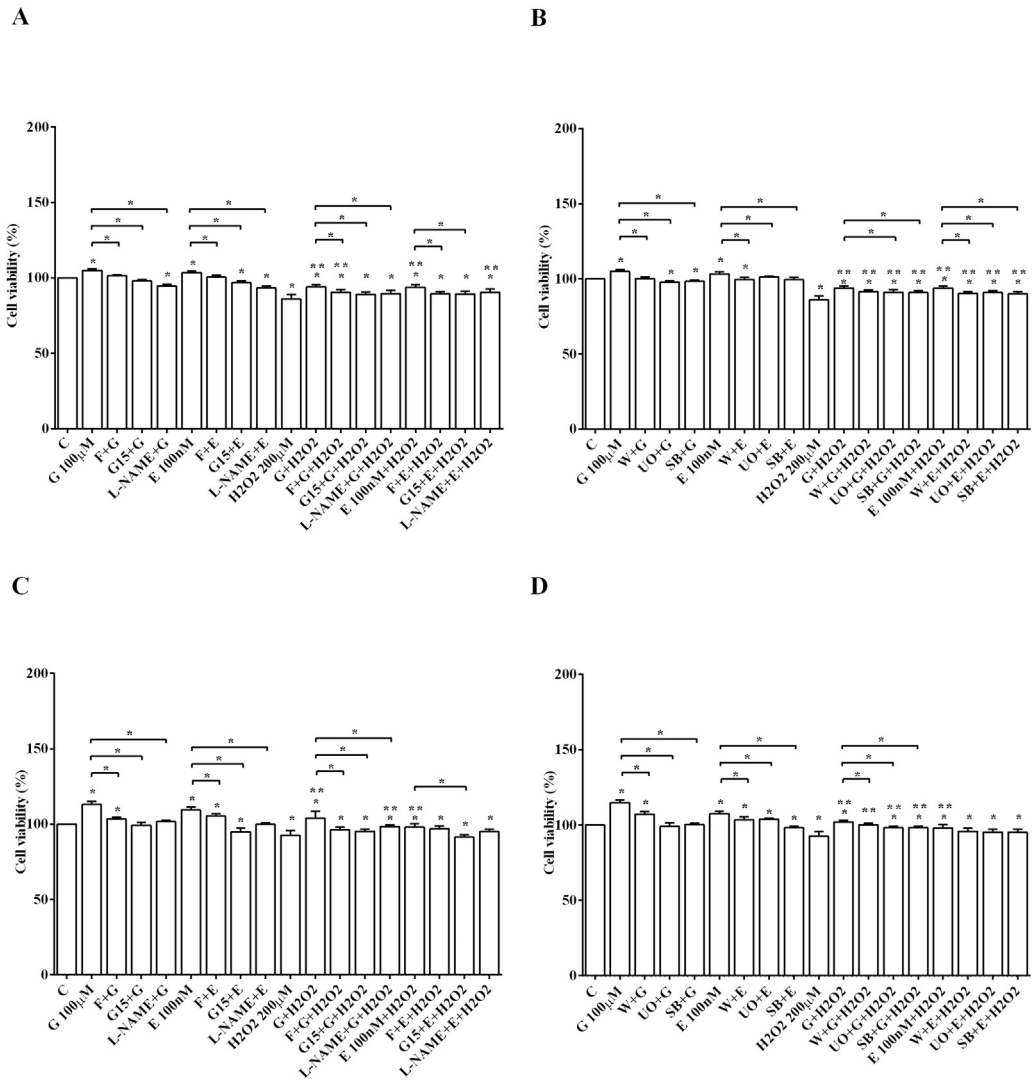

Fig. 6. Effects of genistein and 17 β-estradiol on cell viability in human fibroblasts (A, B) and keratinocytes (C, D) in the presence or absence of various inhibitors. Abbreviations are as in previous Figures. Reported data are means ± SD of five independent experiments. Square brackets indicate significance between groups. * $P < 0.05$. *$P < 0.05$ vs C. **$P < 0.05$ vs H_2O_2.

antioxidant properties [9,41] and has been suggested for topical use in the prevention and treatment of skin aging after menopause [41,49,50].

In our study, 17 β-estradiol was able to cause a dose-related or dose-inverse positive effect on cell viability and proliferation of human keratinocytes (Figs. 4C and 10 C, D) and fibroblasts (Figs. 4A and 10 A, B). It is to note that these results were obtained with concentrations of 17 β-estradiol, amounting to 10^{-9}–10^{-7} M, that were similar to those found in menstrual/menopausal women [50,51] and analogous to those used in similar studies performed in skin fibroblasts and keratinocytes [6,13,41]. The opposite response was observed with genistein, administrated at nutritional concentration, like those previously used in keratinocytes (Figs. 4D and 10 C, D) and fibroblasts (Figs. 4B and 10 A, B) [13]. Notably, both agents were able to improve mitochondrial membrane potential and to increase NO release in physiological conditions and to hinder the fall of mitochondrial membrane potential and the increased NO release caused by hydrogen peroxide.

In this study, we demonstrate that both 17 β-estradiol and genistein reduced ROS release, while increasing GSH level, which was accompanied by the keeping of cell viability and an improvement of the proliferation rate. Since, the up-to-date information about the effects of 17 β-estradiol and genistein on ROS and NO release is scarce, our findings do increase the knowledge about this issue. Moreover, we confirm the cytoprotective effects of estrogens and genistein previously described by other authors [3]; although their precise mechanism of action is unclear, a role could be played by their anti-inflammatory and antioxidant action.

As mentioned above, our results regarding the modulation of NO release and mitochondria membrane potential could suggest a possible novel mechanism through which estrogens/phytoestrogens would exert their protective effects against peroxidation. The fact that NO could exert protection or damage would thus depend on its concentration and on the relative activity of the constitutive or inducible NOS [21]. Moreover, it is to note that eNOS itself could be strongly activated in the presence of peroxidation and change its function. In respect of this issue, it is worth noting that eNOS has been reported to be a redox "hub", being regulated by and contributing to the regulation of intracellular redox homeostasis through mutually interacting tetrahydrobiopterin- and GSH-dependent pathways. Changes of GSH have been reported to

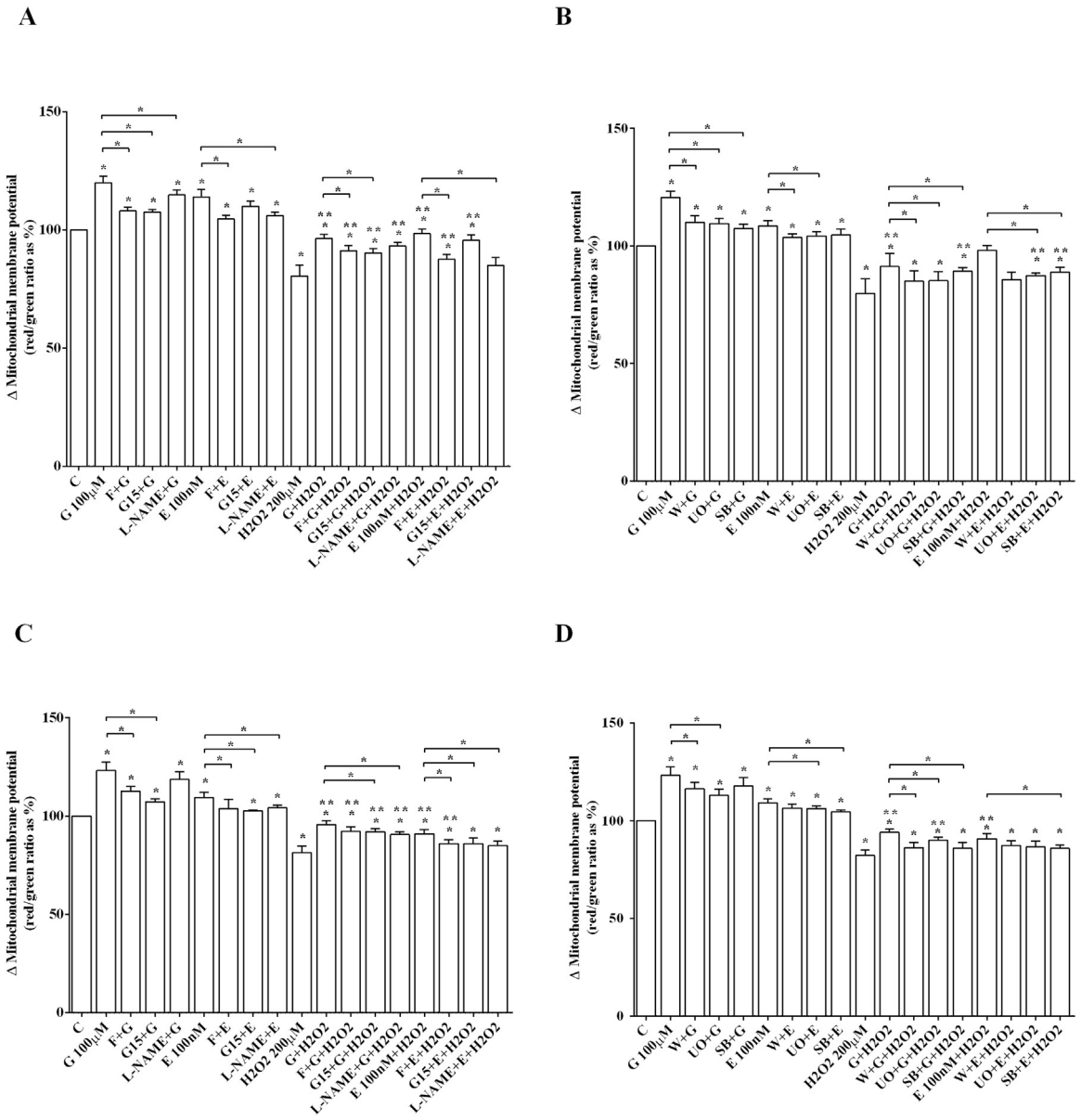

Fig. 7. Effects of genistein and 17 β-estradiol on mitochondrial membrane potential in human fibroblasts (A, B) and keratinocytes (C, D) in the presence or absence of various inhibitors. Abbreviations are as in previous Figures. Reported data are means ± SD of five independent experiments. Square brackets indicate significance between groups. * $P < 0.05$. *$P < 0.05$ vs C. **$P < 0.05$ vs H_2O_2.

cause eNOS uncoupling, which would cause the shift of the enzyme from NO to superoxide production. This could, in turn, increase iNOS activation and strengthen NO release [21].

Thus, the opposite effects of genistein and 17 β-estradiol on NO release in physiological/peroxidative conditions could be related to changes in NOS subtypes recruitment and in eNOS function. While in physiological conditions those agents would act mainly through eNOS subtype, which would cause just minor changes in NO release, in the presence of hydrogen peroxide they would reduce NO release by inhibiting both eNOS and iNOS.

Some speculations could be made about the pathways at the basis of those effects. Hence in our study p-Akt and p38MAPK, which are widely considered as eNOS activators [52], were found to be inhibited and activated, respectively by both estrogens and genistein in fibroblasts and keratinocytes that have undergone peroxidation. Although this point has not been fully examined, we could hypothesize that the reduction of ROS release is involved in the reduction of NOS isoforms activation/expression by the modulation of the crosstalk between Akt/p38MAPK, and/or of pathways related to nicotinamide adenine dinucleotide phosphate oxidase, extracellular signal–regulated kinases 1/2, phospholipase C, 5′ adenosine monophosphate-activated protein kinase and Ca2+/calmodulin-dependent protein kinase II [53–55]. Also, Nf-kb could be involved in the effects of genistein on NOS isoforms. As shown by Duarte et al. [56], flavonoids were found to prevent the inflammatory signaling cascades through downregulation of iNOS associated to NF-kb inhibition. Thus, all the above intracellular signaling cascades could be object of further investigations.

Another key point of our study is represented by the confirmation of the role of mitochondria in aging; this has been highlighted in recent years [57]. Mitochondria could be both sources and targets of damage by free radicals. Furthermore, the fall of mitochondrial membrane potential could act as an initiating event leading to the activation of apoptotic cell death by the release of cytochrome C.

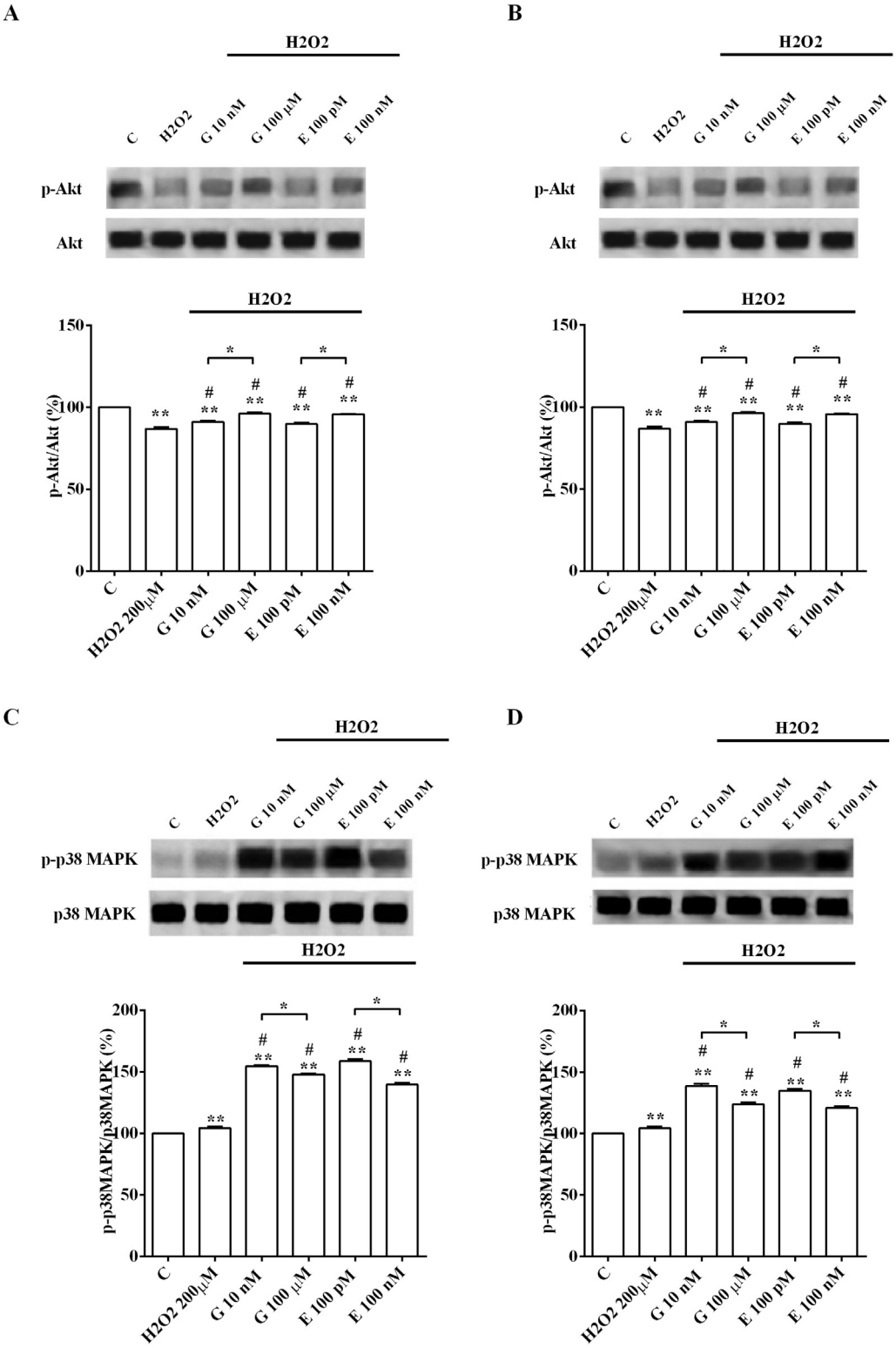

Fig. 8. Effects of genistein and 17 β-estradiol on Akt and p38MAPK activation in human fibroblasts (A, C) and keratinocytes (B, D) cultured in peroxidative conditions. In A–D, densitometric analysis and an example of Western Blot taken from 5 different experiments of p-Akt and p-p38MAPK are shown. p-Akt: phosphorylated Akt; p-p38MAPK: phosphorylated p38MAPK. In each sample, densitometric analysis was performed by taking the percentage change of phosphorylated Akt or p38MAPK vs Akt and p38MAPK. Other abbreviations are as in previous Figures. Reported data are means ± SD of five independent experiments. Square brackets indicate significance between groups. * $P < 0.05$. ** $P < 0.05$ vs control. # $P < 0.05$ vs H_2O_2.

Several evidences of a putative mitochondrial role in aging came from studies on gender-related longevity. Oxidant production by mitochondria is much higher in males than in females [58]; this phenomenon has been found to be related to the action of estrogens [59]. Regarding this issue, estradiol has been reported to prevent the release of cytochrome C from mitochondria and also to increase the

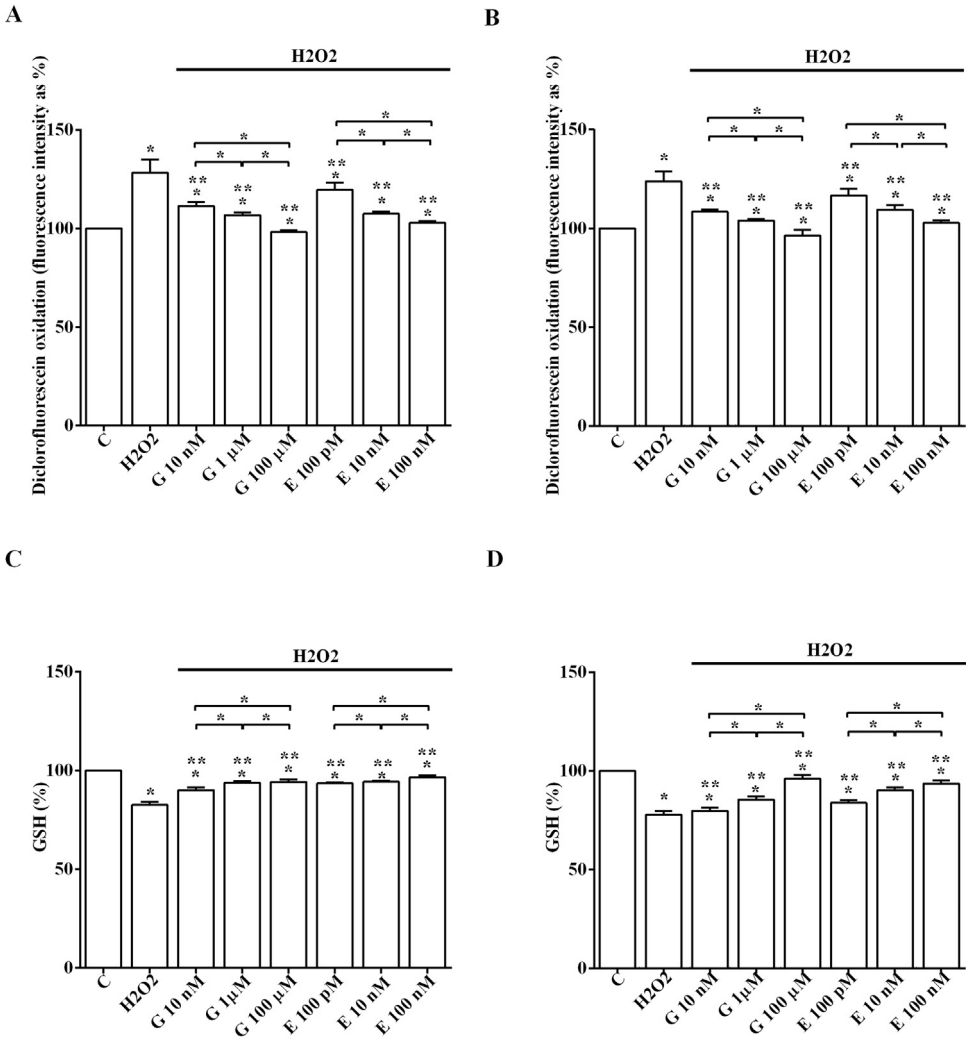

Fig. 9. Effects of genistein and 17 β-estradiol on ROS release and GSH content in human fibroblasts (A, C) and keratinocytes (B, D) cultured in peroxidative conditions. Abbreviations and layout are as in previous Figures. Reported data are means ± SD of five independent experiments. Square brackets indicate significance between groups. *P < 0.05. *P < 0.05 vs C. **P < 0.05 vs H_2O_2.

mitochondrial membrane potential and the efficiency of the respiratory chain. Similar findings have also been described regarding genistein [28]. Thus, our results about mitochondria function would confirm previous observations.

The effects of 17 β-estradiol and genistein on mitochondrial membrane potential could partly explain the discrepancies observed regarding cell viability and cell proliferation. It could be assumed that only strong increases of mitochondrial membrane potential would be needed to obtain beneficial effects on both parameters, with particular reference to cell viability. Hence, in the presence of dose-related increases of mitochondrial membrane potential, in both fibroblasts and keratinocytes a dose-related increase of cell viability was found. Furthermore, epidermal homeostasis is regulated by a balance between proliferation and differentiation. In this context, Notch signaling could play a role in the control of cellular proliferation, differentiation and survival in both physiological and pathological, and would be object of further analysis [60].

Moreover, in our experience almost all effects of genistein and 17 β-estradiol on cell viability and mitochondrial membrane potential were abolished or reduced by fulvestrant, G15, wortmannin, SB203580, and UO126. Those findings highlight the involvement of both ERs and GPER30 receptors and of PI3K-Akt, the p38MAPK and the ERK1/2- related signaling in the mechanisms of action of those agents. Western Blot analysis confirmed the engagement of Akt and p38MAPK.

Previous studies have shown that in keratinocytes and dermal fibroblasts protection against oxidative injuries by estrogens and genistein is mediated by ERβ rather ERα. It is also notable that ERβ has been found to be more expressed in skin cells [61]. Also, the seven-transmembrane receptor, GPER30, has been reported to be involved in the effects of estrogens in human skin cells [62].

The presence of the above membrane estrogen receptors coupled to cytosolic signal transduction proteins could activate direct signaling cascades via conventional second messengers including MAPK producing rapid responses to estrogen [3]. In this context, it is notable that the antioxidant action of estrogens is due to their interaction with estrogen receptors in cells which eventually could lead to the activation of MAPK [28]. However, in a model of ischemia/reperfusion injury, estradiol was found to counteract the activation of p38MAPK [58]. Similarly, genistein could reduce the UVB-activation of MAPK cascade in Wistar rats [59].

Therefore, our findings showing potentiating effects of both estradiol and genistein on p38MAPK would disagree with those

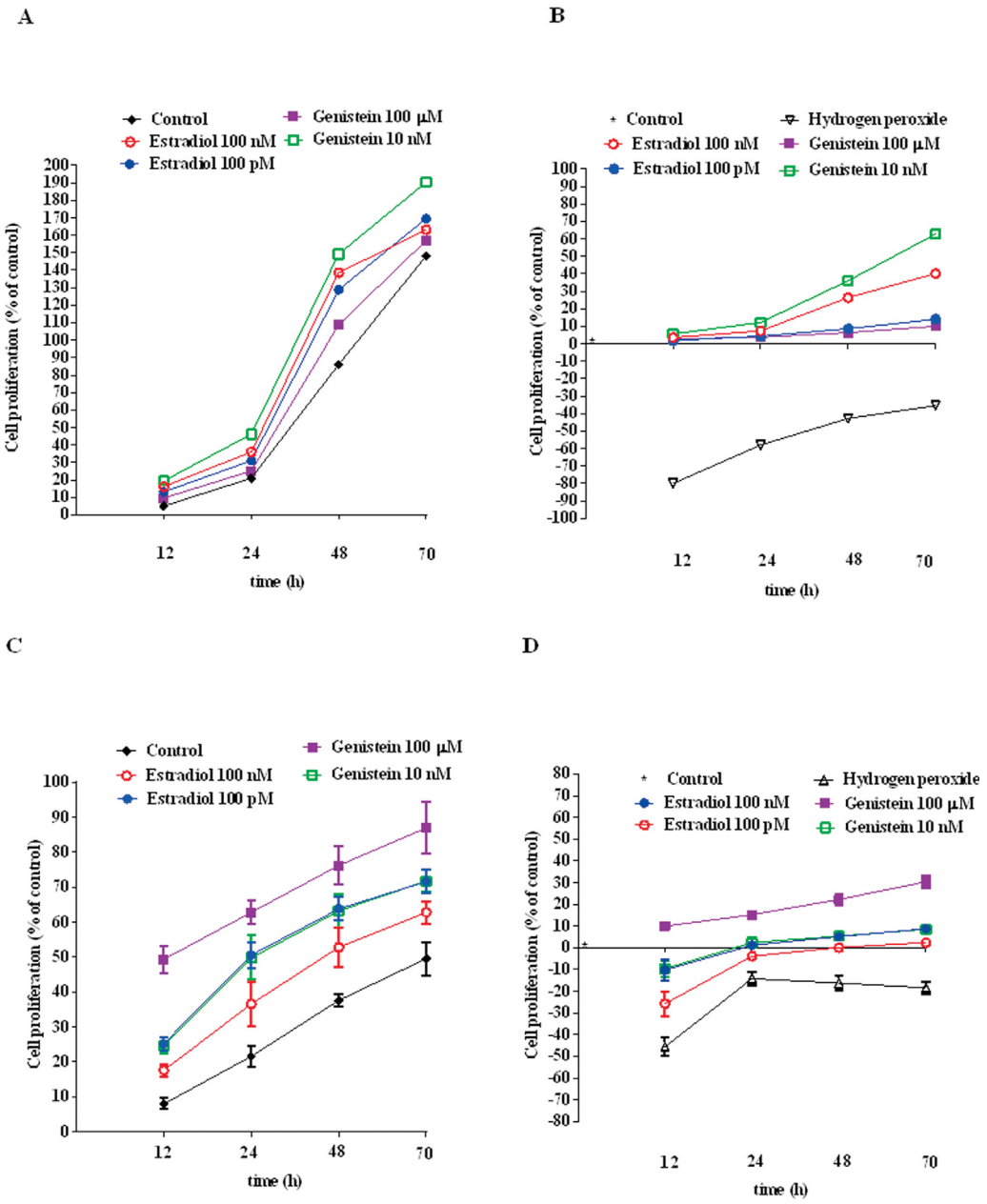

Fig. 10. Effects of genistein and 17 β-estradiol on proliferation of human fibroblasts (A, B) and keratinocytes (C, D) cultured in physiological (A, C) and peroxidative conditions (B, D). Reported data are means ± SD of five independent experiments.

mentioned above. p38 MAPK is activated in response to a wide range of extracellular stimuli, such as inflammation and UV radiation and can influence various cellular processes, including proliferation, differentiation, and apoptosis [63]. About this issue, in human keratinocytes, both anti- and pro-apoptotic responses were found following UV-induced activation of p38MAPK [64]. Moreover, curcuminoid treatment was able to inhibit keratinocyte growth by p38 MAPK activation [64]. The cause of those disparate and opposite effects of p38MAPK is not clear but interplay with other signaling pathways, as well as the nature of p38 MAPK substrates, could account for them.

Regarding the involvement of other intracellular kinases, both ERK1/2 and PI3K-Akt have been reported to be crucial for keratinocyte proliferation induced by the application of exogenous estrogen [6,65]. By this way, our observations obtained with Western Blot about Akt activation could be related to the protective effects exerted by both estradiol and genistein on proliferation rate in fibroblasts and keratinocytes.

The results obtained about MMP1 and 9 expression could be of clinical relevance. Hence, collagen degradation is tightly related to the presence of matrix MMPs, which are mainly secreted by epidermal keratinocytes and dermal fibroblasts. The expression of MMPs, which is quite low in unstimulated cells, can be induced by various extracellular stimuli including UV and infrared radiation [66]. Among various MMPs, MMP-1 is widely considered as the key enzyme responsible for breaking down dermal components in the extracellular matrix. Once the increased levels of MMP-1 has started the degradation of collagen, further processing is followed by MMP-3 and MMP-9. Therefore, MMP-1 plays a significant role in the initiation of UVB-induced wrinkle formation and in photoaging

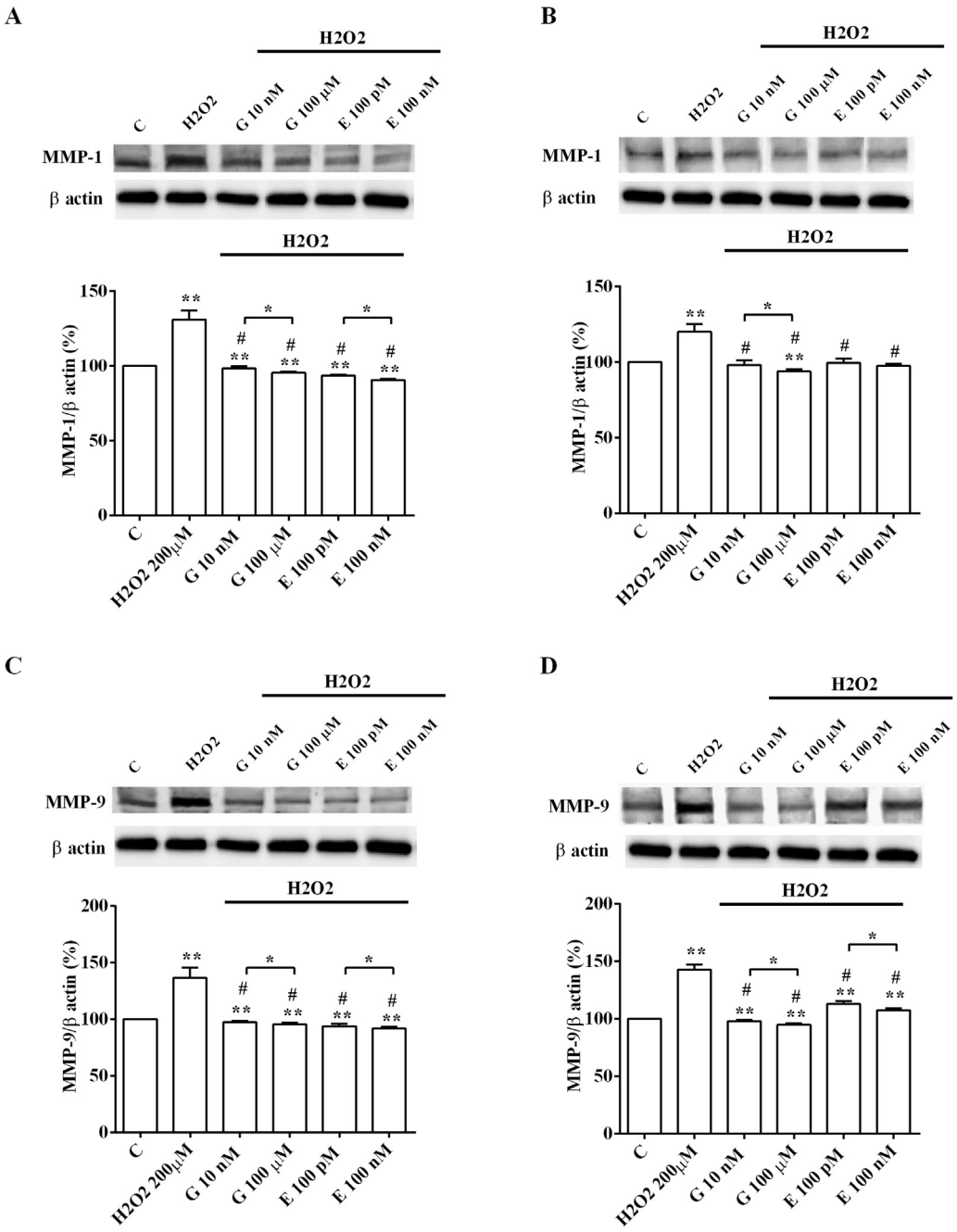

Fig. 11. Effects of genistein and 17 β-estradiol on MMP-1 and MMP-9 in human fibroblasts (A, C) and keratinocytes (B, D) cultured in peroxidative conditions. In A–D, densitometric analysis and an example of Western Blot taken from 5 different experiments of MMP-1 and MMP-9 are shown. In each sample, densitometric analysis was performed by taking the percentage change of MMP-1 and MMP-9 vs β actin. MMP: metalloprotease. Other abbreviations are as in previous Figures. Reported data are means ± SD of five independent experiments. Square brackets indicate significance between groups. *$P < 0.05$. **$P < 0.05$ vs control. #$P < 0.05$ vs H_2O_2.

through matrix degradation [67]. For the above reasons, our results showing inhibitory effects elicited by estrogens and phytoestrogens on MMPs activation caused by peroxidation would highlight their role as potential antiaging tools.

In conclusion, our study demonstrates that 17 β-estradiol and genistein could counteract skin aging by modulating GSH content and ROS release, eNOS/iNOS dependent- NO release, MMPs expression and mitochondria membrane potential in both human fibroblasts and keratinocytes. Through mechanisms involving p38MAPK, Akt, and ERK1/2, as downstream signaling of ERs and GPER30, those agents would keep cell viability and proliferation rate, which would be altered by oxidative stress.

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None.

Conflict of interest

Theauthors have no conflict of interest to declare.

Author declaration

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data.

All authors drafted the article or reviewed it critically for important intellectual content.

All authors gave final approval of the version to be published.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2018.07.007.

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