

Oestriol and oestradiol increase cell to cell communication and connexin43 protein expression in human myometrium

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Oestradiol increases the protein expression of connexin43 (Cx43) gap junctions in myometrium but the effect of oestriol on gap junction expression has not been described previously. Oestriol is the most abundant free oestrogen in pregnant women and there is a marked surge in oestriol concentrations before term and idiopathic preterm labour. In order to determine whether oestriol may have a physiological action on the myometrium, cultured human myometrial cells obtained from non-pregnant hysterectomy specimens were exposed to 10 nmol/l oestradiol or oestriol. Intercellular communication between myometrial cells was investigated by microinjection of confluent cultured cells with the gap junction-permeant tracer Cascade Blue. There was a progressive increase in coupling after exposure to oestradiol or oestriol ($P < 0.0005$). An increase in Cx43 protein expression was demonstrated by immunocytochemistry after 1 h ($P < 0.01$) and 3 days ($P < 0.01$) exposure, and by Western blotting after 1 h ($P < 0.01$) and 3 days ($P < 0.05$) exposure, to both oestradiol and to oestriol. We conclude that oestriol increases gap junction communication in human myometrium by increasing gap junction expression. Elevated oestriol concentrations may thus play a role in the initiation of labour in women, by increasing cell-cell communication in the myometrium.

Key words: connexin43/labour/myometrium/oestradiol/oestriol

Introduction

Preterm delivery is a major cause of perinatal morbidity and mortality, and of long-term disability. In order to decrease the rate of preterm birth it is necessary to have an understanding of the factors that lead to the onset of term and preterm labour. The endocrine events of late pregnancy have been extensively studied in animals. Parturition in sheep and other mammals is preceded by an increase in maternal plasma 17β -oestradiol and a decrease in plasma progesterone concentrations (Liggins *et al.*, 1973). Progesterone maintains uterine quiescence and oestrogen promotes the changes that are necessary for synchronized contractions to occur.

Labour in humans and other mammals is preceded by increases in myometrial gap junction communication which correlate with an increase in connexin43 (Cx43) gap junction mRNA and protein concentrations (Garfield *et al.*, 1977; Hendrix *et al.*, 1992; Tabb *et al.*, 1992; Balducci *et al.*, 1993; Kilarski *et al.*, 1993; Lye *et al.*, 1993). This increased communication is essential for the coordination of uterine

contractions. Oestradiol is known to increase myometrial gap junction formation and Cx43 transcripts (Garfield *et al.*, 1980; Petrocelli and Lye, 1993; Ambrus and Rao, 1994; Kilarski *et al.*, 2000) but, in contrast to that observed in sheep, there is no dramatic rise in oestradiol concentrations before labour in women.

Unlike the sheep placenta, the human placenta cannot convert progesterone to oestradiol as it lacks the 17β -hydroxylase enzyme (Voutilainen and Miller, 1986). The human placenta, however, produces large amounts of oestriol, which is an oestrogen unique to higher primates whose plasma concentrations of oestriol, like those of oestradiol in sheep, rise dramatically in late pregnancy. Oestriol is formed from dehydroepiandrosterone sulphate (DHEAS) which is produced in great quantities by the large fetal zones of the fetal adrenals. DHEAS is 16α -hydroxylated in the fetal liver and then aromatized to oestriol in the placenta. Most of the unconjugated oestriol in plasma is bound to plasma proteins, whereas saliva contains only the unbound unconjugated fraction and thus

reflects the free, biologically active, oestriol concentrations to which cells are exposed. There is a 3-fold increase in oestriol concentrations in saliva during the weeks before the spontaneous onset of labour at term, with a marked increase in the saliva oestriol/progesterone ratio (McGarrigle and Lachelin, 1984; Darne *et al.*, 1987a). Saliva oestriol concentrations are twenty times higher than saliva oestradiol concentrations at term and there is little change in the saliva oestradiol/progesterone ratio in the second half of pregnancy. There is also an inappropriately early rise in the saliva oestriol/progesterone ratio in women prior to the idiopathic onset of preterm labour (Darne *et al.*, 1987b), but no such rise occurs in women who have not entered labour by 42 weeks gestation (Moran *et al.*, 1992). Further studies based on our earlier work (Darne *et al.*, 1987b) have confirmed a rise in oestriol concentrations prior to preterm labour (McGregor *et al.*, 1995; Heine *et al.*, 2000). A saliva oestriol test has been developed (Salest, Biex California USA), and licensed in the United States by the Federal Drug Administration (FDA), as a screening test for preterm labour (Voss, 1999).

These findings led us to speculate that oestriol may play the same role in women that oestradiol does in sheep and other mammals, in preparing the myometrium for labour by increasing gap junction expression and by causing other oestrogenic effects (McGarrigle and Lachelin, 1984). As in other mammals, the signal for the preparation of the myometrium for the onset of labour would come from the fetus; in humans this would be in the form of 16-DHEAS, the precursor of oestriol. Oestriol was long considered to be an impeded (or physiologically weaker) oestrogen, compared to oestradiol, based on dose-dependent uterine weight increases following single injections of oestradiol or oestriol in hypophysectomized female rats (Huggins and Jensen, 1955). However, oestriol was subsequently found to be equipotent to oestradiol in stimulating uterine growth, when administered repeatedly, thus mimicking sustained blood concentrations over a period of time (Martin *et al.*, 1976; Clark *et al.*, 1977; Katzenellenbogen, 1984). Furthermore oestriol has been shown to bind to human myometrial oestrogen receptors *in vivo* (Wiegerinck *et al.*, 1983). More recent studies in women have confirmed the potency of sustained high concentrations of oestriol on myometrial oestrogen and progesterone receptor formation (van Haften *et al.*, 1997). If the rise in oestriol that occurs prior to both labour at term and idiopathic preterm labour (Darne *et al.*, 1987a,b; McGregor *et al.*, 1995) is found to be important in initiating labour, it may be possible to reduce the incidence of preterm birth by counteracting the effect of rising oestriol concentrations by treatment with progesterone or by some other way. Progesterone has been shown to have a tocolytic effect on the myometrium and has been used in the management of preterm labour (Erny *et al.*, 1986); saliva oestriol or oestriol/progesterone measurements may help in the selection of the women who are most likely to respond to treatment.

Uterine contractility is dependent on the propagation of action potentials between myometrial cells, and gap junctions are therefore of primary importance in the regulation of uterine contractile activity (Sims *et al.*, 1982; Miyoshi *et al.*, 1998).

Gap junction channels are composed of two hemichannels (connexons), one provided by each cell. Each connexon is made up from six transmembrane protein subunits (connexins). The docking of two connexons forms a conduit between cells which allows the passage of ions and small molecules between cells (Kumar and Gilula, 1996; Kumar, 1999). Gap junctions have diverse roles throughout the body; in the myometrium, as in the heart, they serve to synchronize muscular contractions. The family of connexin proteins includes at least 16 members, named according to their molecular weight, or by a nomenclature system based on sequence homology using a Greek letter and a number. The major gap junction protein in the myometrium is Cx43/ α 1 (Tabb *et al.*, 1992).

In-vitro studies on human myometrium have shown an increase in Cx43 protein concentrations and gap junction expression with oestradiol treatment (Ambrus and Rao, 1994; Kilarski *et al.*, 2000), but no studies investigating the effect of oestriol on Cx43 expression have been reported. We have therefore explored our hypothesis, that oestriol can stimulate Cx43 protein expression and increase functional communication, by examining the effect of oestradiol and oestriol on gap junctional communication and Cx43 protein expression in cultured human myometrial cells.

Materials and methods

Cell culture

Fresh human myometrium was obtained from the uteri of seven women aged 36–60 years who underwent hysterectomy for benign conditions. Samples were taken from the middle of the anterior uterine wall, ~5 mm away from endometrial or serosal surfaces. The tissue was collected in ice-cold saline, washed and processed for cell dispersion. Myocytes were prepared by enzymatic dispersion of the myometrium as previously described (Phaneuf *et al.*, 1993) with a minor modification. Briefly, pieces of tissue were digested with enzyme solution composed of 1% bovine serum albumin (BSA) and 1 mg/ml collagenase 1A, 1 mg/ml collagenase XI and Dulbecco's modified Eagles' medium (DMEM) (all from Sigma, Poole, UK) for 2–3 h at 37°C. The cell suspensions were dissociated using a Pasteur pipette and undissociated cells were removed by sieving through a 70 μ m cell strainer (Becton Dickinson, Marathon Laboratories, UK). The solution containing free myocytes was centrifuged and the cell pellet was washed twice in DMEM. The final pellet was resuspended and plated in tissue culture flasks. All cultures were grown in Phenol Red-free DMEM, supplemented with 10% charcoal-stripped fetal bovine serum (FBS) (Gibco, Paisley, UK), with 2 mmol/l L-glutamine and 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma), to avoid the oestrogenic effects of Phenol Red (Berthois *et al.*, 1986) and of the endogenous steroids in FBS (Andersen *et al.*, 1993). The growth medium was changed twice a week and the cells were freed from the flasks after reaching confluence, with ethylenediamine tetra-acetic acid (EDTA)-trypsin (Sigma), and passaged to flasks or coverslips for subculture. All experiments were performed on subcultures 2–8.

Oestradiol and oestriol treatment

Oestradiol or oestriol, 10 nmol/l (Sigma), in Phenol Red-free DMEM supplemented with 2% charcoal-stripped FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin was applied to cultured myometrial cells after confluence for either 1 h or 3 days.

Control cells were cultured under the same conditions without oestradiol or oestriol.

Intracellular dye injection and immunocytochemistry

Microelectrodes, with a resistance of 50–60 M Ω in 1 mol/l lithium chloride, were loaded with a 5% solution of a Cascade Blue derivative 8-methoxy pyrene-trisulphonic acid (mol. wt 558) (Molecular Probes, Oregon, USA) and backfilled with 1 mol/l lithium chloride. Confluent cells on coverslips were transferred into a dish containing DMEM on a DMFLS upright fixed-stage fluorescence microscope (Leica, Milton Keynes, UK) for injection. Cells were visualized using infrared optics and penetration was monitored both visually and by changes in membrane potential. Following impalement of a cell by the electrode, Cascade Blue was iontophoresed into the cell, at 1–5 nA, for 1 min. The transfer of Cascade Blue from the injected cell to neighbouring cells was monitored for a further minute on the microscope and the image was recorded using a DC 200 digital camera (Leica). Ten control injections were made in each culture before 10 nmol of oestradiol or oestriol was added to the medium and injections were continued every few minutes for 100 min after the addition. The morphology of the cells was checked and the few non-myometrial cells that had been injected were excluded from the analysis. Data were analysed statistically using Kruskal-Wallis analysis of variance (ANOVA) by ranks and the Mann-Whitney *U*-test.

Immunostaining was performed as previously described (Becker *et al.*, 1995). Cells grown on coverslips and exposed to oestradiol or oestriol for 1 h or 3 days were rinsed in PBS, fixed with 4% buffered paraformaldehyde (pH 7.4), rinsed with PBS and incubated in PBS containing 0.1 mol/l lysine and 0.5% Triton X-100 (Sigma) for 30 min at room temperature. After rinsing, the cells were incubated with monoclonal anti-Cx43 antibody (peptide position 252 to 270; Transduction Laboratories, Exeter, UK) diluted 1:500 in PBS, in a humidified chamber for 2 h at 37°C. After several rinses with PBS, the cells were incubated in the dark for 1 h at room temperature with fluorescent isothiocyanate-conjugated rabbit anti-mouse IgG (Dako, Denmark) diluted 1:25 in PBS. After rinsing with PBS, the coverslips were mounted in Citifluor on slides (UKC Chemical Laboratories, Canterbury, UK).

Images were recorded using a TCS 4D laser scanning confocal microscope (Leica, Milton Keynes, UK). The distribution of gap junction plaques between cultured myometrial cells was heterogeneous, as in the myometrium *in vivo*, and extensive sampling over large areas was required to obtain reliable data. The process of obtaining images was performed according to a published method (Rezapour *et al.*, 1997). Briefly, five non-overlapping but adjacent sets of serial optical sections were recorded from each slide. Each set consisted of five optical sections (158 \times 158 μ m) at intervals of 1 μ m in the *z*-axis. Projections of each set of optical sections were then prepared, and the image data were saved digitally. Images from each slide were recorded under the same brightness and contrast setting. Plaques in thresholded binary images were analysed on a Macintosh computer using the public domain software 'National Institutes of Health (NIH) Image' to measure the size and number of plaques. Data were analysed statistically using Kruskal-Wallis ANOVA by ranks and the Mann-Whitney *U*-test.

Western blotting

Myometrial cells grown in 25 cm² flasks were exposed to oestradiol or oestriol for 1 h or 3 days and then rinsed with cold PBS and scraped off. Cell pellets were lysed in a lysis buffer composed of 20 mmol/l HEPES (pH 7.8), 150 mmol/l NaCl, 0.2 mmol/l EDTA, 0.5% Igepal CA-630, 20% glycerol, 0.5 mol/l dithiothreitol (DTT), 0.5 mmol/l phenylmethylsulphonyl fluoride (PMSF), 0.5 μ g/ml leupep-

tin, 0.5 μ g/ml Sigma protease inhibitor, 0.5 μ g/ml aprotinin and 1 mmol/l diisopropylfluorophosphate (DIFP). Samples were then incubated for 30 min at 4°C and centrifuged at 10 000 *g* for 15 min to pellet the insoluble material. The total protein concentration in the supernatant was determined (Bradford, 1976) using the Bio-Rad protein assay kit (Hertfordshire, UK). Samples from the supernatant were further diluted in 5 \times sample buffer containing 10% β -mercaptoethanol, 10% sodium dodecyl sulphate (SDS), 10% glycerol, 0.004% Bromphenol Blue and 0.125 mol/l Tris/HCl (pH 6.8), boiled for 3–5 min and applied (8 μ g/lane) onto a 10% polyacrylamide gel (Laemmli, 1970). After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, UK) by semi-dry electroblotting. Blots were probed for 3 h with monoclonal anti-Cx43 antibody (peptide position 252 to 270; Transduction Laboratories) diluted 1:250. After washing, blots were incubated for 1 h with peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed, Cambridge Bioscience, UK) diluted 1:2000 in PBS and the bound Cx43 antibodies on the blots were visualised using an enhanced chemiluminescent reagent, ECL-plus (Amersham Pharmacia Biotech). The blots were then re-probed for 3 h with monoclonal anti-desmin antibody (55 kDa; Transduction Laboratories) diluted 1:100 and the process was repeated as above. The immunoblot films were scanned on a flat-bed scanner and band densities were obtained by measuring the area of a density profile curve for each band. Load variations were corrected by calculating the ratio of the densities of Cx43 to desmin, a muscle-specific intermediate filament protein. The ratio of phosphorylated to unphosphorylated forms was also calculated. The data were analysed using Kruskal-Wallis ANOVA by ranks and the Mann-Whitney *U*-test.

The study had the approval of the joint University College London/University College London Hospitals Committee on the Ethics of Human Research.

Results

Intercellular communication after treatment with oestradiol or oestriol

Ten intracellular injections of Cascade Blue dye were performed over a period of ~30 min before the addition of oestradiol or oestriol. Baseline coupling varied from 0–2 cells with a mean of 0.58 cells. Non-myometrial cells had a very different morphology from myometrial cells; they were rarely coupled and were not included in the data analysis. Representative examples of microinjection and coupling are shown in Figure 1 and the time courses of changes in coupling after exposure of cells to oestradiol or oestriol are shown in Figure 2. Following the addition of oestradiol or oestriol, the number of coupled cells gradually increased to an average of 4.3 (oestradiol, *n* = 10) or 4.3 (oestriol, *n* = 12) in the second 60–100 min (*P* < 0.0005).

Changes in Cx43 plaque size and number after treatment with oestradiol or oestriol

Immunostaining of confluent control cultures of myometrial cells with Cx43 specific antibodies revealed gap junction plaques between the cells (Figure 3). Quantification of control concentrations of Cx43 plaques, using NIH Image, revealed an average of 9.3 plaques per 1000 μ m² with an average plaque size of 0.7 μ m² giving a total stained area of 6.9 μ m² per 1000 μ m². After 3 more days in culture (without treatment),

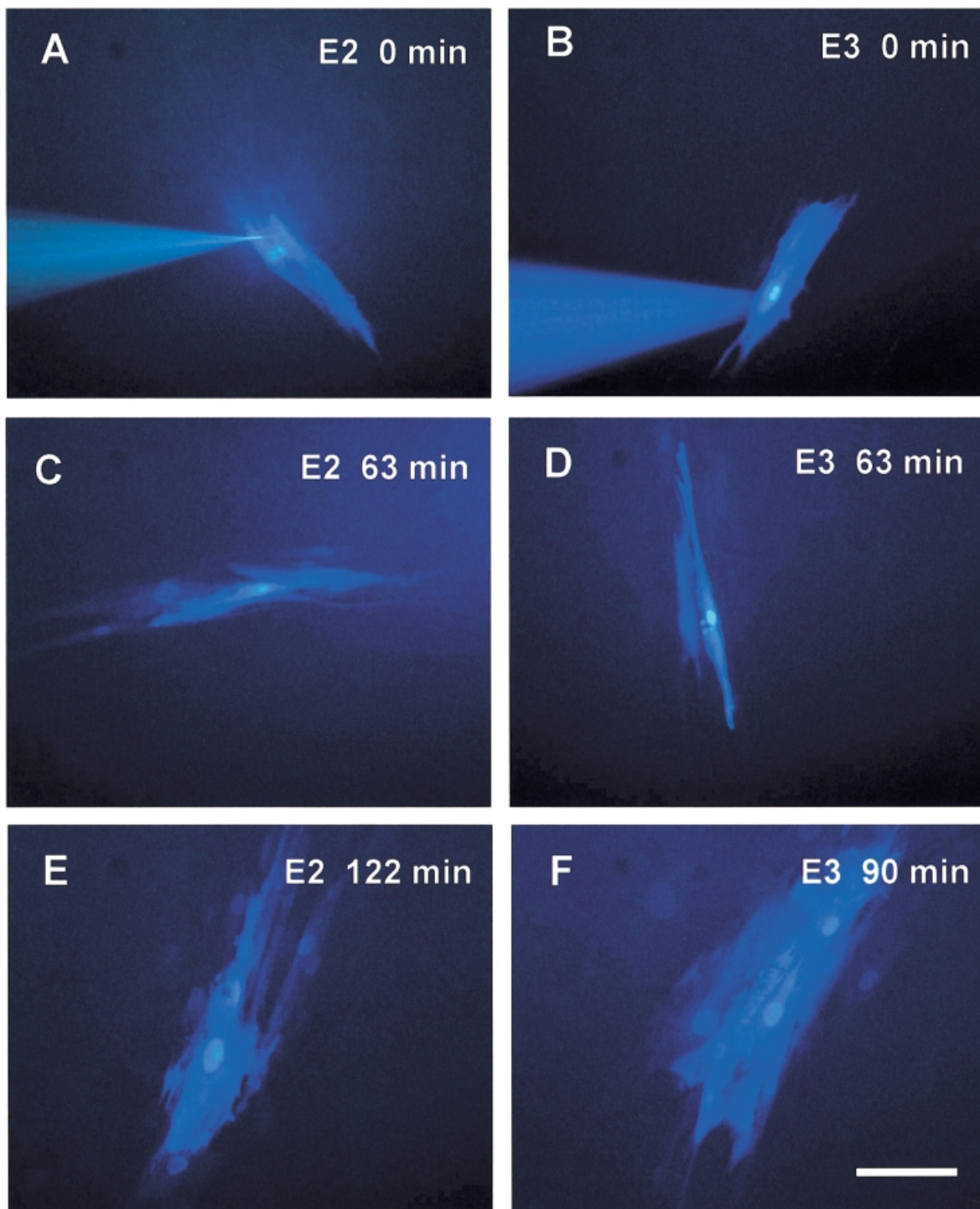


Figure 1. Images of the microinjection of confluent cultures of myometrial cells with the gap junction permeant tracer Cascade Blue. (A and B) In controls prior to treatment, baseline coupling varied from 0 to 2 cells with dye passing to a mean of 0.58 cells. Following exposure to oestradiol (E2) or oestriol (E3) microinjections of dye transfer made at progressive time points showed increased numbers of coupled cells (C and D). After 90 or 120 min (E and F), when an injection was made, clusters of cells filled rapidly with dye, with a mean of more than four cells filled per microinjection. Scale bar = 50 μm .

the size and number of Cx43 positive plaques had increased to an average of 21.9 plaques per 1000 μm^2 with an average size of 1.9 μm^2 and thus a total stained area of 41.3 μm^2 per 1000 μm^2 .

There was a rapid increase in Cx43 protein expression following treatment of confluent cultures with either oestradiol

or oestriol (Figure 3). After exposure to oestradiol for 1 h, the average plaque number was 17.9 plaques per 1000 μm^2 with an average plaque size of 1.0 μm^2 and a total stained area of 17.7 μm^2 per 1000 μm^2 . After exposure to oestriol for 1 h, the average plaque number was 14.2 per 1000 μm^2 and the average plaque size was 0.8 μm^2 with a total stained area of

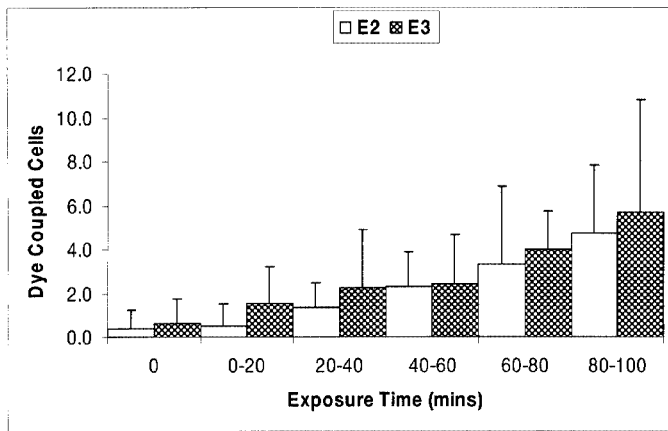


Figure 2. Bar graph showing the increase in dye coupling of myometrial cells, following exposure to oestradiol (E2) or oestriol (E3) over a period of 100 min. Cascade Blue was iontophoretically injected into single myometrial cells, at progressive time points, and the number of coupled cells was recorded after 2 min. Time 0 represents the 10 control injections made before addition of oestradiol or oestriol.

11.9 μm^2 per 1000 μm^2 . The number of plaques was significantly greater in cultures treated with either oestradiol or oestriol than in control cultures ($P < 0.01$) (Figure 4).

Cx43 expression increased further after 3 days exposure to either oestradiol or oestriol (Figure 3). There was an average of 36.9 plaques per 1000 μm^2 with an average size of 2.5 μm^2 and a total stained area of 94.3 μm^2 per 1000 μm^2 with oestradiol treatment. Treatment with oestriol gave an average of 38.2 plaques per 1000 μm^2 with an average size of 1.9 μm^2 and a total stained area of 69.1 μm^2 per 1000 μm^2 . The number of plaques was significantly greater in cultures treated with either oestradiol or oestriol than in 3 day control cultures ($P < 0.01$) (Figure 4).

Changes in Cx43 protein on Western blot after treatment with oestradiol or oestriol

A typical series of Western blots is shown in Figure 5. The total amount of Cx43 protein in all the bands was calculated after correcting for load variations by calculating the ratio of the densities of Cx43 to desmin in each lane. In the majority of cultures, the expression of total Cx43 protein increased significantly, when compared to control concentrations, after the cultured myometrial cells were exposed to oestradiol or oestriol for 1 h ($P < 0.01$) and 3 days ($P < 0.05$). There was no significant difference between the ratios of Cx43 protein expression between oestradiol- and oestriol-treated cells at either 1 h or 3 days, as shown by Western blotting.

Cx43 has several phosphorylated forms running between 42 and 46 kDa on Western blots (Figure 5). The bands were separated according to the area of a density profile curve and termed Cx43-P0 (P0), Cx43-P1 (P1) and Cx43-P2 (P2) (Musil and Goodenough, 1991). We confirmed that these were different phosphorylated forms of Cx43 by incubating lysates with λ -protein phosphatase as previously described (Thomas *et al.*, 1998). In control cells the relationship of the concentrations of phosphorylated forms was generally $P2 > P1 > P0$. The ratio of P1:P0 was unchanged after treatment with oestradiol

or oestriol. In four cases the ratio of P2:P0 decreased after exposure to oestriol for 1 h, but the difference was not statistically significant ($P = 0.08$).

The phosphorylation states were further analysed by probing different fractions of lysate (whole lysate, supernatant and lysis buffer-insoluble pellet) on immunoblots. The three Cx43 forms appeared in all fractions with no ratio changes, i.e. all Cx43 forms were soluble in the lysis buffer containing 0.5% Igepal CA-630. Thus the results of the immunoblots reflect the phosphorylation states of Cx43 and are not due to differential extraction of particular forms of the protein.

Discussion

Oestradiol has been shown to increase gap junction expression in both rat and human myometrium (Garfield *et al.*, 1980; Petrocelli and Lye, 1993; Ambrus and Rao, 1994; Kilarski *et al.*, 2000), but the effect of oestriol on myometrial gap junctions has not been described previously. We chose to study non-pregnant myometrium in order to remove the background influence of the high circulating and tissue steroid concentrations that are present in pregnancy. We used equimolar concentrations of oestriol and oestradiol (10 nmol/l) in order to compare their effects on myometrial gap junctions. This concentration is similar to that of the plasma free (unbound unconjugated) oestriol in women at term but it is $\sim 20\times$ that of plasma free oestradiol at term (Darne *et al.*, 1987a).

Cx43 gap junctions have previously been demonstrated in human non-pregnant myometrial cells in culture (Ciray *et al.*, 2000). Gap junction plaques were clearly visible in our control cultures but the basal level of coupling between cells, as shown by dye transfer, was low before treatment with oestradiol or oestriol. Following the addition of oestradiol or oestriol to the culture medium, intracellular dye injection revealed a progressive, and significant, increase in the level of intercellular dye transfer. The time course of the increase in coupling following oestrogen treatment is more consistent with new gap junction channels being inserted into the membranes than with an increase in the conductance of existing channels, which would be more rapid (Sakai *et al.*, 1992). This is supported by our Cx43 immunostaining studies, which revealed an increase in the number of gap junction plaques within 1 h of treatment with oestradiol or oestriol. An increase in total Cx43 protein concentrations was also demonstrated after treatment with oestradiol or oestriol for 1 h.

Analysis of the immunoblots for the ratios of the different phosphorylation forms of Cx43 showed considerable variation between patients. There was a trend towards a reduced ratio of P2/P0 forms of Cx43 after oestriol treatment for 1 h but this was not statistically significant. Cx43 is synthesized as a single P0 species that runs at 42 kDa and is converted to P1 (44 kDa) and then to P2 (46 kDa) by the addition of phosphate to serine residues (Musil *et al.*, 1990a,b; Musil and Goodenough, 1990). These authors (Musil and Goodenough, 1991) demonstrated that the P0 form is transported to the cell surface where the phosphorylation to P1 and P2 occurs. Other studies have shown that the phosphorylated forms may be functionally involved in the assembly of Cx43 into morpho-

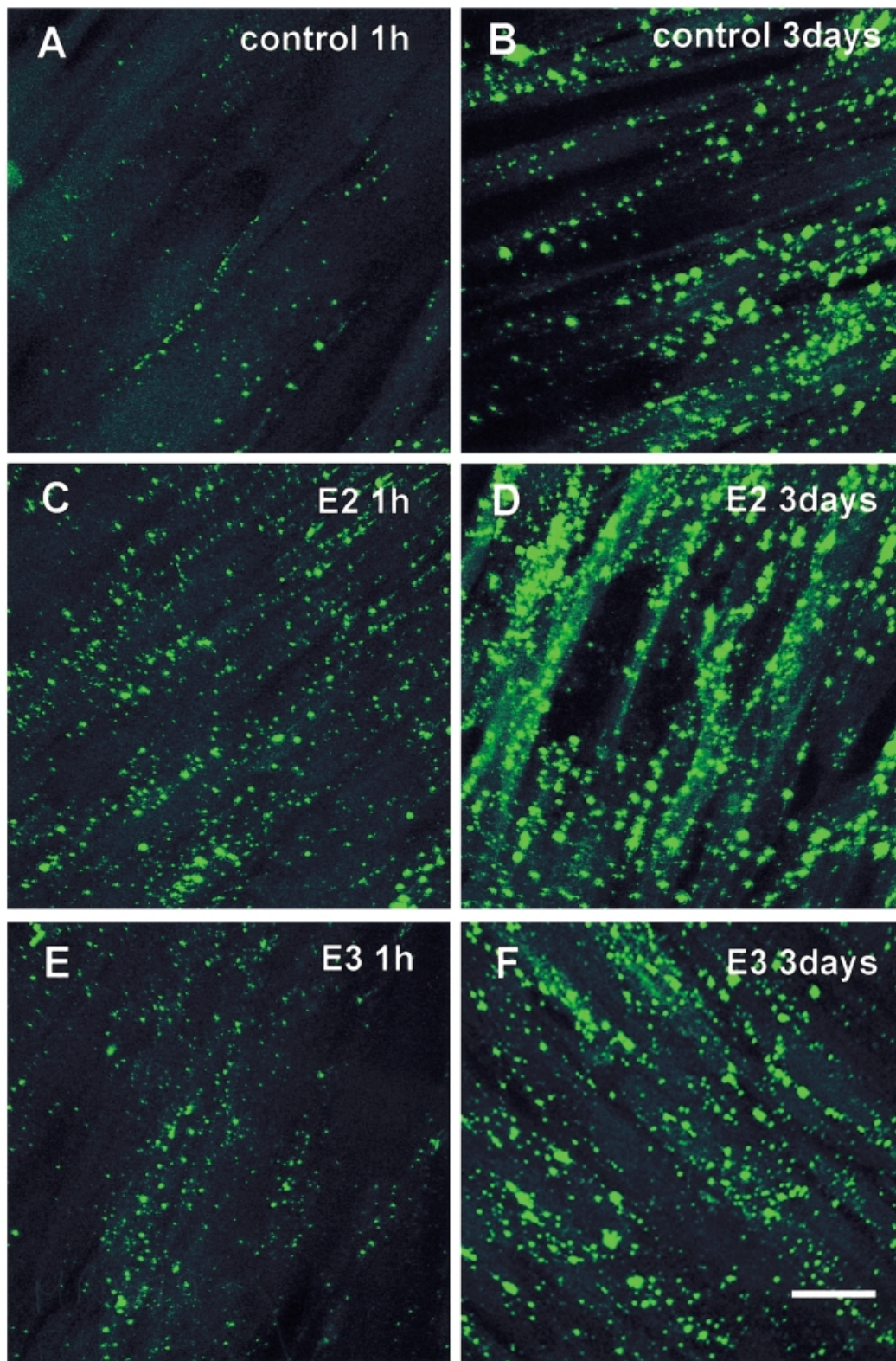


Figure 3. Localization of Cx43 gap junction plaques demonstrated by immunostaining confluent cells from typical myometrial cell cultures. (A and B) Cx43 plaques in untreated (control) cultured cells at 1 h or 3 days respectively. The number of plaques increased within 1 h of treatment with oestradiol (E2) or oestriol (E3). (C and D) Cells treated with oestradiol for 1 h (C) or 3 days (D). (E and F) cells treated with oestriol for 1 h (E) or 3 days (F). Scale bar = 25 μ m.

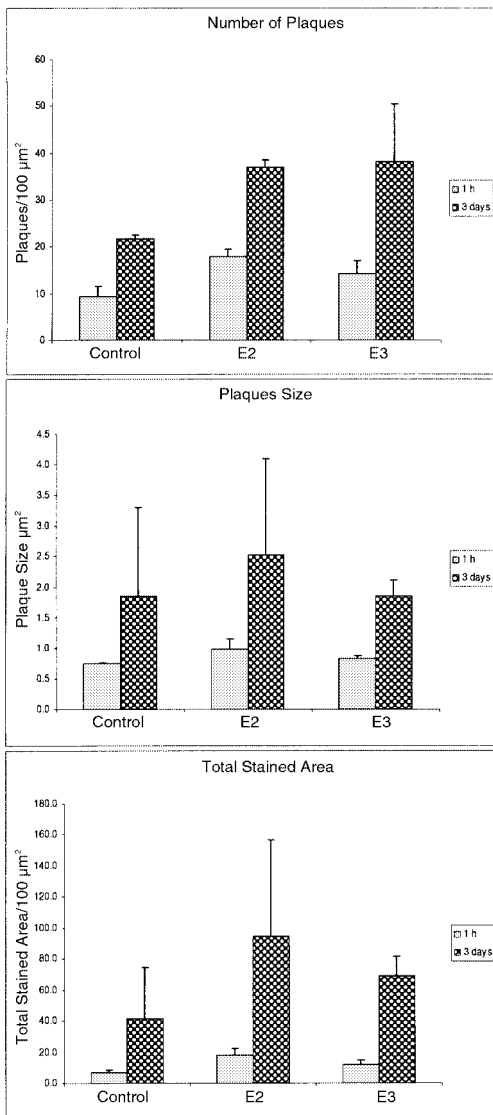


Figure 4. The results of quantitative analysis of Cx43 immunostaining of myometrial cultures exposed to no treatment, or to oestradiol (E2) or oestriol (E3), for 1 h or 3 days. The bar graphs (\pm SD) show the number of plaques/1000 μm^2 , plaque size and the number of positively stained pixels/1000 μm^2 .

gically and physiologically recognisable gap junctional plaques (Musil *et al.*, 1990a,b; Musil and Goodenough, 1990; Moreno *et al.*, 1994; Saez *et al.*, 1997). Thus changes in ratios of the phosphorylated forms of Cx43 may reflect both generation and insertion of new connexons.

Our original interpretation of our findings of a marked increase in oestriol and the oestriol/progesterone ratio before spontaneous labour at term was based on the models available at the time. We envisaged increasing competition between oestriol and oestradiol for ligand binding sites on myometrial oestrogen receptors (ER), with oestriol becoming progressively more dominant in late pregnancy (McGarrigle and Lachelin, 1984; Darne *et al.*, 1987a). Our findings of an inappropriately early rise in the oestriol/progesterone ratio in women in preterm labour, and no change in the ratio in women with prolonged gestation, reinforced our hypothesis of a role for oestriol in the initiation of labour (Darne *et al.*, 1987b; Moran *et al.*, 1992).

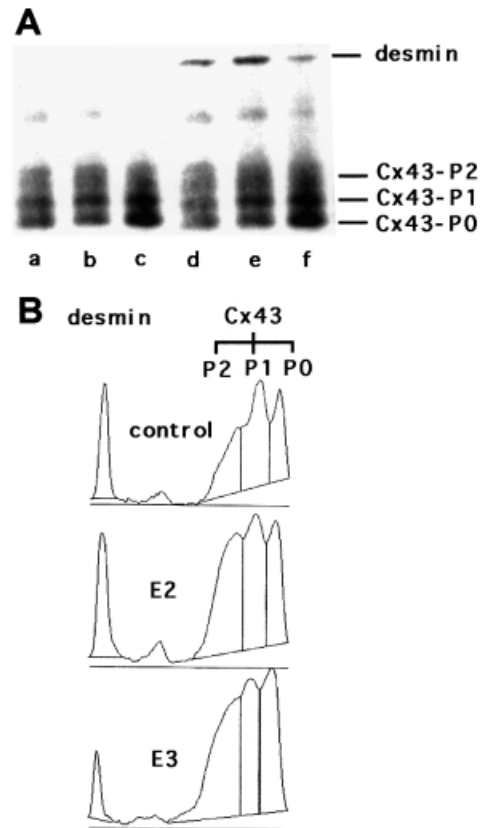


Figure 5. Western blot analysis of Cx43 phosphorylation forms. (A) Western blots showing the total Cx43 concentrations and phosphorylation states in: lane a, control cells; lane b, cells exposed to oestradiol (E2) for 1 h; lane c, cells exposed to oestriol (E3) for 1 h; lanes d, e and f, the same immunoblots reprobed with anti-desmin antibody. (B) Density profile curves of the Western blots from lanes d, e and f which were scanned and the density profile curve then determined using 'NIH Image' software.

ER are now known to exist in at least three forms, ER α , ER β (Kuiper *et al.*, 1996) and its isoform ER β 2 (Petersen 1998), with their own ligand binding characteristics and variable tissue distribution (Kuiper *et al.*, 1997). The potential for oestrone, oestradiol and oestriol to differentially modulate oestrogen stimulated events by effecting ER dimerisation and thus interacting with oestrogen response elements (ERE) on DNA is therefore considerable. For example ER β has greater affinity than ER α for oestriol, whereas the reverse is true for oestrone (Kuiper *et al.*, 1997) and the ER β 1 isoform has much greater affinity for oestriol and oestrone than does the ER β 2 isoform (Petersen *et al.*, 1998). If heterodimerization of the α and β forms also takes place this would further extend the potential for oestrogens such as oestriol to modulate gene transcription. Interestingly, ER β has recently been found to be the predominant form of ER in human myometrium at term (Wu *et al.*, 2000).

Although half-palindromic ERE have been described in the promoter region of the *Cx43* gene (Yu *et al.*, 1994; Piersanti and Lye, 1995; Geimonen *et al.*, 1996), functional proof of activation of these ERE by oestrogen, though possible, has not yet been reported. However, an alternative potential pathway for oestriol influence on Cx43 expression exists. It has

been shown that oestradiol-activated dimers interact with c-fos and c-jun transcription factors at AP-1 sites (Gaub *et al.*, 1990). The direct involvement of the AP-1 site in the transcription of Cx43 has been reported in rat myometrium (Piersanti and Lye, 1995) and in cultured human myometrium (Geimonen *et al.*, 1996; Echeteu *et al.*, 1999). The increased affinity of ER β for oestriol clearly allows oestriol to modulate AP-1 response elements and Cx43 expression, without the need for direct interaction with ERE on DNA.

In summary we have demonstrated, using complementary approaches, that oestradiol and oestriol have a similar effect on Cx43 expression in cultured human myometrial cells. Both oestradiol and oestriol increased intercellular communication within 1 h and increased expression of total Cx43 protein concentrations as demonstrated by immunostaining and immunoblotting. Though the present study was performed *in vitro*, the myometrial cells were responsive to 10 nmol/l of oestriol, a concentration similar to the free plasma oestriol concentration of 5–8 nmol/l in women at term. This is further evidence that oestriol may have a key biological effect in human pregnancy in preparing the myometrium for labour by increasing the expression of Cx43 and thus intercellular communication in the myometrium.

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