Inhibition of XO or NOX attenuates diethylstilbestrol-induced endothelial nitric oxide deficiency without affecting its effects on LNCaP cell invasion and apoptosis

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Abstract

Oestrogen protects cardiovascular health partially via an up-regulation of $NO^\bullet$ (NO radical) production. Its synthetic analogue DES (diethylstilbestrol), used as a potent androgen deprivation therapy for patients with prostate cancer, is however associated with high incidence of thromboembolic events. Exposure of BAECs (bovine aortic endothelial cells) to pharmacologically relevant dosage (12.5 μmol/l, 24 h) of DES resulted in a marked reduction in endothelial $NO^\bullet$ bioavailability determined by ESR (electron spin resonance), while 17β-oestradiol instead increased $NO^\bullet$ production as expected. Intriguingly, endothelial $O_2^{\bullet-}$ (superoxide anion) production was up-regulated by DES *in vitro* and *in vivo*, which was, however, attenuated by the ER (oestrogen receptor) antagonist ICI 182780, the XO (xanthine oxidase) inhibitor oxypurinol or the NOX (NADPH oxidase) inhibitor NSC23766. These agents also restored $NO^\bullet$ production. DES alone in a cell-free system did not produce any ESR-sound $O_2^{\bullet-}$ signal. Of note, eNOS (endothelial NO synthase) mRNA and protein remained unchanged in response to DES. These results suggest that receptor-dependent activation of XO or NOX, and subsequent production of $O_2^{\bullet-}$, mediate DES-induced $NO^\bullet$ deficiency. This could represent a previously unrecognized mechanism that is responsible for cardiovascular complications of DES administration. Importantly, DES-induced suppression of LNCaP cell invasion and apoptosis were not affected by XO or NOX inhibitor. Therefore combinatorial therapy of DES and XO/NOX inhibitor may prove to be an innovative and useful therapeutic option in eliminating cardiovascular complications of DES, while preserving its anti-cancer effects, benefiting patients with advanced cancer who do not respond well to any other treatments but DES.

Keywords

combinatorial therapy; diethylstilbestrol (DES); LNCaP cell; NADPH oxidase (NOX); nitric oxide; reactive oxygen species; superoxide anion; xanthine oxidase (XO)

INTRODUCTION

In postmenopausal women, the incidence of CVD (cardiovascular disease) is much higher than in premenopausal women, implicating a potential impact of oestrogen in protecting

cardiovascular health. Even though oestrogen therapy in treating CVD remains controversial [1], 17β-oestradiol supplementation to postmenopausal women has been shown to have beneficial effects on vascular function [2–4]. These include improvement in $NO^\bullet$ (NO radical)-dependent endothelium-dependent vasodilation as a result of eNOS (endothelial nitric oxide synthase) activation and/or up-regulation of its protein expression [5,6]. Previous findings demonstrated that oestrogen-induced vascularization in response to injury was impaired in eNOS-knockout mice, further implicating a role of eNOS in oestrogen-dependent vascular protection [7]. On the other hand, Wagner et al. [8] and Miller et al. [9] have shown that 17β-oestradiol inhibits expression of NOX (NADPH oxidase) to reduce endothelial production of $O_2^{\bullet-}$ (superoxide anion). Oestrogen has also been shown to inhibit Rac 1 activation, which is required for NOX activation [10,11]. H_2O_2-induced endothelial cell apoptosis in rats was found attenuated by oestrogen [12]. Taken together, these data suggest that 17β-oestradiol is vascular protective via activation of the $NO^\bullet$ pathway and attenuation of ROS (reactive oxygen species) signalling.

DES (diethylstilbestrol), on the other hand, is a synthetic non-steroid oestrogen. DES has been used as a highly effective agent for androgen deprivation therapy in patients with prostate cancer [13], particularly in those who do not respond to other treatments, or those who are castrate-resistant. It is, however, no longer used as a hormone replacement in postmenopausal women, at least in the U.S.A., due to its carcinogenic properties in pregnant women and their daughters (DES was prescribed to pregnant women to stabilize pregnancy). Nonetheless, it is still actively used worldwide, though to a less extent due to the worries from the findings in females, and the cardiovascular side effects. DES not only inhibits DNA synthesis and RNA polymerase in tumour tissues of the prostate but also blocks testosterone production through suppression of LH (luteinizing hormone) secretion, all of which make it highly effective in treating prostate cancer. DES has been reported to induce cytotoxicity and inhibit cell proliferation by cell-cycle arrest in androgen-dependent prostate LNCaP cells [14,15], and induce apoptosis in androgen-independent prostate cancer [16]. A study on gene expression profiling following DES treatment of androgen-dependent prostate LNCaP cells and PC-3 cells showed that expression of many genes responsible for invasion, proliferation and survival were down-regulated more than 5-fold [17], indicating the extensive signalling impact of DES on prostate cancer cells. More importantly, DES is often the last solution when patients are not responding to other alternative treatments [18]. However, DES at the dosage of 5 mg/day has exerted modest to severe thromboembolic cardiovascular events such as myocardial infarction [19]. Although lower doses of DES have been found to be effective for prostate cancer with fewer side effects, thromboembolic toxicity remained [19]. It has been suggested that DES-induced hypercoagulability is associated with different mechanisms including increased coagulation factors, diminished levels of anticoagulators, decreased fibrinolysis and altered platelet function [20]. Due to therapeutic advantages of DES including its low cost and efficacy, combinatorial therapy with other preventive drugs, which may reduce cardiovascular side effects, has received great attention. Indeed, warfarin sodium or aspirin, anti-coagulators, have been tested but shown to be ineffective in preventing cardiovascular complications [19,21], and these treatments are associated with high risk of bleeding.

In the present study, we investigated whether and how DES induces endothelial $NO^\bullet$ deficiency, which could be pro-thrombotic. Of note, $NO^\bullet$ attenuates expression of pro-thrombotic proteins such as PAI-1 (plasminogen-activator inhibitor-1) [22], and up-regulates antithrombotic proteins such as thrombomodulin [23]. Interestingly, we found that DES is able to activate endothelial NOX or XO (xanthine oxidase), leading to increased production of $O_2^{\bullet-}$, which could in turn inactivate $NO^\bullet$ to result in a hypercoagulable state. Combinatorial therapy with DES and NOX/XO inhibitor may therefore prove to be effective in restoring $NO^\bullet$ production, while ideally preserving the anti-cancer effects of DES. Indeed,

we found that the combination of DES with either the NOX inhibitor NSC23766 or the XO inhibitor oxypurinol was highly effective in restoring NO$^\bullet$ production without affecting the reducing effect of DES on the viability of LNCaP cells and PC-3 cells, and the reducing effect of DES on the invasion of LNCaP cells. These effects are specific for prostate cancer cells as these responses were not observed in BAECs (bovine aortic endothelial cells), which served as a control non-cancer cell line. Therefore these findings provide new hope for potentially revitalizing DES for prostate cancer treatment by improving NO$^\bullet$ bioavailability to eliminate cardiovascular side effects, which are often associated with NO$^\bullet$-deficiency related hypercoagulability. It may ultimately benefit a large population of patients with advanced prostate cancer who have to use DES for chemotherapy.

MATERIALS AND METHODS

Materials

DES, oxypurinol and PEG–SOD {PEG [poly(ethylene glycol)]-conjugated SOD (superoxide dismutase)} were purchased from Sigma. NSC23766 and ICI 182780 were purchased from Calbiochem and Tocris Bioscience respectively. DES (7-days release) and 17β-oestradiol tablets were obtained from Innovative Research of America.

Cell culture

BAECs were cultured in M199 containing 10% FBS (fetal bovine serum) and antibiotics as described previously [27]. LNCaP and PC-3 cells were purchased from A.T.C.C., and maintained in RPMI 1640 medium containing 10% FBS. HeLa cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS. Upon confluence, cells were starved with 0.1% FBS-containing medium overnight. Before stimulation with 12.5 μmol/l of DES for 24 h, cells were pre-treated in the presence or absence of 50 μmol/l oxypurinol, 10 μmol/l NSC23766 or 10 μmol/l ICI 182780 for 30 min.

In vivo treatment with DES or 17β-oestradiol

C57BL6 mice were implanted subcutaneously with DES and 17β-oestradiol tablets (controlled even release, 0.5 mg over 7 days). On day 7, freshly harvested aortas were subjected to DHE (dihydroethidium) staining as we described previously [24]. The use of experimental animals and procedures were approved by the Institutional Animal Care and Usage Committee at University of California Los Angeles.

DHE detection of ROS

Endothelial cells were cultured on glass cover slips till confluence, serum deprived and then treated with DES (12.5 μmol/l) for 24 h. Cells were then incubated with fresh DHE solution (2 μmol/l) in the dark for 30 min. After washing with PBS, cells were mounted on glass slides and the fluorescent images were captured using a Zweiss Axioskop inverted fluorescent microscope. Some cells were pre-incubated with PEG–SOD (100 units/ml) for 30 min before DHE incubation. In additional experiments, fresh aortic OCT sections from DES or 17β-oestradiol-treated mice were incubated with DHE for 1 h before analysis of fluorescent images.

ESR (electron spin resonance) detection of endothelial NO$^\bullet$ production

Bioavailable NO$^\bullet$ produced by confluent endothelial cells was detected using ESR as we described previously [27]. In brief, endothelial cells were rinsed with modified Kreb's/Hepes buffer and incubated with freshly prepared NO$^\bullet$-specific spin trap Fe^{2+} (DETC)$_2$ colloid (0.5 mmol/l) for 60 min at 37°C. Gently collected cell suspensions were snap-frozen in liquid nitrogen and loaded into a finger Dewar for analysis with an e-Scan ESR spectrophotometer

(Bruker Biospin) at the following settings: static field 3498.98, field sweep 100, resolution 512, microwave frequency 9.72 GHz, modulation amplitude 9.82, number of X-scan 20, reaction gain 3560.

ESR detection of $O_2^{\bullet-}$ production

Gently collected endothelial cells were suspended in modified Kreb's/Hepes buffer containing deferoxamine (25 μmol/l, metal chelator). Approximately 10^6 cells were mixed with $O_2^{\bullet-}$-specific spin trap CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; 1 mmol/l) in the presence or absence of PEG–SOD (100 units/ml) [25]. The cell mixture loaded into glass capillaries was immediately analysed for $O_2^{\bullet-}$ production kinetically for 10 min. The ESR settings used were centre field, 3475; sweep width, 9G; static field, 3484.981; microwave frequency, 9.75 GHz; microwave power, 21.02 mW; modulation frequency, 86 kHz; modulation amplitude, 2.47 G; resolution in X, 512; and number of x-scans, 10. The SOD-inhibitable $O_2^{\bullet-}$ signals at 10 min time point, normalized by protein concentrations, were compared among different experimental groups.

Amplex Red assay for H_2O_2

Post-stimulation, endothelial cell suspensions were assessed for H_2O_2 production using a fluorometric HRP (horseradish peroxidase) assay (Amplex Red assay; Molecular Probes). Fluorescence was measured at λ_{ex} and λ_{em} of 530 nm and 590 nm respectively, after 1 h incubation at 37°C in the dark. The PEG–CAT (PEG-conjugated catalase; 300 units/ml)-inhibitable fraction, reflective of a specific H_2O_2 signal, was analysed and quantified using a calibration curve.

Northern blotting for eNOS

Total RNA was isolated using TRI reagent. RNA (20 μg) was subjected to gel electrophoresis on 1% agarose gels containing 2.2 mol/l formaldehyde, transferred to nitrocellulose membrane (Optitran), UV immobilized and hybridized with cDNA probe labelled by random primer kit (Stratagene). After hybridization, the membranes were washed twice at 65°C with 1×SSC (0.15 M NaCl/0.015 M sodium citrate) and 0.5% SDS, followed by autoradiography at −80°C.

Western blotting for eNOS

BAECs were lysed in ice-cold Tris buffer (50 mmol/l Tris/HCl, pH 7.4, 2 mmol/l EDTA and 1 mmol/l EGTA) containing 1% Triton X-100, 0.1% SDS, 50 mmol/l NaF, 10 mmol/l $Na_4P_2O_7$, 1 mmol/l Na_3VO_4, 1 mmol/l DTT (dithiothreitol), 1 mmol/l PMSF and 10 μl/ml of the protease inhibitor cocktail (Sigma). Lysate proteins were subjected to SDS/PAGE (7.5% gel) and transferred to NC membrane (Amersham Biosciences). Blots were subjected to immunostaining with anti-eNOS antibody (1:1000 dilution) overnight at 4°C, and subsequent incubation with peroxidase-conjugated secondary antibody for 1 h at room temperature, and proteins were visualized using an enhanced chemiluminescence technique.

Invasion assay

Transwell invasion chambers (8 μm pore size) in 24 well plates (Corning) were used for invasion analyses. Confluent cells were starved with serum-free medium overnight before plating to upper chamber. Transwell was coated with BME (basal membrane extract) overnight. Cells were pre-treated with NSC23766, oxypurinol and ICI 182780 for 30 min before loading to transwell. Cells were loaded into coated transwell at 2.5×10^5 cells/well in serum-free medium. The lower chamber was filled with medium containing 5% FBS in the presence or absence of DES. After incubation at 37°C for 24 h, cells that traversed through the BME-coated membrane were subsequently detached using cell detachment buffer

containing calcein-AM (acetoxymethyl ester) fluorescent dye. The fluorescence was measured in a fluorescence plate reader set at an λ_{ex} of ~485 nm and an λ_{em} of ~520 nm.

Apoptosis detection

Floating or adherent cells were collected after treatment. Apoptosis was assessed with the apo-BrdU (bromodeoxyuridine) TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) Assay kit (Invitrogen), according to manufacturer's instructions. Fragmentation of DNA in apoptotic cells was measured by BrdU incorporation, which was visualized by conjugation to an AlexaFluor® 488 dye-labelled anti-BrdU antibody.

Statistical Analysis

All results are means±S.E.M. from three to eight independent experiments. ANOVA was used to compare means of different experimental groups, and Tukey's multiple test was used as a post-test. A *P* value <0.05 was considered significant.

RESULTS

DES increases endothelial production of ROS

In cultured BAECs, DES (12.5 μmol/l, 24 h) induced a dramatic increase in ROS production (detected by DHE fluorescence), which was attenuated by SOD (Figure 1A). Chronic treatment of C57BL6 mice with subcutaneously implanted DES tablets (controlled release, 0.5 mg over 7 days) also resulted in a striking increase in aortic ROS production. In contrast, the identical treatment of mice with 17β-oestradiol attenuated aortic ROS production.

DES induces NO deficiency

Next, bioavailable NO was measured by ESR in cultured aortic endothelial cells exposed to DES. As shown in Figure 2, DES (12.5 μmol/l, 24 h) induced a marked reduction in NO$^{•}$ bioavailability (0.534-fold of control; *P*<0.001). To investigate whether this response is dependent on ER (oestrogen receptor), endothelial cells were pre-treated with receptor antagonist ICI 182780 before DES stimulation. It turned out that ICI 182780 prevented DES-induced endothelial NO$^{•}$ deficiency (0.914±0.184-fold compared with 0.522±0.04-fold for ICI 182780 compared with DES). Either pre-treatment with the XO inhibitor oxypurinol or the NOX inhibitor NSC23766 significantly alleviated DES-induced endothelial NO$^{•}$ deficiency (0.677±0.044-and 0.683±0.063-fold compared with 0.522±0.04-fold for oxypurinol and NSC23766 compared with DES respectively), indicating that DES induction of NO$^{•}$ deficiency involves ER and activation of XO and NOX.

DES increases superoxide production via NOX and XO

To further confirm whether DES stimulates NOX or XO-dependent $O_2^{•-}$ production that can inactivate NO$^{•}$, we measured $O_2^{•-}$ production from endothelial cells pre-treated with the NOX inhibitor NSC23766 or the XO inhibitor oxypurinol before being exposed to DES stimulation. DES induced a 2-fold increase in $O_2^{•-}$ production in endothelial cells (Figure 3A; *P*<0.05). Either NOX or XO inhibitor abolished DES-induced $O_2^{•-}$ production to near control levels of 1.16- and 1.14-fold respectively. The inhibitors themselves did not affect the $O_2^{•-}$ signal. The spatial distribution of XO and NOX is different within intracellular compartments. NOX is located at the membrane, whereas XO exists in the cytosol. It has been known that $O_2^{•-}$ generated by NOX mediates conversion of xanthine dehydrogenase into XO via oxidation-dependent cleavage of the protein [26].

DES induces superoxide production via ER

The presence of ERα in endothelial cells has been well established. It is known to mediate an oestrogen-induced increase in NO$^{\bullet}$ bioavailability partially via attenuation of ROS. Thus we investigated whether DES increases $O_2^{\bullet-}$ production in a receptor-dependent manner. Intriguingly, the ER antagonist ICI 182780 attenuated DES stimulation of $O_2^{\bullet-}$ production (Figure 3).

DES stimulates endothelial H$_2$O$_2$ production

Endothelial H_2O_2 was detected specifically using an Amplex Red assay. DES treatment led to a 2.4-fold increase in H_2O_2 levels relative to vehicle-treated control cells (Figure 4). Pre-treatment with either NSC23766 or oxypurinol decreased DES-induced H_2O_2 production (1.365 ± 0.226- and 1.495 ± 0.369-fold for NSC23766 and oxypurinol respectively). ER antagonist ICI 182780 also attenuated DES-induced increase in H_2O_2 production (1.342 ± 0.33-fold). These results indicate that DES-induced H_2O_2 production is also dependent on XO and NOX.

DES up-regulates eNOS gene expression

We investigated whether DES treatment affects eNOS gene expression in addition its effects on NO$^{\bullet}$ inactivation via ROS production. In the case of 17β-oestradiol, it up-regulates eNOS expression as well as activating eNOS via receptor-dependent eNOS phosphorylation. As shown by representative Northern and Western blots in Figure 5, DES treatment modestly up-regulates eNOS mRNA and protein expression. However, this may not be beneficial when excessive ROS is produced by DES. eNOS has been shown to become uncoupled to produce $O_2^{\bullet-}$ when its cofactor tetrahydrobiopterin becomes deficient due to oxidation [27,28].

DES invasion of LNCaP cells is not affected by NOX/XO inhibitor

Before proposing the use of combinatorial therapy, it is important to examine whether the anti-cancer effects of DES are preserved when NOX or XO is inhibited. As shown in Figure 6(A), the invasive ability of LNCaP cells was significantly reduced by DES treatment. This inhibitory effect of DES on cell invasion was not altered by pre-treatment with either NSC23766 or oxypurinol. Of note, the effect of DES on invasion was not obvious in PC-3 cells, which are known to be highly metastatic (Figure 6B), there was also no effect on endothelial cells as a non-cancer cell control (Figure 6C). In HeLa cells, DES treatment led to a significant reduction on invasion (Figure 6D). Taken together, these results suggest that combinational therapy of NOX/XO inhibitor and DES is able to preserve the anti-cancer effects of DES.

DES-induced apoptosis of LNCaP and PC-3 cells is not affected by NOX/XO inhibitor

As shown in Figures 7(A) and 7(B), DES induced a significant increase in apoptotic death in both prostate cancer cell line LNCaP and PC-3 cells. These anti-cancer effects were specific for prostate cancer cell lines as there were no effect on endothelial cells (Figure 7C) and non-prostate cancer HeLa cells (Figure 7D). In addition, the apoptosis-inducing effect of DES was not altered by pre-treatment with NSC23766 or oxypurinol. This result is consistent with previous findings that DES-induced ROS does not mediate apoptosis of PC-3 cells [29].

DISCUSSION

The major findings of the present study include: (i) DES induces endothelial NO$^{\bullet}$ deficiency, which is mediated by activation of ROS production; (ii) the source of DES-

induced ROS production appears to be endothelial NOX and XO; and (iii) combinatorial treatment of DES and NOX/XO inhibitor restores NO$^\bullet$ bioavailability without affecting the anti-prostate cancer properties of DES. These results demonstrate that DES-induced cardiovascular side effects may be attributed to oxidative-stress-induced NO$^\bullet$ deficiency and consequent endothelial dysfunction [30].

A growing body of evidence has shown that physiological concentration of oestrogen up-regulates eNOS expression and activation. In addition, it has been reported that oestrogen decreases the generation of ROS and subsequently maintains NO$^\bullet$ bioavailability to protect endothelial function [31,32]. In the present study, however, the oestrogen analogue DES induced a NO$^\bullet$ deficiency, which was not mediated by alteration of eNOS expression, but by an increase in ROS production. We demonstrated further that DES induces ROS production via activation of NOX and XO in endothelial cells. This is interesting as usually one oxidase system is predominantly responsible for agonist-stimulated ROS production. The detailed mechanisms as to cross-talk between the two systems warrant future investigation for a better understanding of vascular oxidant signalling in general.

Of note, DES-induced oxidative stress occurs via ER. Receptor-dependent signalling of DES has been reported in various cells, including endothelial cells, testicular cells and aortic smooth muscle cells [33,34]. In the presence of ICI 182720, DES-induced changes in NO$^\bullet$ and $O_2^{\bullet-}$ levels were reversed. However, another antagonist of ER, tamoxifen, did not affect any of these responses (results not shown). This discrepancy might derive from known differences in properties of these inhibitors. Tamoxifen has been reported to act as a partial agonist accompanied by its own effect on Akt or eNOS activation, whereas ICI 182780 has been shown to have a complete blocking effect of ER [35]. It has been shown previously that the modulatory effects of oestrogen on ROS reduction were completely blocked by ICI 182780, implying an ER-dependent response [36]. In contrast, the effect of tamoxifen on the cardiovascular system is controversial. Although vasodilation induced by tamoxifen has been considered beneficial in postmenopausal women [37], its clinical usage has been linked to increased risk of venous thrombosis [38].

Our present findings provide the first evidence that DES-induced reduction in LNCaP cell invasion is ROS-independent. Even though a detailed mechanism by which DES affects LNCaP cell migration or invasion has not been described, it was shown that DES suppresses expression of several genes, including cadherin, catenein b1, integrin and metalloproteinase domain 9V, all of which are necessary for attachment and invasion [17]. A clinical report has demonstrated that DES therapy might restrict metastasis of prostate cancer [39]. The suppressive ability of DES on PSA (prostate-specific antigen) might be associated with regression of metastasis [40], since PSA has been known to facilitate invasion of prostate cancer cells by degradation of extracellular matrix glycoproteins [41]. Therefore our observation that anti-invasion effects of DES in LNCaP cells was unaffected by NOX or XO inhibitor could prove a valuable foundation for combinatorial therapy enabling protection of the cardiovascular system while preserving the anti-cancer effects of DES. Indeed, DES-induced LNCaP and PC-3 cell apoptosis was not affected by NOX or XO inhibition.

Our results suggest the possibilities of using oxypurinol or NSC23766 for combinatorial therapy with DES, since the combination of these agents with DES improved endothelial NO$^\bullet$ bioavailability while retaining anti-invasion and apoptosis-inducing activities of DES in prostate cancer cells. Chronic therapy with NSC23766 for myelogenous leukaemia in a murine model did not show any toxic effect [42], although its clinical safety remains to be evaluated in humans. Oxypurinol has been approved by the FDA (Food and Drug Administration) and subjected to a Phase II clinical trial for symptomatic heart failure [43]. In this trial, oxypurinol, however, showed neither a beneficial effect nor side effects at the

dosage of 5 mg/kg of body weight per day [43]. It may not prove to be a good agent for heart failure, but its safety makes it a good candidate for combinatorial therapy with DES for patients with prostate cancer.

In summary, the results of the present study have demonstrated that DES induces endothelial $NO^\bullet$ deficiency that is mediated by ROS production derived from activated endothelial NOX and XO. How XO and NOX interact to be both involved in DES stimulation of $O_2^{\bullet-}$, however, requires further investigation. Nonetheless, our findings provide novel insights into molecular mechanisms underlying DES's thromboembolic complications that often occur in prostate patient taking DES. This may ultimately encourage investigation of potential combinatorial therapy of DES and NOX/XO inhibitor, for safer prostate cancer treatment, particularly in those with advanced cancer who do not respond to any other treatments but DES.

Acknowledgments

Abbreviations

BAEC	bovine aortic endothelial cell
BME	basal membrane extract
BrdU	bromodeoxyuridine
CMH	1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine
CVD	cardiovascular disease
DES	diethylstilbestrol
DHE	dihydroethidium
eNOS	endothelial NO synthase
ER	oestrogen receptor
ESR	electron spin resonance
FBS	fetal bovine serum
$NO^\bullet$	NO radical
NOX	NADPH oxidase
$O_2^{\bullet-}$	superoxide anion
PEG	poly(ethylene glycol)
PSA	prostate-specific antigen
ROS	reactive oxygen species
SOD	superoxide dismutase
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
XO	xanthine oxidase

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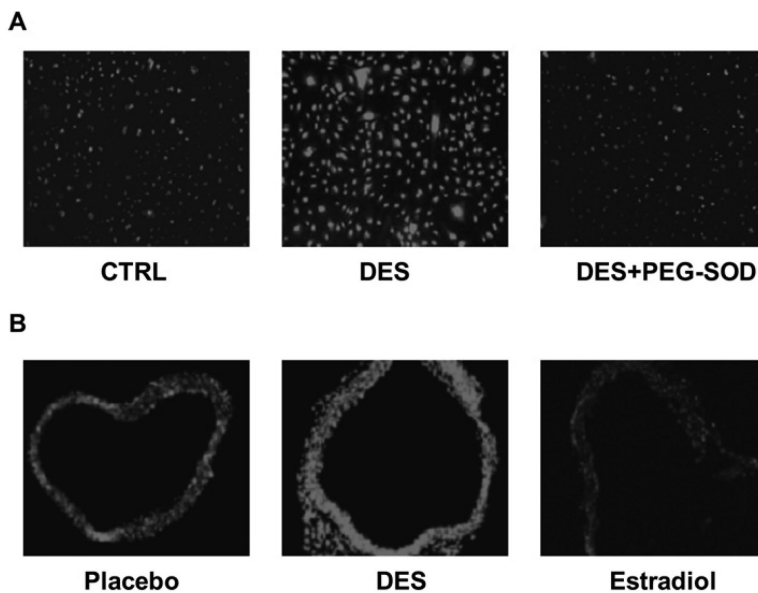
Figure 1. DES stimulates endothelial and vascular production of ROS detected by DHE staining
(A) DES stimulates endothelial $O_2^{\bullet-}$ production. Endothelial cells stimulated with DES
(12.5 µmol/l) for 24 h were incubated with DHE (2 µmol/l) for 30 min in the presence or
absence of PEG–SOD pre-incubation (100 units/ml for 30 min). (B) DES stimulates aortic
$O_2^{\bullet-}$ production. Mice received subcutaneously released DES or 17β-oestradiol (0.5 mg
over 7 days). Fresh aortic OCT sections were stained with DHE for 1 h. The fluorescent
images were captured with a Zweiss Axioskop inverted fluorescent microscope. CTRL,
control.
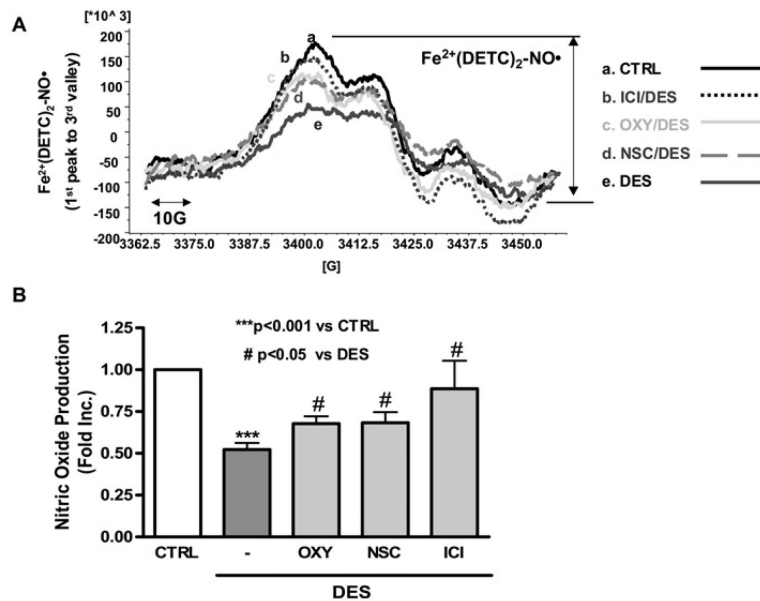
Figure 2. DES induces endothelial NO• deficiency that is attenuated by inhibition of NOX or XO
DES-stimulated (12.5 µmol/l, 24 h) endothelial cells were subjected to ESR detection of NO•. Some cells were pre-incubated with the XO inhibitor oxypurinol (50 µmol/l, 30 min), the NOX inhibitor NSC23766 (10 µmol/l, 30 min) or the ER antagonist ICI 182780 (10 µmol/l, 30 min) before DES stimulation. (A) Representative ESR spectra of bioavailable NO•. (B) Grouped data from six independent experiments. Results are means±S.E.M. ***P<0.001 compared with control; #P<0.05 compared with DES.
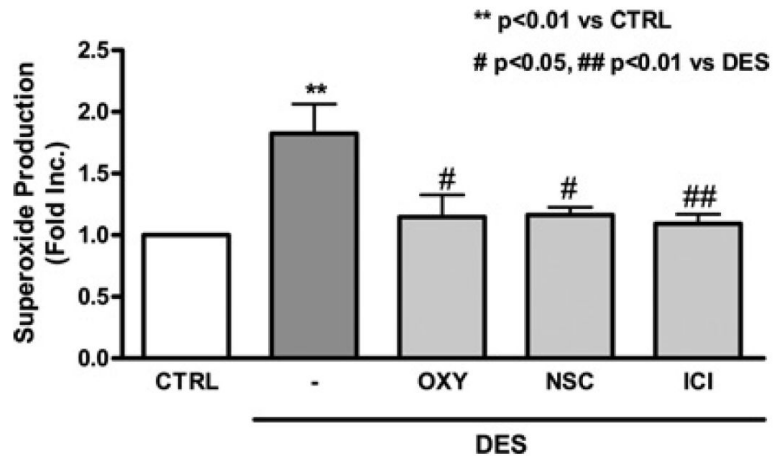
Figure 3. DES stimulates superoxide production via activation of NOX and XO
Endothelial cells were pre-treated with the XO inhibitor oxypurinol (50 µmol/l, 30 min), the NOX inhibitor NSC23766 (10 µmol/l, 30 min) or the ER antagonist ICI 182780 (10 µmol/l, 30 min) before DES stimulation. Superoxide production was determined by ESR. Results are means±S.E.M. **P<0.01 compared with control (CTRL); [#]P<0.05 and [##]P<0.01 compared with DES.
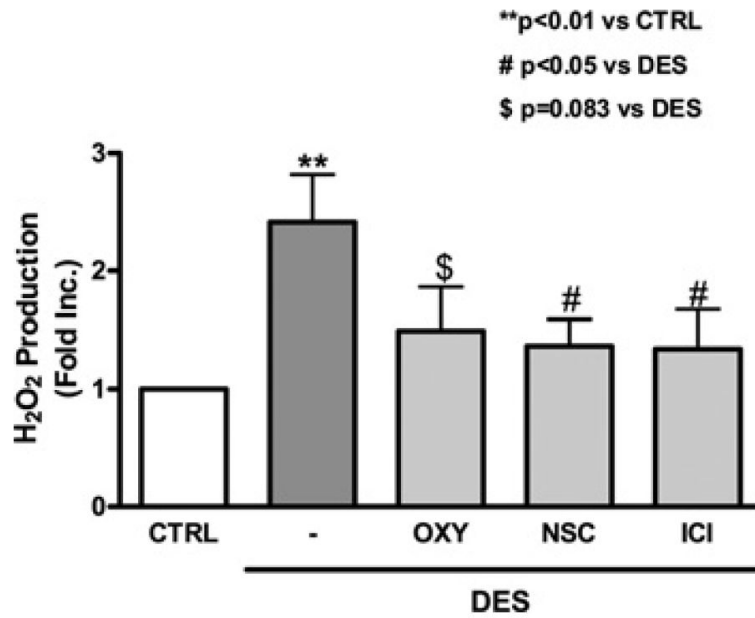
Figure 4. DES-induced H_2O_2 production is attenuated by inhibition of XO, NOX or ER
Endothelial cells were pre-treated with the ER antagonist ICI 182780 (10 µmol/l), the XO inhibitor oxypurinol (50 µmol/l) or the NOX inhibitor NSC23766 (10 µmol/l) for 30 min before DES stimulation (12.5 µmol/l, 24 h). H_2O_2 production was analysed using an Amplex Red assay (Molecular Probes). Results are means±S.E.M. **P<0.01 compared with control; [#]P<0.05 compared with DES; [$]$P$=0.083 compared with DES.
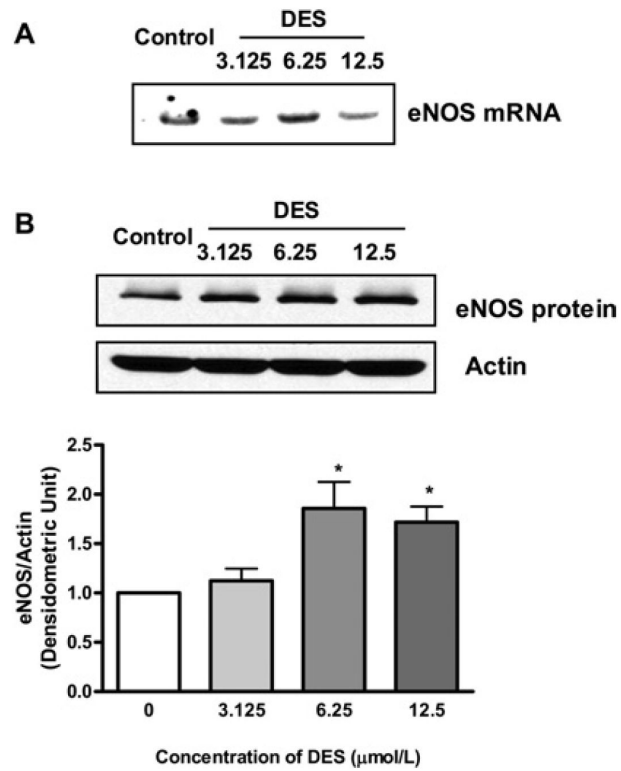
Figure 5. DES up-regulates eNOS mRNA and protein expression
(A) Representative Northern blot of eNOS mRNA expression. (B) Representative Western blot and grouped densitometric data of eNOS protein expression. Results are means±S.E.M. *P<0.05 compared with control.
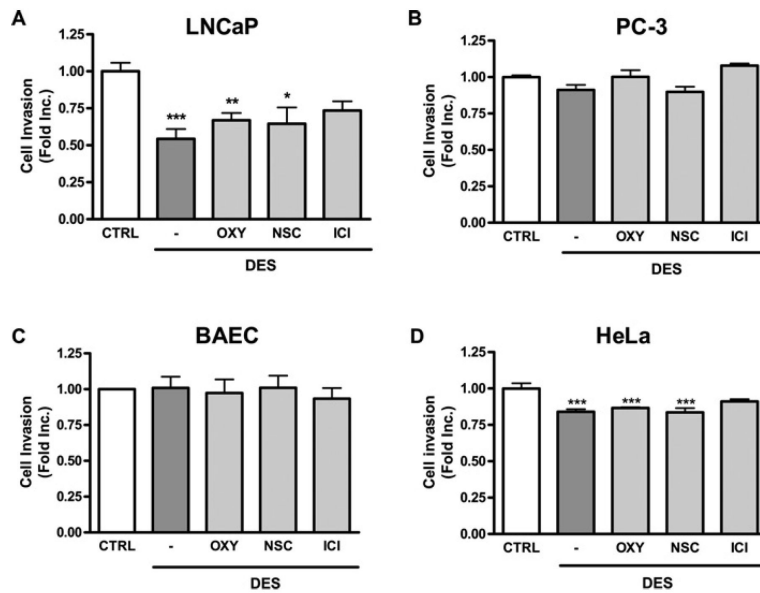
Figure 6. Combined treatment of DES with NOX/XO inhibitor has no effect on DES inhibition of invasion in prostate cancer LNCaP cells
LNCaP, PC-3, HeLa cells or BAECs were placed in BME-coated upper chamber for invasion assay. Cells were pre-treated with the ER antagonist ICI 182780 (10 µmol/l), the XO inhibitor oxypurinol (50 µmol/l) or the NOX inhibitor NSC23766 (10 µmol/l) for 30 min before DES treatment (12.5 µmol/l, 24 h). Invasion ability was determined by BME-coated transwell invasion assay in (A) LNCap cells, (B) PC-3 cells, (C) BAECs and (D) HeLa cells. Results are means±S.E.M. *$P<$0.05, **$P<$0.01 and ***$P<$0.001 compared with untreated cells. CTRL, control.
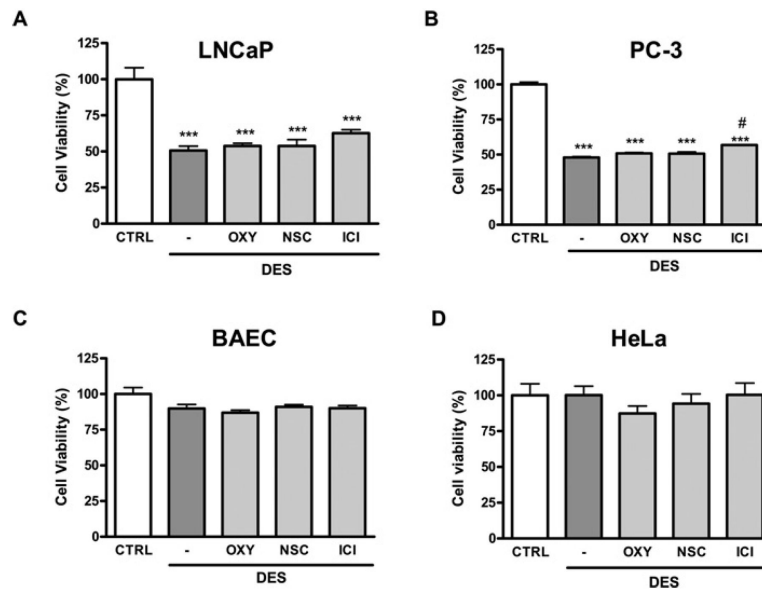
Figure 7. Combined treatment of DES with NOX/XO inhibitor has no effect on DES induction of apoptosis in prostate cancer LNCaP and PC-3 cells
LNCaP, PC-3, BAEC and HeLa cells were pre-treated with the ER antagonist ICI 182780 (10 μmol/l), the XO inhibitor oxypurinol (50 μmol/l) or the NOX inhibitor NSC23766 (10 μmol/l) for 30 min before DES treatment (12.5 μmol/l, 24 h). After treatment, floating and adherent cells were collected and subjected to apo-BrdU TUNEL labelling as described in the Materials and methods section. (A) LNCaP cell, (B) PC-3 cell, (C) BAEC and (D) HeLa cell. ***$P<0.001$ compared with untreated cells; #$P<0.05$ compared with DES.