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Letter to the Editor

Membrane-associated functional estrogen receptors alpha are upregulated in cardiomyocytes under oxidative imbalance

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Dear Editors,

The primary mechanism of 17β-estradiol (E2) activity is mediated by transcriptional actions of the intracellular, nuclear estrogen receptors (ER), ERα and ERβ, to produce genomic effects. However, a variety of cellular responses to physiological concentrations of E2 occurs very rapidly, within seconds to few minutes, so that they cannot be mediated by transcription and protein synthesis. These rapid estrogen-mediated effects (referred to as "nongenomic") are triggered through the activation of non-nuclear membrane-associated ER (mER) [1,2]. These receptors are structurally similar to their intracellular counterparts and, after ligand binding, they activate various protein kinase cascades, including extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), protein kinase A, protein kinase C, Akt, and phosphatidylinositol 3-OH kinase (PI3K) [3].

Cardiac myocytes contain functional ER, and E2 regulates the expression of specific cardiac genes [4]. E2, acting through ERs, also confers critical protection to the cardiovascular system by its pleiotropic effects on cell signaling. E2-mediated outcomes include several important physiological adaptive responses including anti-atherogenic actions, vasodilation and preservation of vascular integrity but, also, a direct activity on cardiomyocytes: the inhibition of apoptosis. This beneficial effect of E2 is apparently due to its antioxidant activity [5]. In fact, generation of reactive oxygen species (ROS) represents a key factor leading to cardiomyocyte apoptosis, e.g. following coronary reperfusion, and the ability of E2/ERs to counteract such redox intermediates is likely to be a key component of the overall protection. In this study, we investigated the cell surface expression of mER in cultured cardiomyocytes in physiological conditions and after hydrogen peroxide exposure, i.e. mimicking the acute oxidative stress occurring in coronary ischemia-reperfusion injury.

Mouse atrial HL-1 cardiomyocytes were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Euroclone, Pero, Milan, Italy), 2 mM L-glutamine (Life Technologies, Milan, Italy), 100 U/ml penicillin and 100 mg/ml streptomycin (Life Technologies), on fibronectin-gelatin coated plates, in a humidified air 5% CO2 chamber at 37 °C. To induce oxidative stress, cells were treated with 50–100 μM H_2O_2 (Sigma-Aldrich, St. Louis, MO) for 30 min. Cells were incubated with membrane-non-permeant E2BSA (molar ratio E2:BSA = 30:1; Sigma) or BSA alone as control, at a concentration of 10 nM (physiological concentration) for 30 min. Cell surface expression of ER was analyzed by flow cytometry after cell staining for 30 min at 4 °C with the following antibodies (Abs): rabbit anti-human ERα MC-20 polyclonal Abs; mouse anti-human ERβ 1531 mAb (Santa Cruz, CA, USA) 1 μg per sample (1×10^6 cells). Equal amounts of appropriate isotype controls (Santa Cruz) were used as negative controls. The primary Abs were visualized by fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated F(ab′)2 fragment secondary Abs (Abcam, Cambridge, UK). ERK phosphorylation was analyzed by flow cytometry and western blot. For the first, cells were fixed with 1.5% formaldehyde followed by permeabilization in methanol and stained with anti-p-ERK1/2 mAb (Cell Signaling). For the second, cell lysates were prepared as previously described [6]. After incubation with peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA, USA), membranes were developed by using SuperSignal West Pico chemiluminescent Substrate (Pierce, Rockford, IL). The membranes were re-probed with anti-β-actin mAb (Amersham, Gent, Belgium). Proteins were quantified by densitometry analysis of the autoradiograms (GS-700 Imaging Densitometer, Bio-Rad).

Cardiomyocytes showed a basal low expression of mERα (Fig. 1), whereas ERβ was undetectable at the cell surface level (not shown). To note, after oxidative stress mERα expression significantly increased and this effect was even more evident after treatment with high concentrations of H_2O_2 (100 mM) (Fig. 1). Furthermore, these surface receptors appeared as functional. In fact, we investigated the effects of a membrane non-permeant form of E2 (E2BSA) and, as a control, of BSA alone, on the activation of a key molecule involved in protein kinase cascades regulating cell homeostasis: ERK/MAPK. We found that E2BSA, activating cardiomyocyte surface-expressed mERα, induced a significant increase of ERK phosphorylation in a dose dependent manner, while BSA did not exert any effect (Fig. 2).

In conclusion, in this study we observed that under oxidative stress, cardiomyocytes can up-regulate mERα expression and that these membrane receptors are capable of triggering critical survival signals into the cell. This could be relevant in the maintenance of cell homeostasis. In fact, mERα activation has been hypothesized to be associated with cell apoptosis hindering [6]. Furthermore, it seems conceivable that the mERα up-regulation, detectable under oxidative stress, could also provide the cell with a mechanism able to counteract subcellular modifications triggered in these conditions, e.g. triggering autophagic cytoprotection [7]. In conclusion, our results highlight the importance of mERα (and estrogens) in regulating the coordinated framework of prompt defense strategies aimed at counteracting oxidative stress, such that induced in vivo by coronary ischemia-reperfusion injury, thus contributing to determine cardiomyocyte survival.

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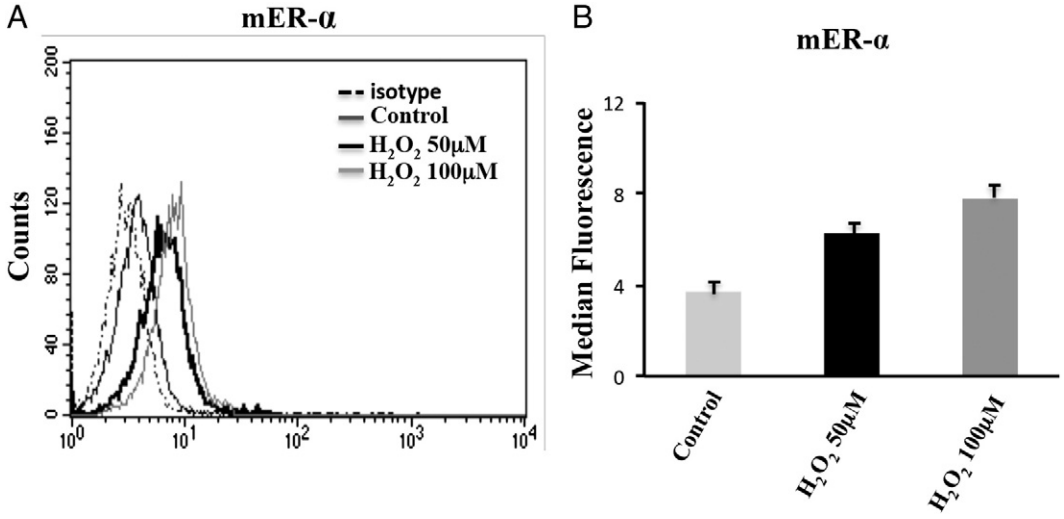

Fig. 1. Flow cytometry analysis of surface ERα expression in cardiomyocytes. Representative flow cytometry histogram plots show the fluorescence intensity of MC20 Abs for mERα compared with isotype controls in: i) untreated, ii) H_2O_2 50 mM, and iii) H_2O_2 100 mM treated cardiomyocytes. Dotted lines indicate isotype control and solid lines indicate anti-ERα Abs staining. (B) Values of mERα/isotype control median fluorescence intensity ratio are reported. The mean ± SD from 5 independent experiments is shown. Control = untreated cells.

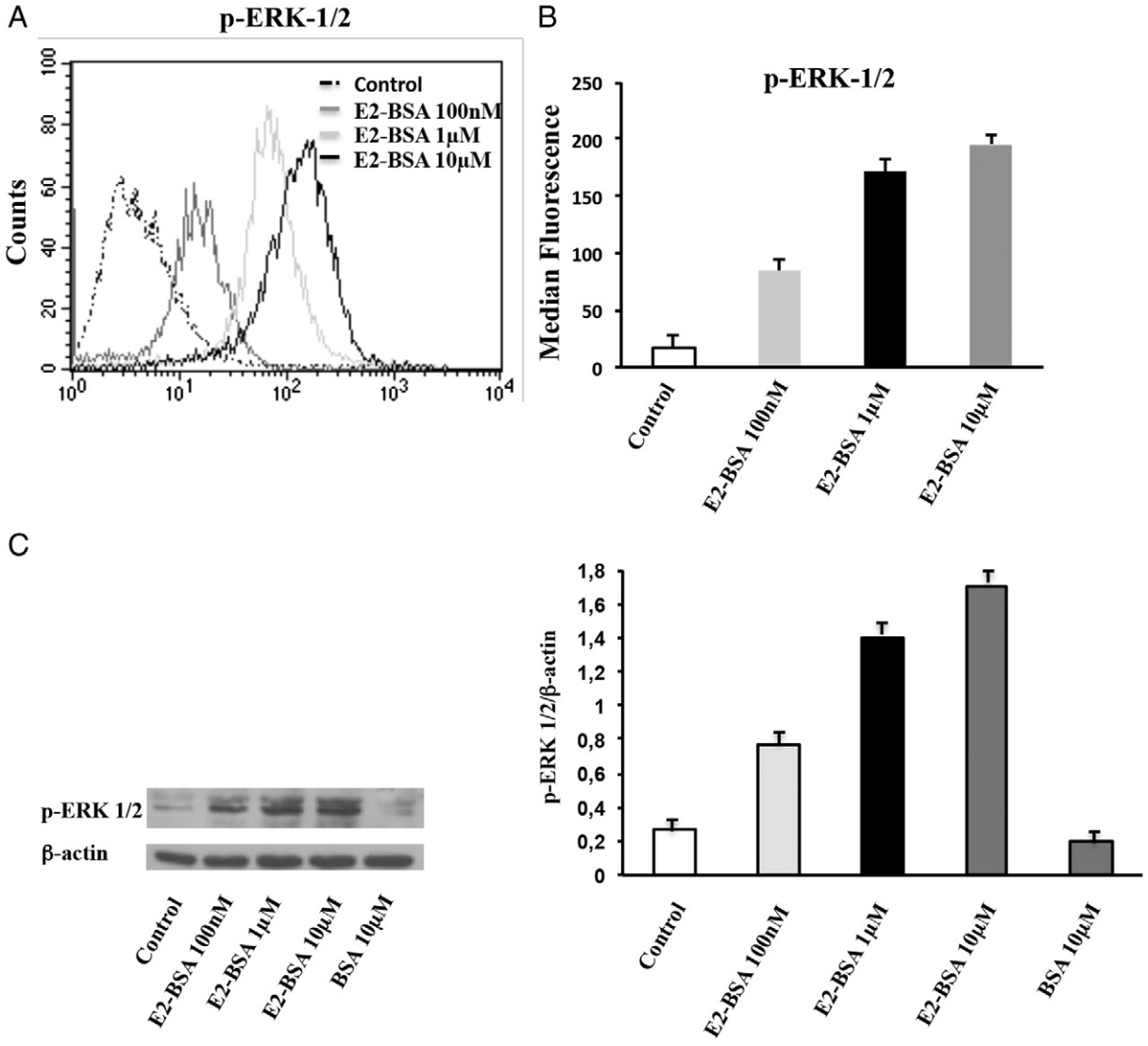

Fig. 2. Effect of E2-BSA on the activation of ERK/MAPK. (A) Representative flow cytometry histogram plots show the fluorescence intensity of p-ERK1/2 compared with isotype controls in untreated and E2-BSA treated cardiomyocytes. Dotted lines indicate isotype control and solid lines indicate anti-p-ERK1/2 Abs staining. (B) Values of p-ERK1/2/isotype control median fluorescence intensity ratio are reported. The mean ± SD from 5 independent experiments is shown. (C) Western blot analysis of p-ERK1/2 in untreated and E2-BSA treated cardiomyocytes. Data from one representative experiment out of 5 are shown (left panel). Densitometric analysis of p-ERK1/2 levels relative to β-actin is also shown (right panel). Control = untreated cells.

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Elena Ortona
Department of Cell Biology, Istituto Superiore di Sanità, Rome, Italy
San Raffaele Institute Sulmona, L'Aquila, Italy

Lucrezia Gambardella
Department of Therapeutic Research and Medicine Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy

Cristiana Barbati
Department of Cell Biology, Istituto Superiore di Sanità, Rome, Italy

Walter Malorni*
San Raffaele Institute Sulmona, L'Aquila, Italy
Department of Therapeutic Research and Medicine Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
E-mail address: malorni@iss.it.
*Corresponding author at: Department of Therapeutic Research and Medicine Evaluation, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy.

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