Nitric oxide regulates oestrogen-activated signalling pathways at multiple levels through cyclic GMP-dependent recruitment of insulin receptor substrate 1

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The gaseous messenger nitric oxide (NO) contributes to biological effects of oestrogen in target tissues, including reproductive organs, bone, cardiovascular and central nervous systems. Vasodilation and anti-atherosclerotic properties of NO have been shown to play a role in these effects. The possibility that NO acts also through regulation of the signal transduction cascade triggered by oestrogen, instead, has never been investigated. To study this we have used the MCF-7 human breast cancer cell line, an established model for oestrogen signalling. Exposure of these cells to 17-β-oestradiol (E_2) in the presence of NO gave rise to activation of signalling events additional to those triggered by E_2 alone, namely tyrosine phosphorylation of specific proteins, including the insulin receptor substrate-1, with recruitment to this adapter of the phosphatidylinositol 3′-kinase and persistent activation of Akt (protein kinase B). Active Akt, in turn, prevented E_2 from activating p42/44 extracellular signal-regulated kinases (ERK 1/2). These effects of NO, which were mediated through generation of cyclic GMP and activation of the cGMP-dependent protein kinase I, initiated in the first minutes after administration of oestrogen. The consequences, however, were long lasting, as modulation of Akt and ERK 1/2 activities by NO was responsible for inhibition of E_2-triggered cell growth and regulation of oestrogen responsive-element dependent gene transcription. Generation of NO is stimulated by both E_2 and growth factors known to contribute to the complex network of intracellular events regulating the biological actions of oestrogen. It is conceivable, therefore, that modulation by NO of E_2 early signalling, here described for the first time, has broad significance in regulating cellular responses to the hormone.

Key words: Akt, mitogen-activated protein (MAP) kinases, NO, oestrogen responsive elements, phosphatidylinositol 3′-kinase, protein kinase.

INTRODUCTION

Oestrogen exerts multiple physiological and pathological effects. On the one hand, it is essential to development and maintenance of reproductive organs; on the other, it may sustain growth of tumours that arise from these tissues [1]. In addition, oestrogen contributes to regulation of the structure of the bone, and to functioning of the cardiovascular and central nervous system [1]. These actions are mediated through activation of various receptors, which include the classical nuclear receptors [1] and receptors at the plasma membrane, whose molecular identity has not yet been fully elucidated [2–5]. Heterogeneity of receptors may explain why oestrogen, together with the well-known ability to initiate gene transcription at the oestrogen responsive elements (ERE), often triggers complex patterns of intracellular signals switched on from the first minutes after hormone administration. These non-genomic actions include activation of adenylate cyclase and phospholipase Cβ, mobilization of intracellular calcium, regulation of ion channels, activation of both the phosphatidylinositol 3′-kinase (PI3K)/protein kinase B (Akt) pathway and the extracellular signal-regulated kinases (ERK 1/2) [6]. Also insulin receptor substrate 1 (IRS-1), a docking protein which acts as a major substrate for growth factors, may be involved in oestrogen signalling [7–11]. This, however, has been reported to occur only in the presence of growth factors, or after long exposure to oestrogen, conditions in which IRS-1 contributes to the cross-talk between the steroid and insulin or insulin-like growth factor-1 (IGF-1) [8–11].

Increasing evidence indicates that an effector molecule crucial for oestrogen signalling is the short-lived messenger nitric oxide (NO). NO participates in cardio-protective and anti-atherosclerotic effects of oestrogen, as well as in the effects of the steroid in the central nervous system and bone metabolism. Until now these effects have been mostly attributed to direct actions of NO on target tissues, including its well-known vasodilating and anti-atherogenic properties [12]. However, the observation that oestrogen not only increases expression of NO synthesizing enzymes (NOS) [13], but also triggers direct activation of endothelial NOS in the first minutes after its addition to cells [3,4,14–16], opens the possibility that part of the NO effects on oestrogen biological actions are also mediated through regulation of the signal transduction cascades initiated by the hormone. So far, this possibility has never been investigated.

Abbreviations used: Ab, antibody; 8 Br, 8-bromo; cGMP, cyclic GMP; DETA-NO, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; DMEM, Dulbecco modified Eagle's medium; E_2, 17β-oestradiol; ECL®, enhanced chemiluminescence; ERE, oestrogen-responsive element; ERK 1/2, p42/44 extracellular signal-regulated kinases; IGF-1 and IGF-1R, insulin-like growth factor 1 and its receptor; IRS-1, insulin receptor substrate 1; mAb, monoclonal antibody; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; NOS, NO synthase; ODQ, H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one; PI3K, phosphatidylinositol 3′-kinase.

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In the present work we have used an established model for studies on oestrogen signalling, the human breast cancer cell line MCF-7, exposed to 17β-oestradiol (E_2) and the NO donor (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)aminodiazen]-1-ium-1,2-diolate (DETA-NO). Our results show that NO renders E_2 competent to trigger rapid tyrosine phosphorylation, and thus activation, of IRS-1. This initiates a complex chain of events that include recruitment of PI3K, persistent activation of Akt and ensuing inhibition of E_2-triggered ERKs activation. These effects of NO on E_2 signalling were found to be mediated through activation of guanylate cyclase, generation of cyclic GMP (cGMP) and ensuing stimulation of the cGMP-dependent protein kinase I (G kinase), and to have long-lasting consequences on cellular responses to the steroid.

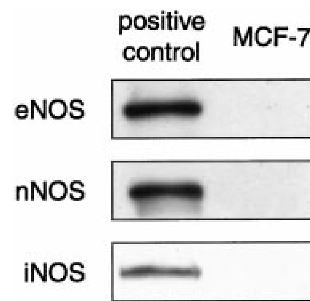

Figure 1 MCF-7 cells do not express NOS isoforms

Expression of endothelial, neuronal and inducible NOS was analysed by Western blotting with specific Abs on 40 μg of cell lysates from subconfluent MCF-7 cells, as detailed in the Experimental section. Positive controls for each NOS isoform (20 μg of lysates from human aortic endothelial cells, rat pituitary cells and activated RAW 264.7 macrophages, for endothelial neuronal and inducible NOS, respectively, all provided by Transduction Labs together with the Abs) were loaded in parallel. The experiments shown are representative of three.

EXPERIMENTAL

Materials

The following reagents were purchased as indicated: H-[1,2,4]-oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) and KT5823 from Calbiochem, Darmstadt, Germany; the Enhanced Chemi-Luminescence (ECL®) kit from Amersham, Little Chalfont, U.K.; DETA-NO from Alexis Co., Nottingham, U.K.; polyclonal antibodies (Ab) anti-Akt and anti-phospho-Akt (Ser 473), monoclonal Ab (mAb) anti-p42/p44 ERK 1/2 and anti-phospho-ERK 1/2 (Thr 202–Tyr 204) from New England Biolabs, Beverly, MA, U.S.A.; mAbs anti-phosphotyrosine (PY20; horseradish peroxidase-conjugated), anti-p85 (PI3K regulatory subunit), anti-endothelial NOS, anti-inducible NOS and anti-neuronal NOS from Transduction Laboratories, Lexington, KY, U.S.A.; anti-rat carboxy-terminal IRS-1 polyclonal Ab from Upstate Biotechnology, Lake Placid, NY, U.S.A.; anti-IGF-1 receptor (IGF-1R) mAb from Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.; calf serum, tissue culture reagents and media from Mascia Brunelli, Milan, Italy. Anti-oestrogen ICI 182,780 was kindly provided by Zeneca, Macclesfield, U.K., and the EREtkLuc plasmid by Dr M. Maggiolini, Dept of Pharmaco-Biology, University of Calabria, Rende, Italy. E_2 and all the other reagents were purchased from Sigma.

Cell cultures and preparation

MCF-7 cells, provided by Dr Eva Surmacz (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA, U.S.A.), were routinely cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) supplemented with 5% calf serum in a 5%-CO_2 humidified atmosphere. These cells were chosen not only because they are an established model for E_2 signalling, but also because they are devoid of any detectable NOS, as demonstrated by both Western blotting (Figure 1) and measurements of NOS activity, carried out as described previously [17]. At the time of experiments, subconfluent cell cultures were detached by trypsinization, cultured in growth medium for 24 h and then for an additional 24 h in DMEM without Phenol Red and serum. Experiments were carried out using 70% confluent cells in DMEM without Phenol Red and serum. All incubations with the various compounds were for 15 min prior to addition of E_2 (time 0).

Immunoprecipitation and Western blotting

Cells, incubated with the various compounds as detailed in the results, were treated with E_2 and washed twice, at the indicated time points, with a cold buffer [150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol, 50 mM Hepes, pH 7.5]. Cells were then lysed for 30 min in the same buffer with the addition of 0.1 mM PMSF, 1% (v/v) Triton X-100, 0.2 mM orthovanadate, 10 μg/ml leupeptin and 10 μg/ml aprotinin (lysis buffer). For immunoprecipitation, lysates were centrifuged at 800 ***g*** for 15 min and the supernatants incubated overnight at 4 °C with 4 μg of either anti-IRS-1 Ab or anti-IGF-1R Ab. Protein G–Sepharose beads were then added to the lysates, which were incubated overnight and then washed three times with the above lysis buffer (see [18] for further details). Protein concentrations in the lysates and immunoprecipitates were assayed using the bicinchoninic acid procedure (Pierce, Rockford, IL, U.S.A.). Western-blot analyses were carried out with either 500 μg of immunoprecipitates or 40 μg of total protein, which were first separated on pre-cast 4–15% gradient SDS/polyacrylamide gels (Bio-Rad, Milan, Italy) and then electrophoretically transferred on to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Non-specific sites were blocked before immunolabelling by incubation for 1 h at 21 °C with blotting buffer [100 mM Tris/HCl, 0.1% Tween 20 and 5% (w/v) non-fat milk, pH 7.4]. The membranes were incubated overnight in blotting buffer with the relevant Ab and washed three times for 1 h with blotting buffer. The secondary anti-rabbit or anti-mouse horseradish peroxidase conjugated Ab was then added for 30 min. After several washes of the membranes in blotting buffer, the antigens were revealed with ECL® according to the manufacturer's instructions. As an internal control, all Western blots in which the phosphorylation levels of either Akt or ERK 1/2 were determined, were subsequently stripped of the first Ab and reprobed with the Ab recognizing both the phosphorylated and non-phosphorylated forms of the enzyme. Similarly, Western blots of IRS-1 and IGF-1R immunoprecipitates analysed with the anti-p85 or anti-phosphotyrosine Abs were reprobed with the anti-IRS-1 Ab or the anti-IGF-1R Ab respectively. Stripping of the first Ab was carried out by incubating nitrocellulose filters for 30 min at 52 °C with a buffer [62.5 mM Tris/HCl, 2% (w/v) SDS, 1% β-mercaptoethanol, pH 6.7]. Filters were then washed twice for 10 min with 100 mM Tris/HCl, 0.1% Tween 20, pH 7.4, before equilibration for 1 h in blotting buffer. Quantitation of the relevant bands was carried out using a Quick Image densitometer equipped with a Phoretix 1-D software image analyser (Canberra Packard, Milan, Italy).

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Transfections and luciferase assay

MCF-7 cells were plated at a concentration of 1×10^5 cells/well the day before transfection into 24-well plates with 500 μl of growth medium. The medium was replaced with DMEM (without Phenol Red and serum) 1 h before transfection. Cells were transfected using FuGENE 6 (Roche, Indianapolis, IN, U.S.A.) according to the manufacturer's instructions. The transfection mixture contained 0.5 mg of EREtkLuc (also called XETL and described in [19]), and 0.05 mg of the *Renilla* luciferase expression vector pRL-CMV (Promega Italia, Milan, Italy), used as a internal control of transfection efficiency. MCF-7 cells were incubated with the DNA mixtures for 8 h, washed and cultured in DMEM (without Phenol Red and serum) for a further 4 h. Cells were then treated with various compounds, as indicated in the Results section, before addition of E_2. After 8 h, cells were lysed (see above procedure) and luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instruction. Luciferase values were divided by the correspondent *Renilla* luciferase values and expressed as relative luciferase units.

Cell-proliferation assays

For the colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [20], MCF-7 cells were plated in 24-multiwell plates at the concentration of 4×10^3 cells/well in growth medium. After 2 days the medium was replaced with DMEM (without Phenol Red and serum) for 24 h. Cells were then incubated with various compounds, as detailed in the Results section, and treated with E_2. At the indicated time points (days 1–3) cells were washed, 0.5 ml of MTT stock solution (2.4 mM in DMEM without Phenol Red, filtered) was added, and the plates were incubated for 1 h at 37 °C. At the end of the incubation the non-transformed MTT was carefully removed and the dye crystals solubilized in 1 ml of 2-propanol. Absorbances were read immediately in a UVKON 941 spectrophotometer using a test wavelength of 570 nm and a reference wavelength of 690 nm.

Statistical analysis

The results are expressed as means $\pm$ standard error of the mean; *n* represents the number of different experiments, which were run in triplicate. Western-blotting data are representative of 3 to 4 experiments, as specified in each Figure legend. Statistical analysis was performed by the Student's *t* test for unpaired data (two-tail). The symbols *, ** and *** or +, ++, +++ in Table 1 and Figure 8 refer to statistical probabilities (*P*) of < 0.05, < 0.01 and < 0.001, respectively, as detailed in the legend to Figure 8. A value of *P* less than 0.05 was considered to be statistically significant.

RESULTS

The MCF-7 cells used in our experiments are devoid of any detectable NOS, as detected by Western blotting with specific Abs recognizing specifically the endothelial, the neuronal or the inducible NOS (Figure 1). In addition, they display no NOS activity, which was assessed by measuring arginine to citrulline conversion as described previously [17] (results not shown). Absence of endogenous NO generation makes these cells a clean system to study the effects of NO. Experiments were carried out using the NO donor DETA-NO which, at the concentration employed (100 μM), released NO at the constant concentration of 0.21 ± 0.2 μM ($n = 4$), as measured by an NO electrode [21]. To investigate the effects of NO on E_2-induced tyrosine phosphorylation, sub-confluent MCF-7 cells were exposed to DETA-NO, E_2 (1 nM) and the oestrogen antagonist ICI 182,780 (100 nM), administered either alone or combined. Tyrosine phosphorylation was measured by Western blotting at different time points after stimulation, as indicated in Figure 2. The effect of E_2 was relatively small and transient, as shown previously in these cells [22]. DETA-NO did not have any effect when administered alone. Combination of E_2 with DETA-NO, instead, resulted in markedly enhanced tyrosine phosphorylation staining which, in addition, became persistent throughout the time-window analysed (1 h). In the presence of ICI 182,780, tyrosine phosphorylation was observed with E_2 neither when administered alone nor when combined with DETA-NO (Figure 2, and results not shown).

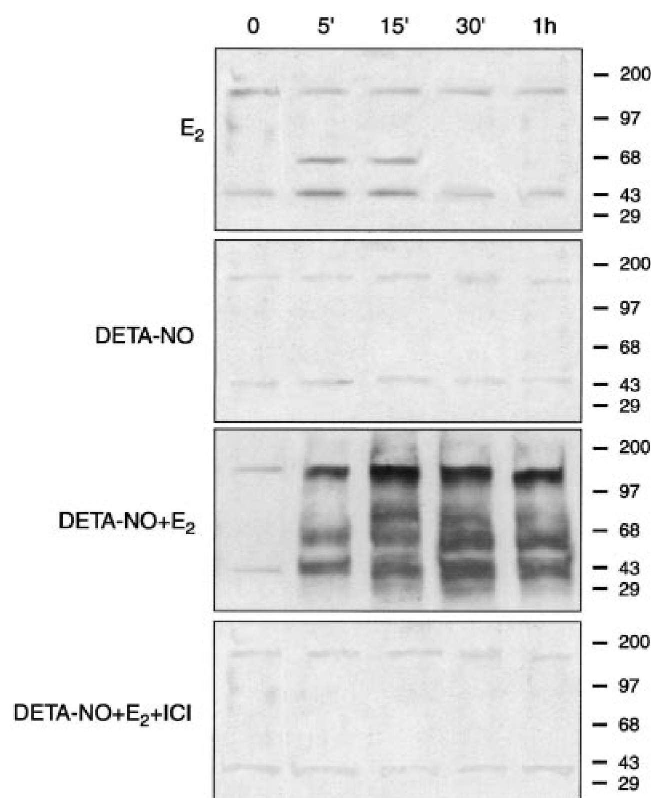

Figure 2 Effects of E_2 and NO on protein tyrosine phosphorylation

Subconfluent MCF-7 cells were incubated for 15 min with or without DETA-NO (100 μM), ICI 182,780 (ICI, 100 nM) or both, as indicated on the left-hand side of each panel, before addition of either E_2 (1 nM) or vehicle (time 0). Cells were collected at the indicated time points, and tyrosine phosphorylation of proteins measured with an anti-phosphotyrosine Ab on Western blots of 40 μg of cell lysates, as detailed in the Experimental section. The positions and molecular mass (kDa) of the molecular-mass markers used (Prestained molecular weight marker, high range, Calbiochem) are indicated on the right-hand side of each panel. The experiments shown are representative of four.

The pattern of tyrosine phosphorylation observed with E_2 and NO included also proteins in the 150–190 kDa range, among which is IRS-1. To verify the involvement of the latter, cells, treated as above, were lysed and IRS-1 immunoprecipitated with an anti-IRS-1 specific Ab. Tyrosine phosphorylation of the precipitates was analysed by Western blotting. As shown in Figure 3, E_2 and DETA-NO had no effect when given alone. By contrast, when administered together, they induced tyrosine phosphorylation of IRS-1, which was already present after 5 min of treatment and maintained throughout the period analysed.

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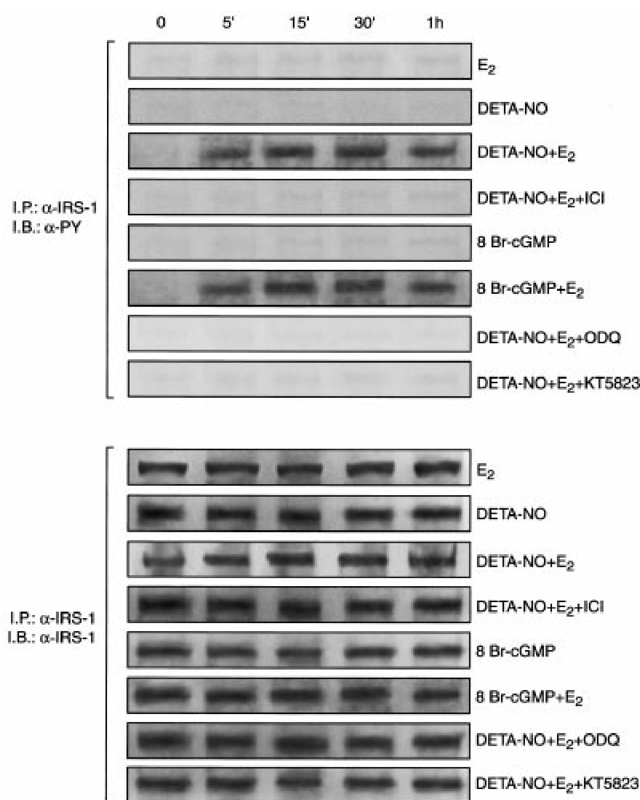

Figure 3 Effects of E_2, NO, cGMP and G kinase on tyrosine phosphorylation of IRS-1

Subconfluent MCF-7 cells were incubated for 15 min with or without DETA-NO (100 μM), ICI 182,780 (ICI, 100 nM), 8 Br-cGMP (1 mM), ODQ (1 μM) or KT5823 (5 μM), in various combinations as detailed on the right-hand side of each panel. Cells were collected at the indicated time points after addition of E_2 (1 nM) or vehicle (time 0), lysed and immunoprecipitated with an anti-IRS-1 Ab. Tyrosine phosphorylation of IRS-1 was measured with an anti-phosphotyrosine Ab (PY) on Western blots of 500 μg of immunoprecipitates (I.P.: α-IRS-1; I.B.: α-PY). As control, the levels of IRS-1 in each immunoprecipitate was measured by reprobing each Western blot with the anti-IRS-1 Ab (I.P.: α-IRS-1; I.B.: α-IRS-1). The experiments shown are representative of four.

This tyrosine phosphorylation was not observed in the presence of ICI 182,780.

NO may exert its effect either through activation of guanylate cyclase, generation of cGMP and activation of G kinase or through alternative pathways [23]. To establish whether NO was acting in a cGMP-dependent manner, experiments were carried out in the presence of the membrane-permeant cGMP analogue, 8-bromo (8 Br)-cGMP (1 mM), the guanylate cyclase inhibitor, ODQ (1 μM), or the selective G kinase inhibitor KT5823 (5 μM) [24]. 8 Br-cGMP mimicked the effect of DETA-NO, as it was ineffective when given alone, while inducing tyrosine phosphorylation of IRS-1 in the presence of E_2 with kinetics similar to those of the NO donor. Consistent with this observation, coincubation in the presence of ODQ abrogated the effect of DETA-NO. Co-incubation in the presence of KT5823 also reversed the effect of DETA-NO (Figure 3). To establish whether the effects observed were due to actual changes in tyrosine phosphorylation of IRS-1 or secondary to changes in IRS-1 protein levels, all Western blots were stripped of the anti-tyrosine Ab and decorated with the anti-IRS-1 Ab. No changes in IRS-1 protein were observed in all cases (Figure 3). Taken together, these results indicate that NO and E_2 synergize to induce tyrosine phosphorylation of IRS-1 and that the effect of the gaseous messenger is dependent on generation of cGMP and activation of G kinase.

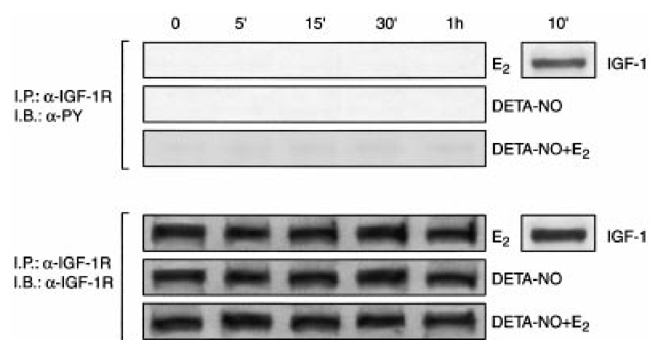

Figure 4 Effects of E_2 and NO on tyrosine phosphorylation of IGF-1R

Subconfluent MCF-7 cells were incubated for 15 min with or without DETA-NO (100 μM) before addition of either E_2 (1 nM) or vehicle (time 0). Cells were collected at the indicated time points, lysed and immunoprecipitated with an anti-IGF-1R Ab. Tyrosine phosphorylation of IGF-1R was measured with an anti-phosphotyrosine Ab (PY) on Western blots of 500 μg of immunoprecipitates (I.P.: α-IGF-1R; I.B.: α-PY). As control, the level of IGF-1 R in each immunoprecipitate was measured by reprobing each Western blot with the anti-IGF-1R Ab (I.P.: α-IGF-1R; I.B.: α-IGF-1R). 10 min stimulation of MCF-7 cells with IGF-1 (1 nM) was carried out in parallel as positive control for IGF-1R tyrosine phosphorylation. The experiments shown are representative of three.

IRS-1 is a major substrate for IGF-1R, and its tyrosine phosphorylation contributes significantly to the cross-talk between the signalling pathways activated by this receptor and E_2. In particular, IRS-1 tyrosine phosphorylation, induced by activation of IGF-1R with either IGF-1 or insulin, appears to be enhanced in the presence of E_2 [9–11]. We investigated, therefore, whether synergism between NO and E_2 in triggering tyrosine phosphorylation of IRS-1 was due to activation of IGF-1R. To this end, cells were exposed to E_2, DETA-NO, or the two together, and IGF-1R tyrosine phosphorylation analysed after receptor immunoprecipitation with an anti-IGF-1R specific Ab. At variance with IGF-1, which induced marked IGF-1R phosphorylation after 10 min treatment, E_2 was ineffective both when administered alone (as already shown in MCF-7 cells, see [9,10] and when combined with DETA-NO (Figure 4). The amount of IGF-1R protein precipitated by the anti-IGF-1R Ab, however, was the same in all conditions (Figure 4). These results indicate that IGF-1R is unlikely to play a role in IRS-1 activation by E_2 in the presence of NO.

IRS-1 acts as an adapter to various downstream effector molecules, among which is PI3K, whose recruitment and activation is increased following IRS-1 tyrosine phosphorylation [25–27]. Recruitment of the PI3K p85 regulatory subunit to IRS-1 was therefore analysed. After administration of E_2 and NO, either alone or combined, IRS-1 was immunoprecipitated and the amount of co-precipitated p85 detected by Western blotting. Treatment with either DETA-NO or E_2 alone revealed no p85 co-precipitation. By contrast, this was clearly observed when the two stimuli were applied together (Figure 5). The amount of IRS-1 protein immunoprecipitated was the same in all conditions (Figure 5).

To investigate whether recruitment of p85 to IRS-1 induced by the combination of NO and E_2 resulted in stimulation of PI3K activity we examined activation of a well-known downstream effector of this enzyme, i.e. Akt, using an Ab recognizing selectively the phosphorylated, active form of the protein (Figure 6). E_2 induced activation of Akt which, however, was transient (up

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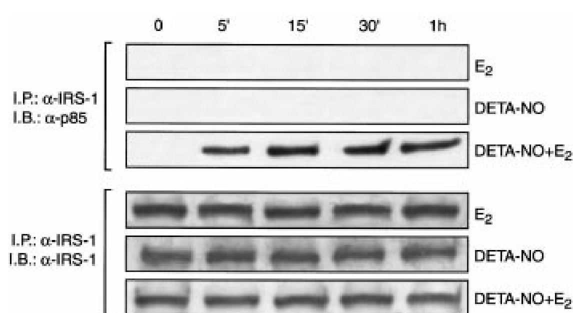

Figure 5 Effects of E_2 and NO on the recruitment of the p85 regulatory subunit of PI3K to IRS-1

Subconfluent MCF-7 cells were incubated for 15 min with or without DETA-NO (100 μM) before addition of either E_2 (1 nM) or vehicle (time 0). Cells were collected at the indicated time points, lysed and immunoprecipitated with an anti-IRS-1 Ab. Co-immunoprecipitation of p85 together with IRS-1 was measured by Western blotting on 500 μg of immunoprecipitates, using an anti-p85 Ab (I.P.: α-IRS-1; I.B.: α-p85). The level of IRS-1 in each immunoprecipitate was measured by reprobing each Western blot with the anti-IRS-1 Ab (I.P.: α-IRS-1; I.B.: α-IRS-1). The experiments shown are representative of four.

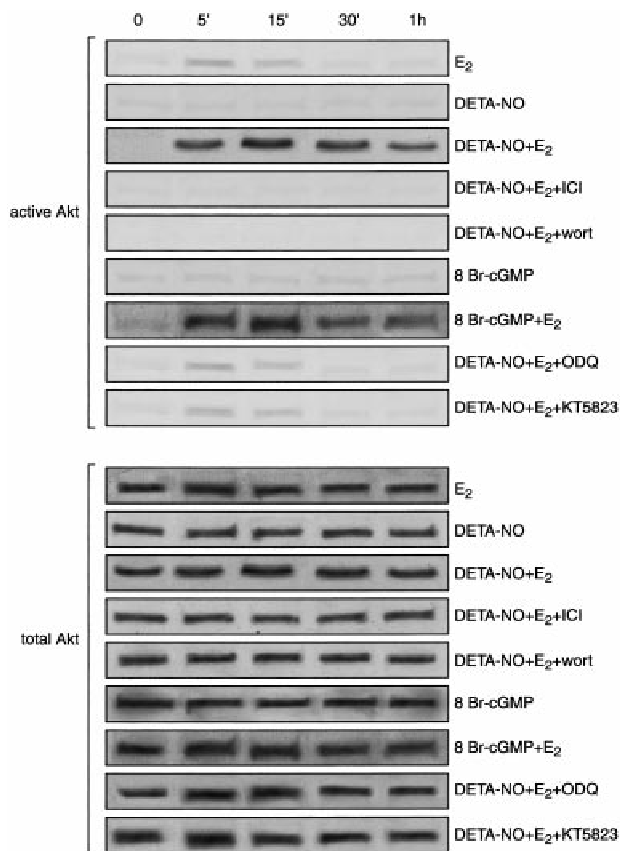

Figure 6 Effects of E_2, NO, cGMP and G kinase on activation of Akt

Subconfluent MCF-7 cells were incubated for 15 min with or without DETA-NO (100 μM), ICI 182,780 (ICI, 100 nM), wortmannin (wort, 20 nM), 8 Br-cGMP (1 mM), ODQ (1 μM) or KT5823 (5 μM), in various combinations as detailed on the right-hand side of each panel. Cells were collected at the indicated time points after addition of E_2 (1 nM) or vehicle (time 0), lysed and samples (40 μg) analysed by Western blotting with an Ab recognizing phosphorylated (Ser 473) Akt (active Akt). As control, each Western blot was reprobed with an Ab recognizing both phosphorylated and non-phosphorylated Akt (total Akt). The experiments shown are representative of four.

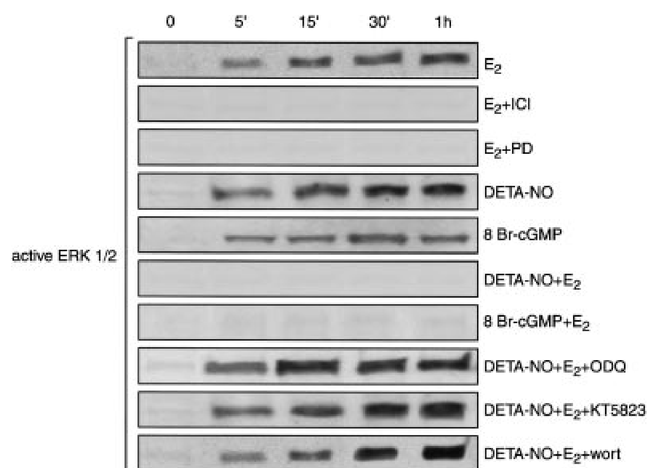

Figure 7 Effects of E_2, NO, cGMP and G kinase on activation of ERK 1/2

Subconfluent MCF-7 cells were incubated for 15 min with or without ICI 182,780 (ICI, 100 nM), PD 98059 (PD, 30 μM), DETA-NO (100 μM), 8 Br-cGMP (1 mM), ODQ (1 μM), KT5823 (5 μM), or wortmannin (wort, 20 nM), in various combinations as detailed on the right-hand side of each panel. Cells were collected at the indicated time points after addition of E_2 (1 nM) or vehicle (time 0), lysed and samples (40 μg) analysed by Western blotting with an Ab recognizing phosphorylated (Thr 202–Tyr 204) ERK 1/2 (active ERK 1/2). As control, each Western blot was reprobed with an Ab recognizing both phosphorylated and non-phosphorylated ERK 1/2 (total ERK 1/2). Ratios of densitometric values measured for active ERK 1/2 to total ERK 1/2 are reported in Table 1.The experiments shown are representative of four.

to 15 min). DETA-NO did not have an effect *per se*, but enhanced Akt activation by E_2; significantly, both in intensity and duration. This effect was abolished when cells were incubated with either ICI 182,780 or a PI3K inhibitor, namely wortmannin (20 nM) or LY 294002 (10 μM), indicating the involvement of the oestrogen receptor and PI3K (Figure 6 and results not shown). The effect of DETA-NO was mimicked by 8 Br-cGMP and reversed by either ODQ or KT5823, demonstrating also that Akt activation by NO in the presence of E_2 depends on cGMP generation and G kinase activation. When Western blots, used to reveal active Akt, were decorated with an Ab recognizing both the active and inactive protein, no difference in total Akt levels was detected in all condition tested (Figure 6). Thus, NO appears capable of enhancing Akt activation by E_2 through the IRS-1/PI3K pathway, turned on in a cGMP/G kinase-dependent fashion.

An early signalling event triggered independently by both E_2 and NO, the latter acting through the cGMP/G kinase pathway, is activation of ERK 1/2 [28–32]. The effects of E_2 and DETA-NO on ERK 1/2 activation were therefore investigated by using Abs recognising specifically either total ERK 1/2 or its phosphorylated, active form. Both E_2 and DETA-NO, when administered alone, induced activation of ERK 1/2, which was persistent throughout the time window analysed (Figure 7 and Table 1). ERK 1/2 activation was inhibited in both cases by co-administration of the MEK (MAP kinase/ERK kinase) inhibitor PD 98059 (30 μM), indicating involvement of this enzyme in ERK 1/2 activation. The effect of E_2 was prevented by ICI 182,780. When we examined the effects of the combined administration of E_2 and NO we found that they were not additive but gave rise to complete abrogation of ERK 1/2 activation. Both the stimulation of ERK 1/2 by NO and their inhibition in the presence of E_2 were found to be dependent on generation of cGMP and activation of G kinase, as these effects of

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Table 1 Effects of E_2, NO, cGMP and G kinase on activation of ERK 1/2 measured as the ratio of active to total ERK 1/2

Subconfluent MCF-7 cells were incubated for 15 min with or without ICI 182,780 (ICI, 100 nM), PD 98059 (PD, 30 μM), DETA-NO (100 μM), 8 Br-cGMP (1 mM), ODQ (1 μM), KT5823 (5 μM), or wortmannin (wort, 20 nM), in various combinations as detailed in the left-hand column. Cells were collected at the indicated time-points after addition of E_2 (1 nM) or vehicle (time 0), lysed and samples (40 μg) were analysed by Western blotting with an Ab recognising phosphorylated, active ERK 1/2. Each Western blot was then reprobed with an Ab recognising both phosphorylated and non-phosphorylated ERK 1/2. Blots were then analysed by densitometry. Shown are the ratios of the values ± standard error of the mean measured for active to total ERK 1/2 ($n = 4$). Asterisks indicate statistical significance versus treatments with E_2 alone at the same time-points, calculated as described in the Experimental section.

	Time (min)				
Compound	0	5	15	30	60
E_2	0.03 ± 0.01	0.41 ± 0.01	0.71 ± 0.03	0.82 ± 0.04	0.91 ± 0.06
E_2 + ICI	0.01 ± 0.01	0.02 ± 0.01	0.06 ± 0.01	0.03 ± 0.01	0.01 ± 0.00
E_2 + PD	0.02 ± 0.01	0.00 ± 0.00	0.04 ± 0.01	0.01 ± 0.01	0.03 ± 0.01
DETA-NO	0.03 ± 0.01	0.48 ± 0.02	0.88 ± 0.05	0.96 ± 0.04	0.97 ± 0.04
8 Br-cGMP	0.00 ± 0.00	0.32 ± 0.01	0.38 ± 0.01	0.68 ± 0.03	0.66 ± 0.03
DETA-NO + E_2	0.03 ± 0.01	0.05 ± 0.01	0.00 ± 0.01	0.04 ± 0.01	0.01 ± 0.01
8 Br-cGMP + E_2	0.01 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.06 ± 0.01	0.09 ± 0.01
DETA-NO + E_2 + ODQ	0.04 ± 0.01	0.56 ± 0.04	0.78 ± 0.06	0.86 ± 0.05	1.07 ± 0.08
DETA-NO + E_2 + KT5823	0.02 ± 0.01	0.51 ± 0.02	0.83 ± 0.04	0.88 ± 0.06	0.93 ± 0.09
DETA-NO + E_2 + wort	0.00 ± 0.00	0.48 ± 0.04	0.80 ± 0.05	1.09 ± 0.04**	1.22 ± 0.03**

NO were mimicked by 8 Br-cGMP and reversed by either ODQ or KT5823 (Figure 7, Table 1 and results not shown).

The mechanism of inhibition of ERK 1/2 in the presence of NO/cGMP and E_2 was next investigated. ERK 1/2 are inhibited by stimulation of the PI3K/Akt pathway [33]. Since this pathway is activated in the presence of NO/cGMP and E_2 (Figures 3 and 4), we assessed its involvement in inhibition of ERK 1/2. When cells were exposed to E_2 together with either DETA-NO or 8 Br-cGMP, and in the presence of wortmannin or LY 294002, ERK 1/2 activation was restored, with enzyme activation greater than that observed with E_2, DETA-NO or 8 Br-cGMP alone (Figure 7, Table 1 and results not shown). These results suggest strongly that the inhibition of ERK 1/2 in the presence of NO/cGMP and E_2 is due to concomitant PI3K/Akt activation.

PI3K/Akt and ERK 1/2 have been shown to enhance the E_2-induced stimulation of ERE-containing genes [34,35]. To evaluate whether the regulation of PI3K/Akt and ERK 1/2 pathways described above influences the ability of E_2 to activate ERE-dependent transcription, cell were transiently transfected with a luciferase reporter gene under the control of a ERE element (EREtkLuc, described in [19]). Exposure to E_2 for 8 h induced EREtkLuc expression, with ensuing luciferase expression, which was abolished by co-incubation with ICI 182,780 (Figure 8A). Treatment with PD 98059, wortmannin, or LY 294002 resulted in inhibition of E_2 effects on EREtkLuc, that was more pronounced when PD 98059 and either PI3K inhibitor were both present (Figure 8A and results not shown).

We then examined the effects of DETA-NO and 8 Br-cGMP. Results were similar with both compounds, therefore only those obtained with 8 Br-cGMP are described in detail (Figure 8A). The cyclic nucleotide had no effects on EREtkLuc. Likewise, it did not modify EREtkLuc response to E_2 administration when co-administered with the steroid. As demonstrated above, however, E_2 signalling is profoundly modified by cGMP, with PI3K/Akt greatly stimulated and ERK 1/2 inhibited. We investigated the role of these changed kinase activities. In the presence of wortmannin or LY 294002, luciferase expression induced by co-administration of 8 Br-cGMP with E_2 was not modified, presumably because of the concomitant greater activation of ERK 1/2 observed when PI3K inhibitors are administered prior to 8 Br-cGMP and E_2 (see above). Consistently, addition of PD 98059 together with either wortmannin or LY 294002 inhibited to a great extent luciferase expression induced by co-administration of 8 Br-cGMP with E_2, with values similar to those observed with E_2 in the presence of PD 98059 and either PI3K inhibitor (Figure 8A and results not shown). This suggests that PI3K/Akt activation and concomitant ERK 1/2 inhibition by 8 Br-cGMP compensate each other, resulting in no changes in E_2-stimulated ERE-dependent transcription.

Activation of ERK 1/2 and gene transcription are relevant to mitogenic effects of E_2 [35–37]. We therefore evaluated whether the NO-induced, cGMP-dependent regulation of E_2 signalling influences the mitogenic properties of the steroid. Since NO also acts on growth independently of cGMP ([24] and references therein), we analysed specifically the effect of the cyclic nucleotide. Cell proliferation was measured using an established technique, i.e. conversion of MTT into its derivative, formazan [20].

Administration of E_2 induced cell proliferation, which was completely inhibited by ICI 182,780. Blockade of ERK 1/2 activation with PD 98059 resulted in reduced E_2-dependent cell proliferation, whereas wortmannin or LY 294002 modified the effect of neither E_2 nor E_2 plus PD 98059 (Figure 8B, and results not shown). These results are consistent with the involvement of ERK 1/2 in E_2-induced cell proliferation already described in these cells [37]. 8 Br-cGMP itself induced a small, yet significant increase in cell proliferation (Figure 8B). The effect of the cyclic nucleotide, however, was not additive with that of E_2. On the contrary, when E_2 and 8 Br-cGMP were administered together, cell proliferation was inhibited to a large extent. This inhibition was likely due to PI3K/Akt-dependent inhibition of ERK 1/2, as it failed to appear when 8 Br-cGMP and E_2 were administered in the presence of wortmannin or LY 294002 (Figure 8B and results not shown). Consistent with this, administration of E_2 and 8 Br-cGMP together with PD 98059 did not result in further inhibition (Figure 8B). These results indicate that regulation by NO/cGMP of E_2 signalling pathways results in modulation of one of the important functions of the steroid, i.e. cell proliferation.

DISCUSSION

A fascinating aspect of E_2 is its ability to trigger complex patterns of cell responses that contribute to co-ordinate growth, development and differentiation of organs as diverse as those forming the reproductive systems of both females and males, mammary glands, bone, cardiovascular and central nervous systems. The discovery that E_2 interacts with distinct membrane and nuclear receptors to trigger both rapid signal transduction

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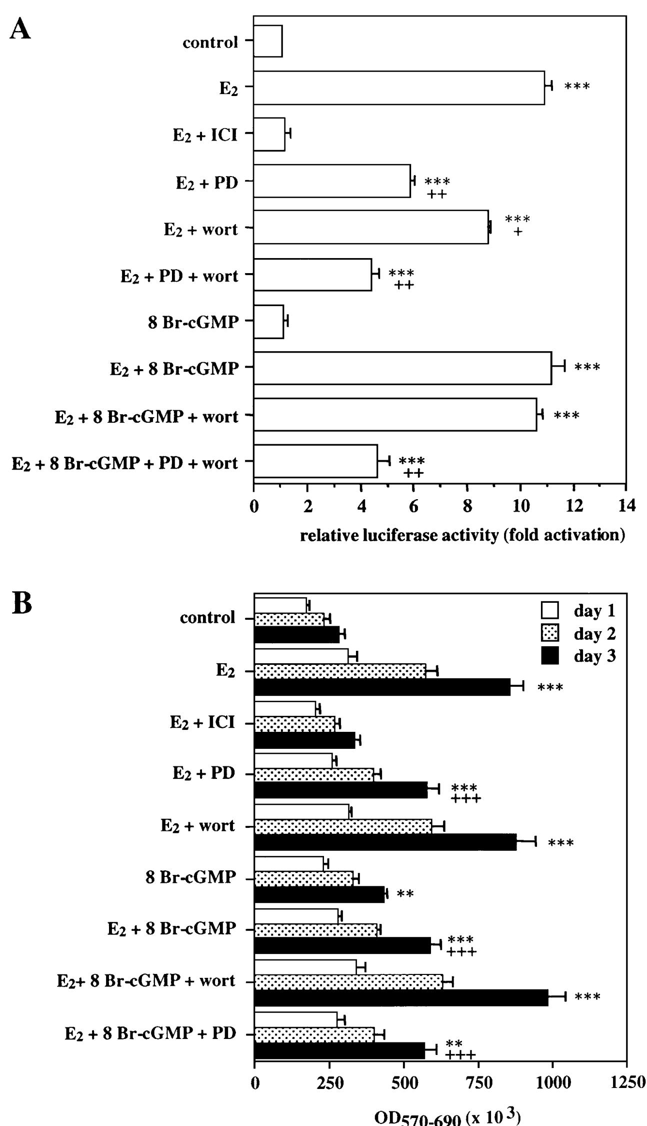

Figure 8 Effects of E_2 and cGMP on activation of ERE-dependent transcription and cell proliferation

(**A**) Subconfluent MCF-7 cells, transiently transfected with EREtkLuc, were incubated for 15 min with or without ICI 182,780 (ICI, 100 nM), PD 98059 (PD, 30 μM), wortmannin (wort, 20 nM) or 8 Br-cGMP (1 mM) in various combinations as detailed on the left-hand side. Cells were then incubated for a further 8 h with either E_2 (1 nM) or vehicle, after which luciferase activity was determined as detailed in the Experimental section. The normalized luciferase activity of the unstimulated cells was arbitrarily assigned the value of 1 (control). Values are expressed as fold increase over control $\pm$ standard error of the mean. (**B**) MCF-7 cells, incubated as indicated in (**A**), were treated with or without E_2 for 1–3 days, after which the MTT assay was carried out as described in the Experimental section. Absorbances were measured using a test wavelength of 570 nm and a reference wavelength of 690 nm. In both panels, statistical probability versus untreated controls is indicated by the asterisks, that versus cells treated with E_2 alone with crosses, and both calculated as described in the Experimental section ($n = 6$).

events and more prolonged regulation of gene transcription, and that these signals integrate and partially overlap with those of growth factors and cytokines, provides a molecular explanation to such complex effects of the steroid [1,6]. The efficiency of E_2 signalling, however, together with the pathological consequences arising from its disregulation (tumours, for example) suggest that its signal transduction pathways are exquisitely co-ordinated. Our results show that NO contributes substantially to such co-ordination, in analogy with similar actions by NO on signalling pathways activated by other receptor agonists [17,38].

Indeed, NO modifies profoundly the whole pattern of early signalling events triggered by E_2. A first effect is that of increasing E_2 ability of phosphorylating proteins on tyrosine. In particular, in the presence of NO, E_2 becomes competent to induce an early (min) phosphorylation, and thus activation, of IRS-1. We have investigated possible mechanisms underlying such effect of NO. Previous reports indicated that tyrosine phosphorylation of IRS-1 in the presence of E_2 occurs as a consequence of synergistic action between the hormone and agonists of IGF-1R [9–11]. Our results, however, indicate that the involvement of this receptor in mediating the effects of NO is unlikely, because co-administration of E_2 and NO did not result in increased IGF-1R activation. In contrast, we found that the effect of NO is mediated through generation of cGMP and activation of G kinase, a member of the serine/threonine family of protein kinases known to mediate several physiological effects of NO [23]. Phosphorylation of IRS-1 by other members of this family of kinases, namely protein kinase Cs, Akt [possibly via activation of mTOR (mammalian target of rapamycin)], stress and mitogen-activated protein kinases, was shown in various cell systems. This resulted, however, in impairment of IRS-1 function [39–42]. The ability of G kinase to activate, rather than inhibit, IRS-1 function might be due to direct phosphorylation on specific sites different from those affected by these other kinases, or mediated through phosphorylation of intermediate, as yet unidentified, molecules.

Until now involvement of IRS-1 in signalling by E_2 had been recognized to be limited to specific conditions, because it appeared to require either long-term exposure to the steroid, with increased expression of the adapter, or the combined administration of E_2 with insulin and IGF-1 [8–11]. The finding that NO enables activation of IRS-1 by E_2 independently of IGF-1R activation suggests that the role for the adapter in oestrogen signalling is broader than previously recognized. In particular, IRS-1 might be ordinarily involved in transducing E_2 signals in cells endowed with the E_2-activatable, endothelial NOS. This is of biological relevance in view of our findings that IRS-1, when activated, is central to regulation of the signalling pathways activated by E_2.

The first important consequence of IRS-1 involvement in E_2 signalling is the recruitment of PI3K and the ensuing activation of Akt. A stimulation of Akt by E_2 due to direct interaction of PI3K with the oestrogen receptor, i.e. without involvement of IRS-1, has already been described [4,15,16]. We found, however, that the two modes of Akt activation, i.e. IRS-1-dependent and independent, differ profoundly in terms of kinetics. IRS-1-independent activation is small and transient (see also [4] and [15]), whereas that induced by recruitment of IRS-1 through the NO/cGMP/G kinase pathway is persistent and more conspicuous.

There are important implications of this persistent Akt activation. Akt is a messenger through which E_2 activates endothelial NOS to generate NO [4,16]. In cells endowed with this enzyme NO might therefore act in a Akt-centred, feed-forward loop aimed at amplifying the initial steps of E_2 signalling. In addition, Akt has been recently shown to be among the signals through which various growth factors trigger phosphorylation of the activation function-1 domain of oestrogen receptor α, thereby enhancing receptor activity [43]. This, together with the observation that growth factors also generate NO/cGMP [38] suggests that the gaseous messenger does contribute substantially to the cross-talk between the signalling pathways activated by E_2 and growth factors. This might be of relevance in view of the regulatory role that such cross-talk plays in modulating biological functions of tissues target of oestrogen [1].

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Change in ERK 1/2 function is another important consequence of the E_2/NO-induced recruitment to E_2 signalling of the IRS-1–PI3K/Akt pathway. Activation of ERK 1/2, which is marked when either E_2 or NO/cGMP are administered independently ([28–32], and this paper), was inhibited when they were combined. This was consequent to activation of PI3K, because inhibition of PI3K with either wortmannin or LY 294002 reverted this effect, enabling additive activation of ERK 1/2 by the steroid and the gaseous messenger. Akt stimulation by PI3K is known to impair ERK 1/2 activation through inhibition of the Raf proteins [33]. Since both E_2 and NO (via G kinase) activate ERK 1/2 through the Raf/MEK pathway ([28,31,32] and this paper), it is conceivable that Akt-dependent inhibition of Raf is the mechanism underlying the switch-off of ERK 1/2 stimulation observed in the presence of E_2 and NO. Activation of ERK 1/2 is relevant to mitogenic effects of E_2 [37], which, however, involve not only physiological stimulation of growth but also its tumourigenic development, especially in reproductive organs [1]. Activation of the NO/cGMP pathway by E_2, and ensuing inhibition of ERK 1/2, might therefore contribute to regulate E_2 signalling and inhibit generation of abnormal mitogenic signals. This hypothesis is substantiated by the observation that MCF-7 cell growth triggered by E_2 was inhibited in the presence of cGMP. This inhibition was due to the cGMP-dependent blockade of ERK 1/2, mediated via activation of the PI3K/Akt pathway.

Akt and ERK 1/2 phosphorylate oestrogen receptor α, thereby enhancing the ability of E_2 to promote transcription of ERE-containing genes [34,35,44]. Consistent with these observations we found that inhibition of either kinase resulted in reduced activation of ERE by E_2, measured using the EREtkLuc reporter gene. The IRS-1-dependent changes in PI3K/Akt and ERK 1/2 activities induced by the concomitant administration of NO/cGMP together with E_2, instead, did not modify significantly the effects of E_2 on ERE, most likely because of the balance between inhibition of ERK 1/2 and stimulation of Akt. Thus, while inhibiting E_2-dependent cell growth, NO/cGMP appear to maintain the ability of the steroid to stimulate gene transcription. It must be noted, however, that gene transcription by E_2 involves interaction of oestrogen receptors with a vast array of co-activators and co-repressors [45]. Direct effects of NO/cGMP on these proteins, with changes in their ability to interact with oestrogen receptors or target sequences, cannot be excluded by our experiments. In addition, NO/cGMP activates the AP-1 transcription complex, which may contribute to E_2-dependent gene transcription [24,46,47]. Thus, the combination of E_2 with NO/cGMP might also lead to changes in expression of specific genes. E_2 sustains differentiation of target tissues, especially reproductive organs, to which NO also contributes [1,48]. Cell growth and gene expression are relevant to this function of the steroid. In view of this, the dual action by NO on E_2 signalling described here might have evolved to contribute to the developmental actions by E_2.

Activation of IRS-1 by the combined action of E_2 and the NO-stimulated cGMP/G kinase pathway may result in downstream signalling events additional to those reported here. IRS-1 has over 20 potential tyrosine-phosphorylation sites, which serve as binding sites to a variety of SH2 domain-containing messenger molecules [7]. Whether the pattern of tyrosine phosphorylation induced by co-stimulation with E_2 and NO leads to recruitment of PI3K/Akt only or also of any of these other effectors remains to be investigated.

In conclusion, our results show that NO, through generation of cGMP and activation of G kinase, modifies profoundly E_2 early signalling and contributes to regulate long-term actions of the steroid. Generation of NO, formation of cGMP and activation of G kinase is triggered in most cells by a variety of receptors, including tyrosine kinase and G-protein-coupled receptors, as well as receptors for cytokines, in both physiological and pathological conditions [17,23,38]. It is conceivable, therefore, that the overall regulation of E_2 signalling by NO described here is a common pathway regulating oestrogen biological activity.

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