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Rapid paper

Progesterone effect on intracellular inorganic sulphate in uterine epithelial cells

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Summary

The effect of progesterone on the available intracellular sulphate pool in subcultured glandular epithelial cells from guinea-pig endometrium is reported. Progesterone in concert with 17β-estradiol was shown to cause an increase in the available intracellular sulphate pool. The maximum effect was obtained for 10^{-8} M and 10^{-7} M progesterone. This effect of progesterone on the available intracellular sulphate pool essentially concerned the intracellular inorganic sulphate and was inhibited by the antiprogesterone steroid RU 486 (5×10^{-7} M). Sulphate incorporation into the endometrial epithelial cells was suppressed by the inhibitor of anion transport diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and the protein synthesis inhibitor, cycloheximide. These results would suggest that a sulphate transport system may be involved in the accumulation of the intracellular sulphate, stimulated by progesterone. This phenomenon could be an early process in the preparation of the endometrium for implantation.

Introduction

The uterus synthesizes and secretes sulphated components which can play an important role in the transport and implantation of the blastocyst (Tang et al., 1987; Morris et al., 1988; Kubushiro et al., 1989; Cidadao et al., 1990). Steroid hormones have been shown to increase synthesis and secretion of uterine sulphated proteins (Takata and Terayama, 1977; Munakata et al., 1985). This effect of steroid hormones is specially marked in uterine epithelial cells (Tang et al., 1987; Carson et al., 1988; Morris et al., 1988; Aplin et al., 1989). As previously reported, progesterone increased the secretion of sulphated proteins (Thiard et al., 1989a) and the synthesis and secretion of tyrosine sulphated proteins (Thiard et al., 1989b) in guinea-big uterus.

To determine the contribution of epithelial cells in this uterine response, this study was aimed at investigating the progesterone effect on the endogenous sulphate concentration in guinea-pig endometrial glandular epithelial cells (GEC) subcultured on a basement membrane matrix in a serum-free, chemically defined medium. We demonstrate that progesterone increases intracellular inorganic sulphate concentration in glandular epithelial cells and this effect is inhibited by the antiprogesterone steroid, RU 486.

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Materials and methods

Materials. Mature female guinea-pigs of the albino Dunkin Hartley strain were obtained from Charles River France (St Aubin, France). Tissue culture media and supplies were obtained from Vietech (St Bonnet, France). Tissue culture dishes and supplies were obtained from Falcon (Illkirch, France). [^{35}S]Na_2SO_4 carrier-free (41 TBq/mmol) was from NEN (Paris, France). 17β-Estradiol (E_2), progesterone, mouse EGF, diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), cycloheximide, PEI cellulose pre-coated plates and other chemicals were from Sigma Chimie (La Verpillière, France). RU 486 was donated by Roussel-Uclaf Laboratories (Romainville, France). Engelbreth Holm Swarm Tumor Matrix (Matrigel) was from Collaborative Research (Velizy, France). Sephadex G-25 (fine) was from Pharmacia-France (St Quentin, France). Mouse monoclonal antibody raised against guinea-pig progesterone receptors (mPR II) was purchased from Transbio (Paris, France).

Cell culture. Endometrial epithelial glands were isolated from uteri of mature guinea-pigs as described by Chaminadas et al. (1986) and cultured as recently reported by Mahfoudi et al. (1991). Briefly, epithelial glands were cultured in phenol red-free Ham's F12 medium containing 5% (v/v) fetal calf serum (FCS). Subconfluent primary cultures were obtained after 5–7 days, then the cells were subcultured at a density of 5×10^4 cells/cm^2 on 35 mm plastic plates coated with Matrigel, in a chemically defined medium (CDM) composed of phenol red-free supplemented Ham's F12 containing 10^{-8} M E_2. This medium was renewed after 2 days and subcultured cells reached confluency within 4 days of culture. The percentage of epithelial cells in the confluent subcultures was determined by indirect immunostaining with an anticytokeratin monoclonal antibody (Chaminadas et al., 1989). The progesterone receptor content in the cultured cells was analysed using the method described by Perrot-Applanat et al. (1985) modified as previously reported by Chaminadas et al. (1989). Briefly, cells were fixed in picric acid-paraformaldehyde, postfixed in methanol and treated with 5% (v/v) FCS in PBS to minimize the nonspecific binding of reagents in subsequent steps. Excess FCS was removed by three washings in PBS then cells were incubated for 18 h at 4 °C with a nonspecific antibody or mouse monoclonal antibody mPR II. Specifically bound mPR II was revealed with a peroxidase-conjugated antimouse IgG antibody. The percentage of labelled nuclei was determined with a Zeiss reticular micrometer (10×10 mm square) at $250 \times$ magnification, in a set of four randomly chosen confluent subcultures.

Hormone treatment and metabolic labelling. When subcultured cells reached confluency on day 4, the medium was removed, and cell layers rinsed twice with CDM. Then the cells were incubated with CDM containing 10^{-8} M E_2 alone or with progesterone. Untreated control cells were incubated with CDM containing vehicle alone (0.01% ethanol). At various times after the hormone treatment, the medium was replaced with Na_2SO_4-free CDM containing [^{35}S]Na_2SO_4 (15.4 MBq/ml) and the corresponding hormones or vehicle alone.

Sulphate incorporation measurement. Labelling media (1 ml) were centrifuged at $3500 \times g$ for 20 min and aliquoted (200 μl). Cell layers were washed 3 times with 5 ml PBS and total cellular material was obtained by incubating cell layers for 2 h with lysis solution: 9 M urea, 3% (v/v) Nonidet P-40 and a mixture of protease inhibitors as reported by Dutt et al. (1986). [^{35}S]SO_4-labelled macromolecules (eluting at the void column volume) were isolated by gel filtration chromatography from media (secreted macromolecules) (200 μl) or from total cellular material (cellular macromolecules) (200 μg proteins) on a 16×1 cm column of Sephadex G-25 (fine) equilibrated with 6 M urea, 0.2% (v/v) Triton X-100, 0.02% (w/v) sodium azide in 20 mM Tris-acetate (pH 7.0). Radioactivity was measured in aliquots from total cellular material and from cellular and secreted macromolecules by liquid scintillation counting and expressed as cpm per μg of cellular proteins quantified by the method of Lowry et al. (1951). The available cellular sulphate pool (ACSP) was expressed as [^{35}S]Na_2SO_4 measured in total cellular material minus [^{35}S]Na_2SO_4 incorporated in cellular macromolecules.

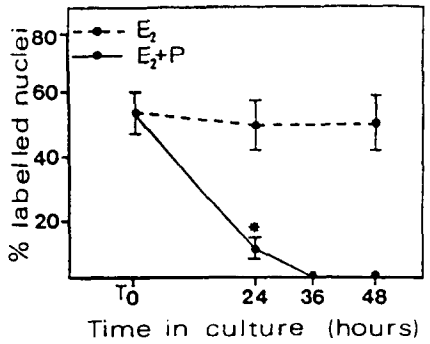

Fig. 1. Progesterone effect on the percentage of progesterone receptors in GEC. The percentage of progesterone receptor-immunostained nuclei was calculated in confluent (T_0) and postconfluent (24, 36, 48 h) subcultures on Matrigel-coated dishes in hormonally defined medium. Progesterone (10^{-7} M) was added (———) or not added (- - - - -) to the culture media containing E_2 (10^{-8} M) at T_0. Data are the mean ± SEM from quadruplicate cultures. * $P < 0.05$ vs. E_2 treatment.

Statistical analysis. Statistical differences were analysed using a one-way analysis of variance with a multiple comparison test. Significance was taken as $P < 0.05$.

Results

All studies were conducted with confluent cells after 4 days of subculture in CDM containing 10^{-8} M E_2. The percentage of confluent cells exhibiting positive immunostaining with an anti-cytokeratin antibody ranged from 90 to 95%. In order to evaluate the progesterone responsiveness of GEC, progesterone receptor content was determined in the subcultured cells. After the first 4 days of subculture, the percentage of progesterone receptor-immunostained cells averaged 50% (Fig. 1). Immunostaining was restricted to nuclei and no significant variation in the percentage of immunostained nuclei was observed in subcultures in the presence of 10^{-8} M E_2 prolonged for 48 h. When 10^{-7} M progesterone was added after 4 days of subculture in CDM containing 10^{-8} M E_2, a significant decrease in immunostained nuclei was observed after 24 h and no immunostained nuclei were detected after 36 h. Consequently, the effect of progesterone on GEC was tested within the first 24 h after adding progesterone when the percentage of cells exhibiting progesterone receptors was still high.

In preliminary studies the incorporation of $[^{35}S]Na_2SO_4$ into GEC was measured. Subcultured cells in Na_2SO_4-free CDM containing 10^{-8} M E_2 were incubated with $[^{35}S]Na_2SO_4$ (15.4 MBq/ml) for increasing periods of time. The maximum sulphate incorporation into GEC was obtained after a 15 h incubation period (data not shown).

In order to study the dose-dependent effect of progesterone on sulphate incorporation in GEC, subcultured cells were incubated with CDM plus 10^{-8} M E_2 containing increasing concentrations of progesterone (10^{-10} to 10^{-6} M). Control cells were incubated with CDM plus 10^{-8} M E_2 alone. After 8 h of incubation, the medium was replaced with Na_2SO_4-free CDM containing the corresponding hormones plus $[^{35}S]Na_2SO_4$ (15.4 MBq/ml) and left for 15 h. Results are reported in Fig. 2. A dose-dependent effect of progesterone on sulphate incorporation was observed in all fractions tested, i.e. total cellular material, both secreted and cellular macromolecules and in ACSP. The maximum effect was obtained for 10^{-8} and 10^{-7} M progesterone. Compared with control cells without progesterone, a 2.9- and 4.5-fold increase was observed respectively in cellular material and ACSP for 10^{-8} M progesterone. As progesterone increased the ACSP fraction the most, it was decided to focus the subsequent studies on the effect of progesterone on ACSP.

ACSP consists of the free inorganic sulphate in the cell and the organic sulphate donor, 3'-phosphoadenosine-5'-phosphosulphate (PAPS). The effect of progesterone on the intracellular inorganic sulphate pool was studied. For this purpose subcultured cells in CDM containing 10^{-8} M E_2 were incubated with 5×10^{-8} M progesterone for 8 h, then $[^{35}S]Na_2SO_4$ (15.4 MBq/ml) was added to the culture medium for a pulse-labelling period of 30 min to appreciate the early effect of progesterone. Untreated cells and cells treated with 10^{-8} M E_2 were processed in the same way. At the end of the incubation period with $[^{35}S]Na_2SO_4$, PAPS constantly represented 7.5% of the sulphate present in the cells as determined by thin-layer chromatography on

PEI-cellulose plates according to the method of Bochner and Ames (1982) in a solvent system containing 0.5 M LiCl (data not shown). Consequently, under our experimental conditions, the measurement of $[^{35}S]Na_2SO_4$ was representative of the inorganic sulphate in the cells. $[^{35}S]Na_2SO_4$ amounts per μg of protein were not statistically different in untreated and E_2-treated cells (Fig. 3). Progesterone (5×10^{-8} M) in concert with 10^{-8} M E_2 significantly increased the amount of $[^{35}S]Na_2SO_4$ (1.4-fold) as compared with untreated or E_2-treated cells. The effect of progesterone was suppressed by the addition of 5×10^{-7} M RU 486 into CDM during hormone treatment and the labelling period. In this case, the intracellular inorganic sulphate amount was not significantly different from that obtained in untreated or E_2-treated cells. The effect of DIDS, an anion transport inhibitor, was tested in hormone-treated and untreated cells. DIDS (5 mM) was added simultaneously with $[^{35}S]Na_2SO_4$. Whatever the hormone treatment, DIDS constantly decreased the intracellular inorganic sulphate amount. The DIDS-sensitive sulphate fraction (intracellular $[^{35}S]Na_2SO_4$ without DIDS minus intracellular $[^{35}S]Na_2SO_4$ with DIDS) was 1.9-fold higher in the E_2 plus progesterone-treated cells than in untreated or E_2-treated cells. Cycloheximide (10 μg/ml) was added to the incubation medium of E_2 plus progesterone-treated cells 3 h before the metabolic labelling. Cycloheximide induced a significant decrease in the intracellular $[^{35}S]Na_2SO_4$ which was reduced to the same level as in the DIDS-treated cells.

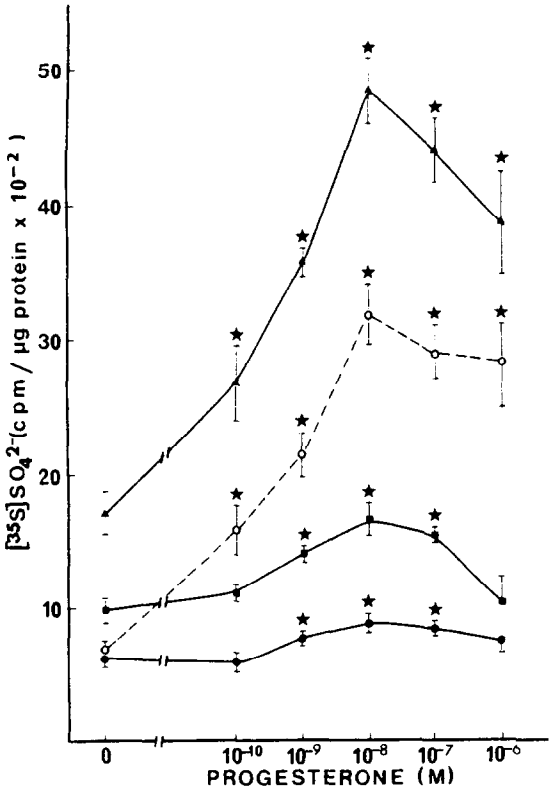

Fig. 2. Dose-dependent effect of progesterone on sulphate incorporation in GEC. When the cells reached confluence, media were removed and replaced with media containing 10^{-8} M E_2 alone (0) or with progesterone at increasing concentrations (10^{-10} to 10^{-6} M). Sulphate incorporation was measured in total cellular material (▲) or in secreted (●) and cellular (■) macromolecules as described in Materials and Methods. Available cellular sulphate pool (○) was calculated by subtracting the sulphate incorporated in cellular macromolecules from that in total cellular material. The mean ± SEM of triplicate determinations at each point from at least three separate cultures are presented. * $P < 0.05$ vs. E_2 treatment (0).

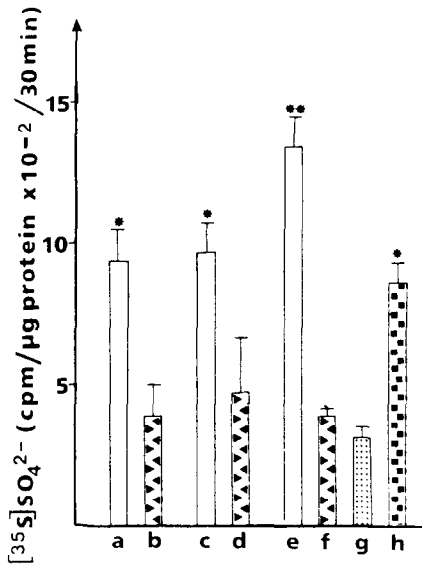

Fig. 3. Sulphate uptake in GEC. Sulphate uptake was measured in GEC untreated (a, b), treated with 10^{-8} M E_2 alone (c, d) or with 5×10^{-8} M progesterone (e, f, g and h) in the presence of 5 mM DIDS (b, d and f) or 10 μg/ml cycloheximide (g) or 5×10^{-7} M RU 486 (h). Data are the mean ± SEM from triplicate determinations from at least three separate cultures. * $P < 0.05$ vs. DIDS treatment. ** $P < 0.05$ vs. no treatment or other treatments.

Discussion

The results presented in this paper demonstrate for the first time that progesterone increases the size of the intracellular pool of inorganic sulphate in uterine glandular epithelial cells. In whole guinea-pig uterus, we have previously demonstrated a low but significant increase in the available sulphate pool induced by progesterone (Thiard et al., 1989b). To investigate this process in endometrial glandular epithelial cells we took advantage of the culture system we have recently described (Mahfoudi et al., 1991). This culture system consisted of homogeneous populations (> 90%) of endometrial glandular epithelial cells subcultured on a basement membrane matrix in a chemically defined medium. Moreover, these cells contained progesterone receptors whose amounts decreased after progesterone treatment, demonstrating progesterone responsiveness of the subcultured GEC. Sulphate incorporation was compared in GEC treated or not treated by sex steroid hormones. When progesterone was added to the media containing E_2, optimal doses of progesterone produced a 4.5-fold increase in the available cellular sulphate pool while only a weak increase was obtained in cellular and secreted macromolecules. Therefore, the glandular epithelial cells from endometrium seem to be a specific target for this progesterone effect. However, the available cellular sulphate pool in stromal cells, under progesterone control, must be investigated to ascertain the epithelial cell specificity.

We have pointed out that the available cellular sulphate pool represents intracellular inorganic sulphate pool plus its activated form, PAPS. By reducing the metabolic labelling to a 30 min period we aimed at investigating the size of the intracellular pool of inorganic sulphate under progesterone control. As expected, the available intracellular sulphate was composed for the most part of inorganic sulphate after the 30 min labelling period. It is to be noted that progesterone in combination with E_2 significantly increased the size of this intracellular inorganic sulphate pool, in comparison with untreated or E_2-treated cultures. Glandular cells treated simultaneously with RU 486, E_2 and progesterone had similar amounts of intracellular inorganic sulphate to E_2-treated or control cells. As RU 486 binds nuclear progesterone receptors but not estrogen receptors (Philibert, 1984), it was concluded that the action of progesterone on the intracellular inorganic sulphate was mediated by its binding to nuclear receptors. Moreover, the known anion transport inhibitor, DIDS and the protein synthesis inhibitor, cycloheximide markedly reduced the amount of intracellular inorganic sulphate, whatever the hormonal treatment. These results suggest that a typical sulphate transport system may be involved in the intracellular accumulation of inorganic sulphate and this transport system may be affected by progesterone.

Interesting questions remain regarding the function of the intracellular inorganic sulphate in GEC and its progesterone control. In guinea-pig uteri, progesterone increases the secretion of sulphated proteins (Thiard et al., 1989a) and the synthesis and secretion of tyrosine-sulphated proteins (Thiard et al., 1989b). During the secretory phase of the estrous cycle, when the circulating level of progesterone is high, numerous changes take place in the endometrium including increased synthesis of sulfogalactoccramides (Kubushiro et al., 1989) and two classes of polypeptide-associated keratan sulphate are characterized in epithelial secretions from human endometrium (Hoadley et al., 1990). The sulfotransferase for the synthesis of estrogen sulphate has been shown to be induced in uterine endometrium in the secretory phase (Buirchell and Hahnel, 1975) by progesterone (Clarke et al., 1982). Therefore, in the secretory phase, endometrium requires various sulphated components. The degree of final protein sulphation or other sulphated components depends on the concentration of active PAPS, which in turn may be governed by the concentration of inorganic sulphate since the sulphation of entactin varies with sulphate concentration (Campbell et al., 1987). Similar effects on sulphation of proteoglycans have already been observed (Humphries et al., 1986). It is possible that in E_2-primed cells progesterone increases the available sulphate pool which in turn induces the sulphation of components such as sulphated glycolipids or tyrosine-sulphated proteins necessary for the transport and the implantation of the blastocyst. Sulphated

glycolipids (Roberts and Ginsburg, 1988) and some tyrosine-sulphated proteins including entactin (Paulsson et al., 1985), fibronectin (Liu and Lipmann, 1984) or collagen (Fessler et al., 1986) play a key role in the cell matrix and cell–cell interactions. Fessler et al. (1986) suggested that the tyrosine sulphate residues influence the interaction of these molecules with other extracellular matrix components. Therefore, an intracellular inorganic sulphate increase followed perhaps by the activation of sulfotransferases responsible for the synthesis of sulfatides and tyrosine-sulphated proteins are two processes that are needed to prepare these components for the initial implantation phase, namely the transport and the adhesion of the blastocyst to the uterine epithelium. Thus, an increase in the intracellular inorganic sulphate could be an early effect of progesterone in the preparation of the endometrium for implantation. Studies are now in progress in our laboratory to investigate the sulphate transport system in endometrial glandular epithelial cells.

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References

Aplin, J.D., Hoadley, M.E. and Seif, M.W. (1989) Biochem. Soc. Trans. 17, 136–137.

Bochner, B.R. and Ames, B.N. (1982) J. Biol. Chem. 257, 9759–9769.

Buirchell, B.J. and Hahnel, R. (1975) J. Steroid Biochem. 6, 1489–1494.

Campbell, A.G., Fessler, L.I., Salo, T. and Fessler, J.H. (1987) J. Biol. Chem. 262, 17605-17612.

Carson, D.D., Tang, J.P., Julian, J. and Glasser, S.R. (1988) J. Cell Biol. 107, 2425–2435.

Chaminadas, G., Propper, A.Y., Royez, M., Prost, O., Rémy-Martin, J.P. and Adessi, G.L. (1986) J. Reprod. Fertil. 77, 547–558.

Chaminadas, G., Alkhalaf, M., Rémy-Martin, J.P., Propper, A.Y. and Adessi, G.L. (1989) J. Endocrinol. 123, 233–241.

Cidadao, A.J., Thorsteinsdottir, S. and David-Ferreira, J.F. (1990) Eur. J. Cell Biol. 52, 105–116.

Clarke, C.L., Adams, J.B. and Wren, B.C. (1982) J. Clin. Endocrinol. 55, 70–75.

Dutt, A., Tang, J.P., Welply, J.K. and Carson, D.D. (1986) Endocrinology 118, 661–673.

Fessler, L.I., Brosh, S., Chapin, S. and Fessler, J.H. (1986) J. Biol. Chem. 261, 5034–5040.

Hoadley, M.E., Seif, M.W. and Aplin., J.D. (1990) Biochem. J. 266, 757–763.

Humphries, D.E., Silbert, C.K. and Silbert, J.E. (1986) J. Biol. Chem. 261, 9122–9127.

Kubushiro, K., Kojima, K., Mitami, M., Mozawa, S., Iizuka, R., Iwamori, M. and Nagai, Y. (1989) Arch. Biochem. Biophys. 268, 129–136.

Liu, M.C. and Lipmann, F. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3695–3698.

Lowry, O.H., Rosebrough, M.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.

Mahfoudi, A., Nicollier, M., Propper, A.Y., Coumes-Marquet, S. and Adessi, G.L. (1991) Biol. Cell (in press).

Morris, J.E., Potter, S.W. and Gaza-Bulseco, G. (1988) Endocrinology 122, 242–253.

Munakata, H., Isemura, M. and Yosisawa, Z. (1985) Tohoku J. Exp. Med. 147, 73–75.

Paulsson, M., Dziadek, M., Suchanek, C., Huttner, W.B. and Timpl, R. (1985) Biochem. J. 231, 571–579.

Perrot-Applanat, M., Logeat, F., Groyer-Picard, M.T. and Milgrom, E. (1985) Endocrinology 116, 1473–1484.

Philibert, D. (1984) in Adrenal Steroid Antagonism (Agarwal, M.K., ed.), pp. 77–101, Walter de Gruyter, Berlin.

Roberts, D.D. and Ginsburg, V. (1988) Arch. Biochem. Biophys. 267, 405–415.

Takata, K. and Terayama, H. (1977) Biochim. Biophys. Acta 500, 333–343.

Tang, J.P., Julian, J., Glasser, S.R. and Carson, D.D. (1987) J. Biol. Chem. 262, 12832–12842.

Thiard, M.C., Nicollier, M., Mahfoudi, A. and Adessi, G.L. (1989a) J. Reprod. Fertil. 87, 687–697.

Thiard, M.C., Nicollier, M., Mahfoudi, A., Cormery, B. and Adessi, G.L. (1989b) Cell. Mol. Biol. 35, 199–205.