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Both estrogen receptor subtypes, ERα and ERβ, prevent aldosterone-induced oxidative stress in VSMC via increased NADPH bioavailability

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ABSTRACT

Activation of vascular mineralocorticoid (MR) or estrogen receptors (ER) exerts opposing effects on vascular remodeling. As we have previously shown, activation of either estrogen receptor subtype, ERα or ERβ, is fully sufficient to attenuate vascular remodeling in aldosterone salt-treated rats. To further elucidate the underlying mechanism(s) we tested the hypothesis that ER and MR activation might differentially modulate vascular reactive oxygen species (ROS) generation. In support of this concept, aldosterone increased ROS generation in vascular smooth muscle cells as determined by quantitative dihydroethidium fluorescence microscopy. Co-treatment with the selective ERα agonist 16α-LE2, the selective ERβ agonist 8β-VE2 or the non-selective ER agonist 17β-estradiol (E2) significantly reduced aldosterone-induced ROS generation. The pure ER antagonist ICI 182,780 completely blocked these salutary effects of E2, 16α-LE2 and 8β-VE2. Activation of ERα or ERβ fully blocked the reduction of intracellular nicotinamide adenine dinucleotide phosphate (NADPH) levels observed in aldosterone treated vascular smooth muscle cells. Intracellular NADPH levels were closely associated with expression and activity of the NADPH generating enzyme glucose-6-phosphate dehydrogenase. In conclusion, estrogens attenuate the detrimental vascular effects of excessive MR activation at least in part by preventing the depletion of intracellular NADPH levels.

1. Introduction

The mineralocorticoid aldosterone (ALDO) regulates electrolyte and volume homeostasis via ligand-dependent activation of the mineralocorticoid receptor (MR) [1]. However, excessive or disproportional MR activation in vascular smooth muscle cells (VSMC) causes elevated oxidative stress, increased NADPH oxidase activity and decreased nitric oxide (NO) bioavailability which together promote adverse vascular remodeling and dysfunction [2–4].

Reactive oxygen species (ROS) are by-products of mitochondrial energy metabolism, NADPH and xanthine oxidase activity as well as uncoupled endothelial NO synthase activity. ROS also originate from host defense mechanisms against infectious agents [5–7]. Excessive oxidative stress shifts the delicate balance between ROS generation and ROS elimination towards predominant ROS generation [5]. Intracellular ROS are eliminated by superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) [5,8,9]. Of note, the activity of these enzymes critically depends on NADPH bioavailability and impaired bioavailability of NADPH due to decreased glucose-6-phosphate dehydrogenase (G6PDH) expression and/or activity is thought to limit the activity of these enzymes [10].

In analogy to the MR, estrogen receptors (ER) also act as ligand-activated transcription factors [11,12]. But in contrast to ALDO, estrogens confer largely beneficial cardiovascular effects via two distinct intracellular ER subtypes, termed ERα and ERβ. The cardio- and vasoprotective effects of estrogens in part result from enhanced NO bioavailability and hence improved NO-dependent vasodilatation [12,13]. Furthermore, estrogens confer antioxidative effects in the cardiovascular system [14,15].

Recently, we reported that selective activation of either ER subtypes protects female rats against ALDO-induced hypertension, cardiac hypertrophy and adverse vascular remodeling [16]. We have now addressed the question whether the opposing effects of ALDO and estrogens on vascular remodeling might converge on vascular ROS generation as a common cellular target. Specifically, we hypothesized that ER and MR agonists might regulate intracellular ROS levels in opposite directions by reducing (MR) or enhancing (ER) antioxidant capacity in VSMC.

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2. Materials and methods

2.1. Cell culture

VSMC were isolated by enzymatic digestion of aortic segments from female Wistar-Kyoto rats (6–10 weeks; Harlan-Winkelmann, Germany). In brief, rat aortas were quickly excised and placed in 0.9% sodium chloride. After removing the adventitia and scraping off the endothelial cell layer, proximal aortic sections were cut into segments and washed 3 times in PBS containing 2.0 U/ml penicillin, 0.5 mg/ml streptomycin (Sigma–Aldrich, Germany) and 0.5 μg/ml fungizone (Gibco, Germany). Aortic segments were digested in a solution containing 920 U/ml collagenase type II (Worthington, USA), 20 mg/ml BSA (Sigma–Aldrich), 20 U/ml penicillin, 20 μg/ml streptomycin and 0.5 μg/ml fungizone and gently shaken at a rate of 50 cycles/min for 30 min. The supernatant was transferred into fetal bovine serum (FBS, Biochrom AG, Germany) and centrifuged for 10 min at 1000 rpm. The cell pellet was resuspended in Medium 199 with Earle's Salts (Invitrogen, Germany) supplemented with 100 U/ml penicillin, 2.5 mg/ml streptomycin, 2.5 μg/ml fungizone and 10% FBS. Cells were grown in a 5% CO_2 atmosphere at 37 °C and medium was changed every second day. Experiments were performed with VSMC from passages 5–12.

2.2. Hormone treatment of VSMC

Rat VSMC were grown to semi-confluence in Medium 199 with Earle's Salts supplemented with 100 U/ml penicillin, 2.5 mg/ml streptomycin and 10% FBS free of steroid hormones (c.c. Pro GmbH, Germany). Subsequently, cells were treated with ALDO (100 nM; Acros Organics, Belgium), ALDO + E2 (100 nM each; E2; Sigma–Aldrich), ALDO + 16α-LE2 (100 nM each; selective ERα agonist; Bayer Pharma AG, Germany), ALDO + 8β-VE2 (100 nM each; selective ERβ agonist; Bayer Pharma AG) or vehicle for 48 h. Hormones were dissolved stepwise; first in dimethylsulfoxide (5 mg/ml), followed by 2-Hydroxypropyl-β-cyclodextrin (200 μM; Sigma–Aldrich) and finally in PBS (10 μM). In selected experiments, cells were co-incubated with either the pure estrogen receptor antagonist ICI 182,780 (100 μM; Sigma–Aldrich) or NADPH (100 μM; Sigma–Aldrich).

2.3. Measurement of intracellular ROS generation

Intracellular ROS generation in VSMC was evaluated by dihydroethidium (DHE) fluorescence. DHE, a cell permeable dye that reacts with superoxide ($O_2^{\cdot-}$) to yield ethidium, binds to nuclear DNA and generates a bright red nuclear fluorescence signal. The fluorescence intensity of DHE indicates the relative levels of $O_2^{\cdot-}$ production [17]. In brief, monolayers of VSMC were grown on chamber slides and treated for 48 h with hormones or vehicle. Subsequently, the cells were incubated in the presence of DHE (100 μM, Molecular Probes, Germany) in a light-protected humidified chamber (37 °C, 30 min) and washed three times with PBS before fixation with 4% paraformaldehyde and co-staining with DAPI to visualize cell nuclei. After an additional PBS washing, VSMC were mounted in VectaShield mounting medium (Vector Laboratories, USA); imaging was performed on a fluorescence imaging system (Keyence/Biozero, Germany). Special care was taken to ensure identical visualization parameters such as exposure time, contrast and brightness for all treatment groups. DHE staining was quantified by analyzing the pixel density of 200–400 cells per group in each individual experiment ($n = 4$/group; $n = 3$/group in experiments with ICI) using the BZ Analyzer Software (Keyence/Biozero). Mean fluorescence intensity (MFI) of ethidium in each image was normalized to MFI of DAPI staining. $O_2^{\cdot-}$ generation was quantified as fold change in MFI versus vehicle group [18].

2.4. Intracellular NADP and NADPH content

The intracellular redox state of VSMC was analyzed spectrophotometrically by quantification of total NADP ($NADP^+$ + NADPH) and NADPH content using a commercial kit (NADP+/NADPH Quantification Assay Kit; BioVision, USA) according to the manufacturer's instructions. Unfractionated cell lysates were prepared from hormone and vehicle treated cells. Protein content of each sample was adjusted to 500 μg/ml to ensure all measurements were performed within the linear range of the NADPH standard curve. The assay specifically quantified NADPH by an enzyme cycling reaction in which $NADP^+$ reacts to NADPH. To selectively quantify NADPH content, samples were heated to 60 °C for 30 min to eliminate $NADP^+$. Intracellular NADP and NADPH levels are expressed as ng/mg protein.

2.5. Protein expression

Hormone and vehicle treated VSMC were homogenized in RIPA buffer containing protease inhibitors (1:25; Roche). Crude protein lysates were analyzed by standard Western blotting techniques. VSMC protein extracts were separated on 10% SDS–PAGE and electrophoretically transferred onto nitrocellulose membranes. Immunoblotting was performed using anti-G6PDH antibody (Sigma–Aldrich, 1:2000 rabbit polyclonal); GAPDH (Chemicon, Germany, 1:5000 mouse monoclonal) served as a loading control. Immunoreactive proteins were visualized by HRP-coupled secondary antibodies and the enhanced chemiluminescence kit (GE Healthcare, Sweden). The ImageQuant software (Scan Pack 3.0; Biometra) was used for densitometric analysis based on peak area.

2.6. G6PDH activity assay

G6PDH catalyzes the conversion of glucose-6-phosphate and $NADP^+$ to 6-phosphogluconolactone and NADPH. G6PDH activity was measured using a specific kit according to the manufacturer's instructions (Worthington Biochemical Corporation). In brief, crude cell lysates of hormone and vehicle treated VSMC were resuspended in 5 mM glycine buffer, pH 8.0 and the protein content for each sample was adjusted to 4 mg/ml. Ten micro liters of samples were added in duplicate to 96-well plates containing 270 μl 55 mM Tris–HCl buffer, pH 7.8 with 3.3 mM $MgCl_2$, 10 μl 0.1 M D-glucose-6-phosphate and 10 μl 6 mM $NADP^+$ (Sigma–Aldrich) at 30 °C. Enzyme activity of G6PDH was measured as the increase of absorbance at 340 nm due to conversion of $NADP^+$ to NADPH. Measurements were performed on a Dynex MRX absorbance reader over a time period of 15 min to obtain ΔA_{340nm}/min. To ensure assay specificity for G6PDH activity, the absorbance of each sample was measured again in the presence of the G6PDH inhibitor 1-fluoro-2,4-dinitrobenzene (100 mM, Sigma–Aldrich) [19]. Data are expressed in mU/mg protein.

2.7. Statistical analysis

Results are presented as mean ± SEM. Multigroup comparisons were performed by one-way analysis of variance followed by Student–Newman–Keuls post hoc all pair wise testing using SigmaStat 2.03 software. p-values < 0.05 were considered as significant.

3. Results

3.1. Intracellular ROS generation in rat VSMC

Intracellular ROS content assessed by quantitative DHE fluorescent microscopy was significantly higher in ALDO compared to

vehicle treated VSMC ($p < 0.05$; Fig. 1A and B). Co-treatment with E2 ($p < 0.05$ vs. ALDO), 16α-LE2 ($p < 0.05$ vs. ALDO) or 8β-VE2 ($p < 0.05$ vs. ALDO) significantly blocked ALDO-induced ROS generation. In line with an ER mediated mechanism, the pure ER antagonist ICI 182,780 fully blocked the reduction of ROS content that was observed upon co-treatment with E2 ($p < 0.05$), 16α-LE2 ($p < 0.05$) and 8β-VE2 ($p < 0.05$) as shown in Fig. 2A and B.

3.2. Intracellular NADP and NADPH levels

Total intracellular NADP content (reduced plus oxidized NADP) tended to be lower in ALDO compared to vehicle treated VSMC (Fig. 3A). Co-treatment with E2 (481 ± 68 ng/mg; $n = 5$, $p < 0.05$), 16α-LE2 (678 ± 106 ng/mg; $n = 5$, $p < 0.05$) or 8β-VE2 (688 ± 75 ng/mg; $n = 5$, $p < 0.05$) significantly increased intracellular NADP content in ALDO treated VSMC. Intracellular NADPH levels which were lower in ALDO compared to vehicle treated VSMC (78 ± 19 $n = 5$ vs. 214 ± 24 ng/mg, $n = 3$, $p < 0.05$; Fig. 3B) increased significantly upon co-treatment with E2 (184 ± 28 ng/mg, $n = 5$, $p < 0.05$), 16α-LE2 (246 ± 28 ng/mg, $n = 5$, $p < 0.05$) and 8β-VE2 (238 ± 54 ng/mg, $n = 4$, $p < 0.05$).

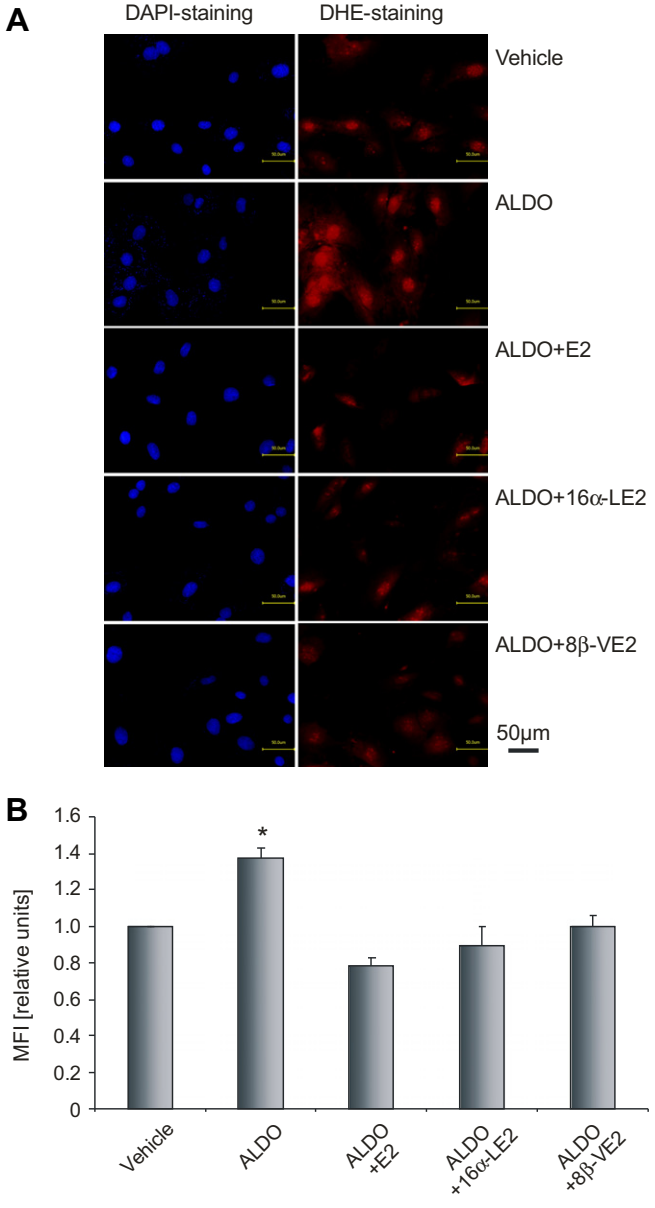

Fig. 1. Simultaneous and selective activation of ERα and ERβ estrogen receptors blocks ALDO-induced ROS generation in VSMC. Representative DHE stainings of VSMC (A) and quantification of nuclear ethidium staining (B) illustrate increased intracellular ROS generation in ALDO compared to vehicle treated VSMC and to cells co-treated with ALDO and E2, 16α-LE2 or 8β-VE2. The bar graph (B) illustrates mean fluorescent intensity (MFI) in relative units ± SEM. $n = 4$ independent experiments/group, $^{*}p < 0.05$.

3.3. NADPH supplementation

Increased DHE fluorescence intensity in cultured VSMC treated with ALDO compared to vehicle was completely blocked by direct supplementation of cultured VSMC with NADPH as shown in Fig. 3C and D (0.84 ± 0.05 vs. 1.26 ± 0.08 MFI; $n = 4$, $p < 0.05$).

3.4. G6PDH protein expression levels and activity

G6PDH protein expression tended to be lower in ALDO compared to vehicle treated VSMC as shown in Fig. 4A. Co-treatment with E2, 16α-LE2 or 8β-VE2 alleviated the tendency towards decreased G6PDH expression in ALDO treated VSMC without reaching full statistical significance. G6PDH enzyme activity (Fig. 4B) was significantly lower in ALDO compared to vehicle treated VSMC (26.9 ± 1.8 vs. 32.6 ± 0.6 mU/mg, $n = 7$, $p < 0.05$). G6PDH activity was significantly higher and not different from baseline in VSMC co-treated with ALDO plus either E2 (32.8 ± 0.8 mU/mg $n = 7$, $p < 0.05$ vs. ALDO), 16α-LE2 (30.9 ± 0.9 mU/mg $n = 7$, $p < 0.05$ vs. ALDO) or 8β-VE2 (32.3 ± 1.1 mU/mg $n = 7$, $p < 0.05$ vs. ALDO).

4. Discussion

The present study shows for the first time that both ER subtypes, ERα and ERβ, antagonize ALDO-dependent ROS generation in VSMC not by reversing NADPH oxidase activation (supplementary data) but by maintaining G6PDH enzyme activity and cellular NADPH levels in ALDO treated VSMC. These findings indicate that the opposing effects of ALDO and estrogens on ROS generation in VSMC converge on G6PDH activity as a common cellular target.

Epidemiological studies have shown that premenopausal women carry a lower risk for cardiovascular disease compared to age matched men. However, these protective effects are lost after menopause which is associated with a decline of endogenous estrogen levels [12,20]. The protective cardiovascular functions of estrogens are partially mediated by effects on the blood vessel wall including enhancement of NO bioavailability, a decrease of total serum cholesterol and a decrease of plasma fibrinogen concentrations [12,13]. Furthermore, estrogens possess known antioxidative effects in cardiovascular tissues [12,13,21] as shown by Dantas et al. who observed a reduction of $O_2^{\cdot -}$ generation in estrogen treated female spontaneously hypertensive rats [22]. In addition, Wing et al. demonstrated that E2 attenuates the development of atherosclerotic lesion in apoE-deficient mice via decreased aortic ROS production [23].

In contrast to estrogens, excessive MR activity confers largely unfavorable effects on the cardiovascular system including hypertension and adverse cardiac and vascular remodeling which arise at least in part from increased oxidative stress and decreased NO bioavailability [24,25]. Mechanistically, excessive MR activation enhances the expression and the activity of vascular and cardiac NADPH oxidase which causes increased ROS production and oxidative stress [25,26]. The NADPH oxidase subunit $p67^{phox}$ appears to play an important role in these processes since Pechánová et al. observed an up-regulation of $p67^{phox}$ in the left ventricle of spontaneous hypertensive rats and a reduction of cardiac ROS generation

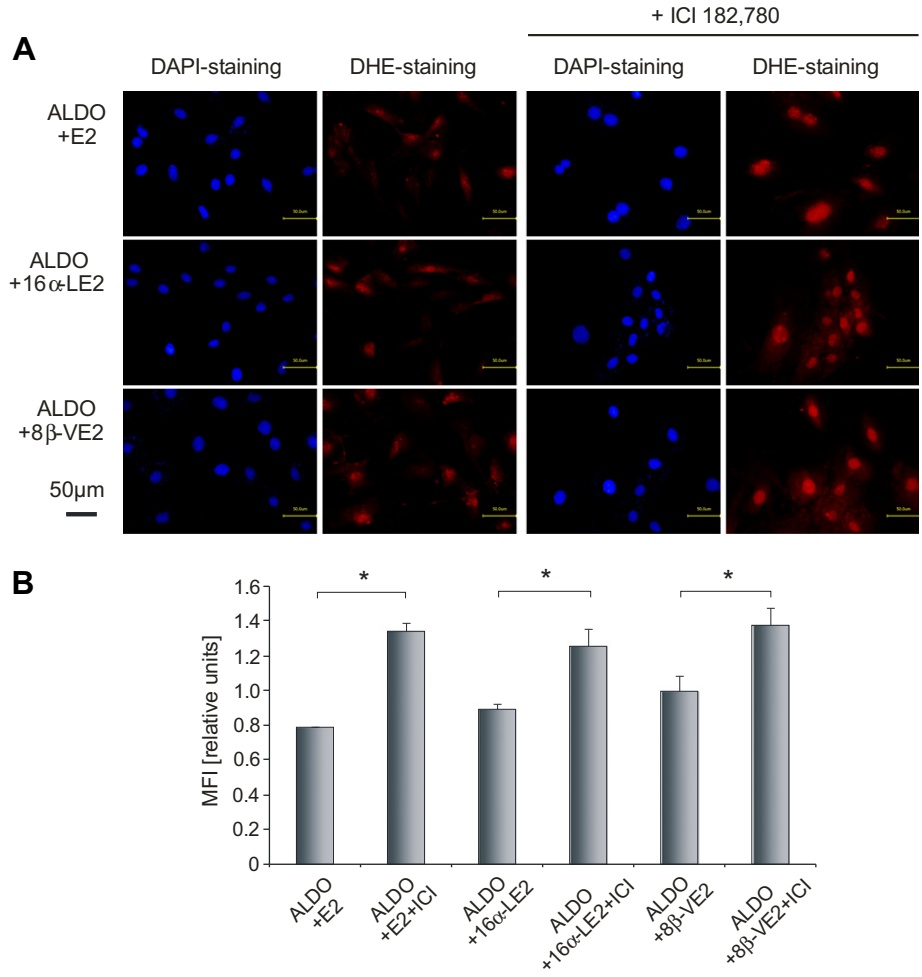

Fig. 2. ERα and ERβ estrogen receptors attenuate ROS generation in VSMC via ER-dependent mechanism(s). Representative images of VSMC stained with the fluorescent dye DHE (A) and quantification of DHE fluorescence intensity (B) illustrate that the addition of the pure ER antagonist ICI182,780 (ICI) fully prevented the decrease of ROS generation that was observed in VSMC co-treated with ALDO and different estrogen receptor agonists. Values indicate mean fluorescent intensity (MFI in relative units) ± SEM. $n = 3$ independent experiments/group, $^{*}p < 0.05$.

and elevated blood pressure upon pharmacological inhibition of NADPH oxidase [27].

In agreement with these findings, increased ROS generation in ALDO treated VSMC was associated with an enhanced $p67^{phox}$ expression and an up-regulation of NADPH oxidase activity. Although it appears conceivable that increased vascular ROS generation might have contributed to vascular fibrosis in our previous study [16], we could not confirm the concept that E2 or selective activation of the ERα or ERβ receptor blocked the adverse vascular effects of ALDO on the level of cellular $p67^{phox}$ expression and NADPH oxidase activity, because both parameters remained elevated despite co-treatment of VSMC with ALDO and estrogens (supplementary data). It is also unlikely that differential expression of other NADPH subunits such as $p47^{phox}$ and rac1 might have affected ROS generation in ALDO and estrogen treated VSMC, first because both NADPH oxidase subunits were expressed at comparable levels among all treatment groups (data not shown) and second, because NADPH oxidase activity remained elevated despite estrogen co-treatment.

Thus, it appears likely that increased ROS generation via MR activation and enhanced ROS elimination upon estrogen treatment arise – at least in part – from independent mechanisms. To address this hypothesis, we analyzed the expression and the activity of several key antioxidative enzymes because these might have been increased in response to estrogen treatment. However, comparable protein expression of several key enzymes such as MnSOD and catalase as well as the activity levels of total SOD, GPx and catalase (data not shown), do not support this hypothesis. The current results contrast with studies by Strehlow et al. reporting a reduction of angiotensin II-induced ROS generation in VSMC by E2 via increased activity and expression of ecSOD and MnSOD [21]. The fact that we did clearly not observe such an effect in this study might be dose related because Strehlow et al. used a 10-fold higher concentration of E2 (1 µM). In addition, the authors did not analyze MnSOD and ecSOD expression and activity in VSMC co-treated with E2 and angiotensin II [21]. Taken together, the current observations indicate that the activity of antioxidative enzymes including total SOD, GPx and catalase is fully preserved in ALDO and in estrogen treated VSMC. However, these *in vitro* measurements do not formally rule out a lower activity of these enzymes within the context of a living cell because intracellular levels of important co-factors such as NADPH might be decreased *in vivo* whereas NADPH needs to be provided in excess for *ex vivo* assays of enzyme activity.

To address this issue, we measured intracellular NAPDH levels in ALDO and in estrogen treated VSMC. NADPH plays a key role in numerous and fundamental biological processes by acting as an electron source [28]. NADPH is an essential co-factor for fatty acid, steroid and DNA synthesis and for the activity of cellular antioxidative defense mechanisms [10,28]. In particular, NADPH is

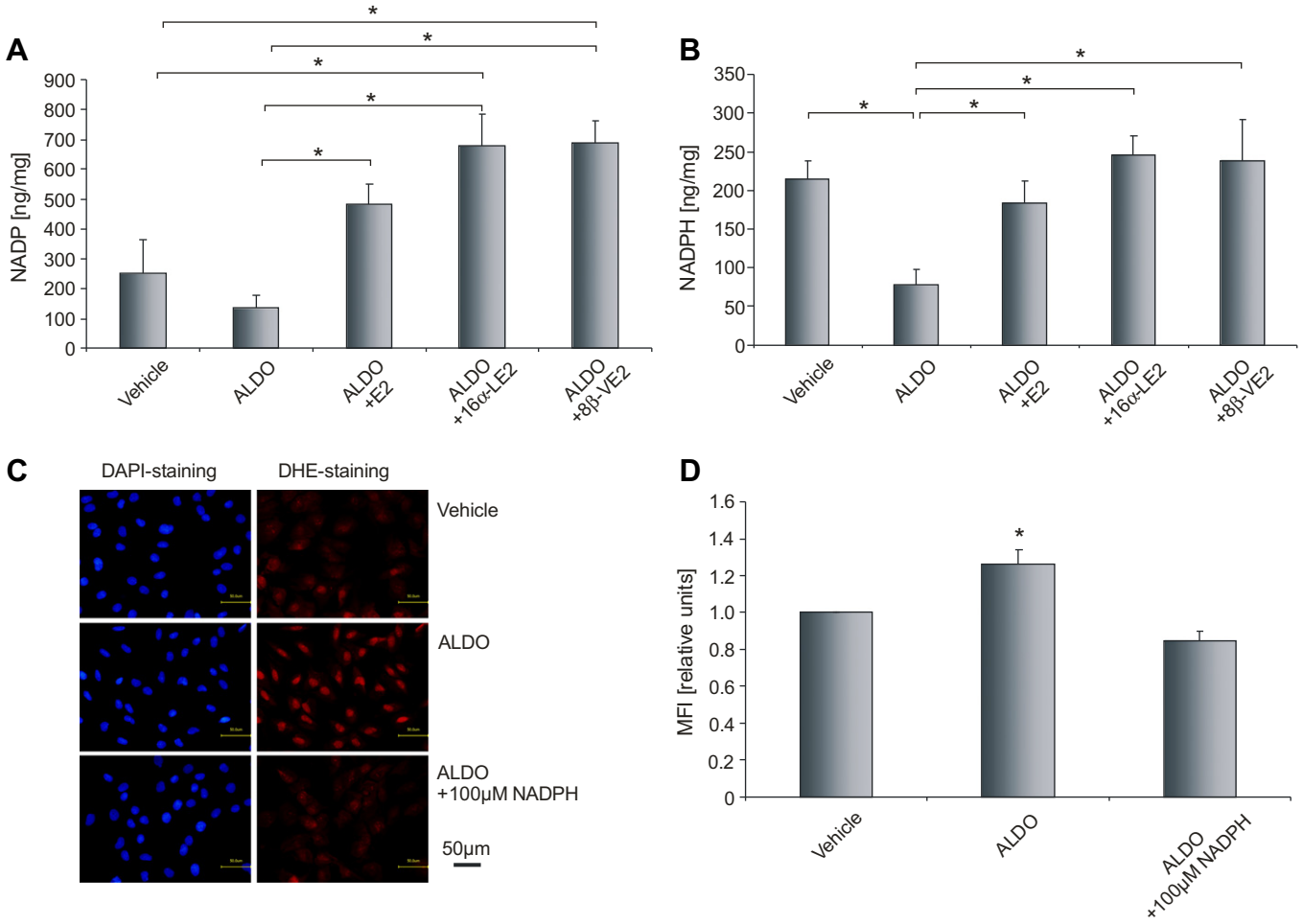

Fig. 3. ERα and ERβ activation augments decreased cellular NADP and NADPH content in ALDO treated VSMC which block ALDO-dependent ROS generation. The bar graphs illustrate decreased total NADP (A) and NADPH (B) content of VSMC treated with ALDO that rose to levels above baseline upon co-treatment with E2, 16α-LE2 or 8β-VE2. Values indicate total NADP (A) and NADPH (B) content in ng/mg protein ± SEM. $n = 5$ experiments/group; $^*p < 0.05$. Representative images of VSMC stained with DHE (C) and quantification of nuclear ethidium staining (D) illustrate increased intracellular ROS generation in ALDO treated VSMC that was fully blocked by supplementation of NADPH. Bar graphs (D) indicate mean fluorescent intensity (MFI in relative units) ± SEM. $n = 4$ independent experiments/group; $^*p < 0.05$.

strictly required to regenerate reduced glutathione (GSH) from oxidized glutathione via glutathione reductase. In turn, GSH is essential for the function of GPx and glutathione S-transferase [29] and the H_2O_2-disposing enzyme catalase – which contains four tightly bound molecules NADPH that provide protection of the enzyme against inactivation [10,30]. Also, thioredoxin, the major ubiquitous disulfide reductase, is reduced to its active form by electrons from NADPH via thioredoxin reductase [10,31]. Lower intracellular NADPH levels in ALDO compared to vehicle treated VSMC indicate a depletion of intracellular reducing equivalents which is likely to impair the activity of antioxidative enzymes and thus to aggravate oxidative stress. Interestingly, activation of both ER subtypes by E2 as well as selective activation of ERα or ERβ more than fully restored intracellular NADPH content to levels even above baseline. This essentially new finding supports the hypothesis that estrogens enhance ROS elimination in ALDO treated VSMC specifically by preserving intracellular NADPH levels as an essential co-factor of ROS eliminating enzymes [10].

This interpretation is further supported by our current observation that direct addition of an excess of NADPH fully prevented ALDO-dependent ROS generation. Although it had been reported that exogenously added nucleotides are generally not taken up by living cells, Lu et al. confirmed at least that the transport of NADH across the plasma membrane is possible via the purinergic receptor P2X7 [32]. Because the P2X7 receptor is expressed in VSMC (supplementary data) it is conceivable that NADPH is transported via this receptor into the cells.

NADPH is generated from $NADP^+$ via $NADP^+$-dependent enzymes including G6PDH, 6-gluconate-phosphate dehydrogenase, isocitrate dehydrogenases and malic enzymes [10]. To elucidate the mechanism(s) by which ERα and ERβ activation preserved NADPH levels in ALDO treated VSMC we examined G6PDH expression and activity since G6PDH is not only the first and rate limiting enzyme of the pentose phosphate pathway but also a major source of NADPH [33,34]. Previously, Leopold et al. reported that ROS accumulation and oxidative stress are increased in ALDO treated bovine aortic endothelial cells as a result of decreased G6PDH expression and activity. Interestingly, the authors were able to show that ALDO impaired vascular reactivity via decreasing G6PDH activity *in vivo* [34]. Consistent with their findings, we observed a trend towards decreased G6PDH protein expression in aortic VSMC upon exposure to ALDO that was no longer detected upon co-treatment with estrogens. However, as an essentially new finding, we were able to show that co-treatment with E2 and with selective ER agonists for ERα and ERβ fully prevented the down-regulation of G6PDH enzyme activity in ALDO treated

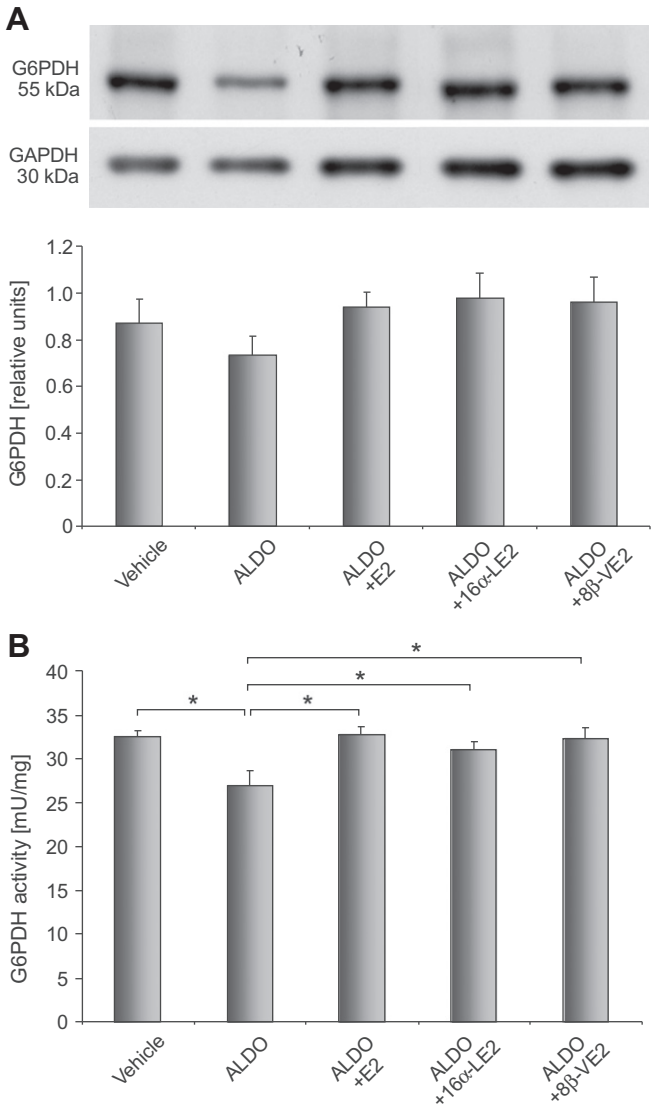

Fig. 4. ERα and ERβ activation restores impaired G6PDH expression and activity in ALDO treated VSMC. The representative Western blot (A) illustrates decreased G6PDH expression in ALDO treated VSMC that returned to baseline levels upon co-treatment with E2, 16α-LE2 or 8β-VE2. Values indicate mean ± SEM (relative units) normalized to GAPDH. $n = 7$ independent experiments/group. Panel (B) illustrates decreased G6PDH activity in ALDO treated VSMC that returned to baseline upon co-treatment with E2, 16α-LE2 or 8β-VE2. Values indicate mean ± SEM (mU/mg protein) $n = 7$ independent experiments/group, $^{*}p < 0.05$.

VSMC. Because G6PDH acts as a major source of NADPH it appears conceivable that preservation of G6PDH activity by ERα and ERβ activation might have contributed to maintain physiological NADPH levels in ALDO treated VSMC. This implication is affirmed by analyzing expression and activity of other NADPH-generating enzymes, namely isocitrat dehydrogenase (supplementary data) and malic enzyme (data not shown), which were not altered by hormone treatment.

In summary, the present study shows for the first time that ligand-dependent activation of ERα and of ERβ prevents VSMC against ALDO-induced intracellular ROS generation not via reversal of increased $p67^{phox}$ expression and NADPH oxidase activity, but by preserving the intracellular NADPH pool that is essential for the activity of ROS eliminating enzymes. G6PDH appears as a common target of ER and MR activity that is regulated in opposite directions by estrogens and by mineralocorticoids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.053.

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