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QUARTERLY

Contribution of protein kinase A and protein kinase C signalling pathways to the regulation of *HSD11B2* expression and proliferation of MCF-7 cells✪

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Contribution of the protein kinase A (PKA) and protein kinase C (PKC) signalling pathways to the regulation of 11β-hydroxysteroid dehydrogenase type II (HSD11B2) gene expression was investigated in human breast cancer cell line MCF-7. Treatment of the cells with an adenylyl cyclase activator, forskolin, known to stimulate the PKA pathway, resulted in an increase in HSD11B2 mRNA content. Semi-quantitative RT-PCR revealed attenuation of the effect of forskolin by phorbol ester, tetradecanoyl phorbol acetate (TPA), an activator of the PKC pathway. It was also demonstrated that specific inhibitors significantly reduced the effect of activators of the two pathways.

Stimulation of the PKA pathway did not affect, whereas stimulation of the PKC pathway significantly reduced MCF-7 cell proliferation in a time-dependent manner. A cell growth inhibitor, dexamethasone, at high concentrations, caused a 40% decrease in proliferation of MCF-7 cells and this effect was abolished under conditions of increased *HSD11B2* expression.

It was concluded that in MCF-7 cells, stimulation of the PKA signal transduction pathway results in the induction of *HSD11B2* expression and that this effect is mark-

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Abbreviations: 4α-PDD, 4α-phorbol didecanoate; GC, glucocorticoids; HA1004, *N*-(2-guanidinoethyl)-S-isoquinolinesulfonamide hydrochloride; HSD11B2, 11β-hydroxysteroid dehydrogenase type II; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylfarmazan; PKA, protein kinase A; PKC, protein kinase C; TPA, teradocanoyl phorbol acetate.

edly reduced by activation of the PKC pathway. Activation of the PKC pathway also resulted in inhibition of cell proliferation, while activation of the PKA pathway abolished the antiproliferative effect of dexamethasone. These effects might be due to oxidation of dexamethasone by the PKA-inducible HSD11B2.

In vitro studies have shown that glucocorticoids (GC) are inhibitors of breast cancer cell growth (Lippman *et al.*, 1976; Osborne *et al.*, 1979; Zhou *et al.*, 1989; Poulin *et al.*, 1991; Goya *et al.*, 1993). This effect, however, has not been confirmed *in vivo*. It has been reported that in other tissues (kidney, liver, foetal lung) the antiproliferative effect of glucocorticoids is dependent not only on their concentration in serum but also on their effective intracellular content (Penning & Ricigliano, 1991; Roy, 1992; Hundertmark *et al.*, 1994). One of the enzymes involved in GC metabolism, 11β-hydroxysteroid dehydrogenase type II (HSD11B2) catalyses oxidation of hormonally active glucocorticoids, cortisol and corticosterone, to the much less active 11-keto-metabolites, cortisone and 11-dehydrocorticosterone, respectively. The enzyme is localised in mineralocorticoid target cells (distal nephrons, large intestine, salivary glands) where it protects the non-selective mineralocorticoid receptor from binding of glucocorticoids (Krozowski *et al.*, 1995).

This enzyme has a high affinity for its natural substrates (K_m for cortisol is about 50 nM) and requires NAD^+ as a cofactor (Albiston *et al.*, 1994). It also catalyses oxidation of synthetic glucocorticoids including dexamethasone (Pasquarette *et al.*, 1996; Hundertmark *et al.*, 1997).

It has been postulated that the antiproliferative effect of GC on breast cancer cells is attenuated as a consequence of high activity of HSD11B2 in these cells (Hundertmark *et al.*, 1997). Although the level of HSD11B2 in MCF-7 is relatively low (Nawrocki *et al.*, 2002), inhibition of HSD11B2 with glycyrrhetinic acid results in a significant increase in the antiproliferative action of GC in these cells (Hundertmark *et al.*, 1997).

We have previously reported (Lecybył *et al.*, 2003) that in renal epithelial cells an activator of the protein kinase A (PKA) pathway, forskolin, induces *HSD11B2* gene expression, whereas an activator of the protein kinase C (PKC) pathway, teradecanoyl phorbol acetate (TPA), significantly reduces the stimulatory effect of forskolin. These results suggest that activators of the PKC pathway inhibit the expression of *HSD11B2* by interfering with the PKA pathway.

The present investigation was designed to explore, with the use of semi-quantitative RT-PCR, the effect of modulators of the signal transduction pathways involving PKA or PKC, on the accumulation of the HSD11B2 transcript in MCF-7 cells. The effects of signal transduction pathway activators, as well as of dexamethasone, on the proliferation of these cells were also investigated.

MATERIALS AND METHODS

Cell culture. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine. The medium was supplemented with 10% foetal calf serum (FCS) and antibiotic/antimycotic (ABAM). After reaching confluence, culture medium was replaced with the same medium containing insulin, transferrin and sodium selenite mixture (ITS) instead of FCS. All reagents were from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.).

Cells were incubated for 24 h, unless otherwise indicated, without or with test substances: forskolin (12.5 μM), 12-*O*-tetradecanoyl-phorbol-13-acetate, TPA (10 nM), 4α-phorbol didecanoate, 4α-PDD (10 nM) or a combination of forskolin and TPA. The effect of inhibitors of adenylyl cyclase (50 μM *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride, HA1004) and protein kinase C (5 μM chelerythrine) were also in-

vestigated. All test substances were from Sigma-Aldrich (St. Louis, MO, U.S.A.).

RNA isolation, reverse transcription and amplification of HSD11B2 cDNA. Total RNA was isolated from the cells by TRI reagent/chloroform extraction (Chomczyński & Sacchi, 1987) and poly(A)$^+$ RNA was reverse transcribed, followed by amplification using primers specific for human *HSD11B2*:

F:5′-TCTAGAGTTCACCAAGGCCCA-3′ and
R:5′-GCAAGTGCTCGATGTAGTCCT-3′.

Amplification of HSD11B2 standard (using RedTaq Polymerase, Sigma-Aldrich) was conducted in a Perkin-Elmer thermocycler for 30 cycles including: denaturation at 94°C for 20 s, annealing at 59°C for 20 s and elongation at 72°C for 25 s, with preliminary denaturation at 94°C for 4 min and final elongation at 72°C for 7 min. For standard curve preparation, different concentrations of the template in the range of 0.05 to 1.0 amol/μl were used.

Simultaneously, a fragment of β-actin cDNA was amplified and used as an internal standard. The standard curve for β-actin was prepared as described above with the annealing temperature of 64°C. Electrophoresis of the amplification products was conducted in 1.5% agarose gels and was followed by staining with ethidium bromide. Gels were then photographed and subjected to densitometric analysis using a BandLeader computer program (Version 3.00).

Proliferation assay. The MCF-7 cells were cultured in DMEM supplemented with glutamine and FCS. When 30% confluence was reached, test substances: 12.5 μM forskolin, 10 nM TPA, 10 nM 4αPDD, 50 μM HA1004, 5 μM chelerythrine and 10^{-10} to 10^{-7} M dexamethasone were added and cell proliferation was monitored. At different time intervals, 5% MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) was added and after 2 h supernatant was removed and the cells were treated with Me_2SO in order to visualise metabolised MTT. All reagents were from Sigma.

Statistical analysis. Each experiment was performed at least in triplicate, the results were evaluated by Student's *t*-test, and $P < 0.05$ was considered as the level of significance.

RESULTS AND DISCUSSION

Incubation of breast cancer MCF-7 cells for 24 h with the adenylyl cyclase activator, forskolin, resulted in an about 3-fold ($P < 0.05$) increase of HSD11B2 mRNA accumulation above the control level, and this effect was attenuated ($P < 0.05$) by an inhibitor of adenylyl cyclase, HA1004. Incubation of the cells with an activator of PKC, phorbol ester TPA, significantly ($P < 0.05$) reduced the basal level of HSD11B2 mRNA, while an inhibitor of protein kinase C, chelerythrine, reversed ($P < 0.05$) this effect (Fig. 1 A and B). Phorbol ester, 4α-PDD, which does not activate PKC, did not influence the basal level of *HSD11B2* transcript (Fig. 1 A and B), suggesting that the effect of TPA was due to PKC activation. TPA also significantly attenuated forskolin-induced transcription of *HSD11B2* (Figs. 2 and 3) indicating that like in renal epithelial cells (Lecybył *et al.*, 2003), it interferes with the PKA pathway. Treatment of the cells with the test substances did not alter the level of β-actin mRNA (Figs. 1A and 2A), indicating that the effects of forskolin and TPA on the expression of *HSD11B2* were specific.

In forskolin-treated cells there was no significant change in cell proliferation up to 96 h (Fig. 4). In cells treated with TPA, cell proliferation was slightly decreased after 24 h, and a significant inhibition ($P < 0.05$) was observed after 48 and 96 h (Fig. 4). The phorbol ester 4α-PDD which does not affect the PKC activity, had no influence on cell proliferation (not shown). This implied an indirect involvement of the PKC pathway in the inhibition of MCF-7 cell proliferation.

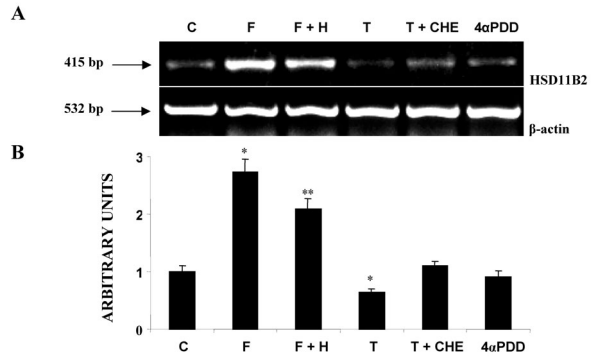

Figure 1. The effects of activators and inhibitors of PKA and PKC pathways on the level of *HSD11B2* transcript in breast cancer cell line MCF-7.

Cells were incubated for 24 h in the absence or presence of test substances. After incubation total RNA was isolated and poly(A)$^+$ RNA was reverse transcribed. Specific cDNAs were amplified by PCR using primers specific for HSD11B2 and β-actin and the amplification products were subjected to electrophoresis in 1.5% agarose gel. (A) Ethidium bromide staining. (B) Densitometric analysis of the bands normalised against β-actin cDNA. C, control; F, forskolin; F + H, forskolin + HA1004; T, tetradecanoyl-phorbol acetate; T + CHE, tetradecanoyl-phorbol acetate + chelerythrine; 4αPDD, 4 alpha-phorbol didecanoate. *Statistically significant relative to control, ($P < 0.05$); **statistically significant relative to F ($P < 0.05$).

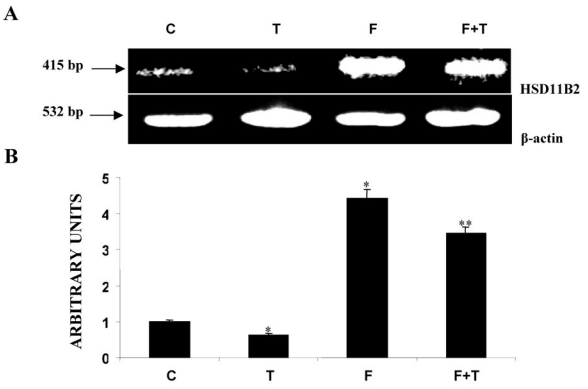

Figure 2. The effects of TPA and forskolin on the level of *HSD11B2* transcript in breast cancer cell line MCF-7.

Cells were cultured as described in the legend to Fig. 1. (A) Ethidium bromide staining. (B) Densitometric analysis of the bands normalised against β-actin mRNA. C, control; T, tetradecanoyl-phorbol acetate; F, forskolin; F + T, forskolin + tetradecanoyl-phorbol acetate. *Statistically significant relative to control, ($P < 0.05$); **statistically significant relative to F ($P < 0.05$).

Dose-dependency experiments demonstrated that at 48 h of incubation, dexamethasone on its own affected MCF-7 cell proliferation only at the 100 nM concentration (Fig. 5). Since dexamethasone is oxidised by HSD11B2 (Ferrari *et al.*, 1996), this suggests that the activity of this enzyme in MCF-7 cells is significant. Treatment of the cells with dexamethasone and an activator of PKA pathway, forskolin (Fig. 5) resulted in abolition ($P < 0.05$) of the antiproliferative action of dexamethasone that might be caused by an increased expression of *HSD11B2* and thus oxidation of dexamethasone.

Although forskolin treatment resulted in attenuation the antiproliferative action of dexamethasone, TPA on its own did not affect the action of dexamethasone (Fig. 5) suggesting

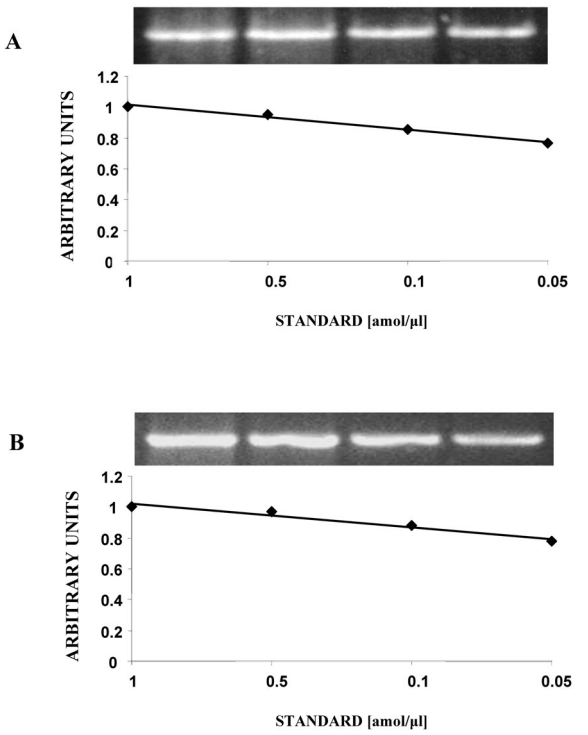

Figure 3. Semi-quantification of HSD11B2 (A) and β-actin (B) standards.

Samples containing different amounts of appropriate DNA templates were amplified, visualised with ethidium bromide, and standard curves were drawn.

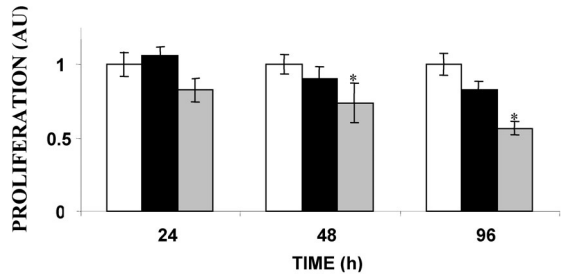

Figure 4. Time course of the effect of activators of the PKA and PKC pathway on the proliferation of breast cancer cell line MCF-7.

Cells were grown in culture, and when 30% confluence was reached, test substances: forskolin (black bars) or tetradecanoyl-phorbol acetate (gray bars) were added. At time intervals cell proliferation was determined by the MTT test. Open bars represent cells cultured without additions of test substances (control). AU, arbitrary units. The results are mean ± S.E.M. for at least three experiments. *Statistically significant difference relative to control ($P < 0.05$).

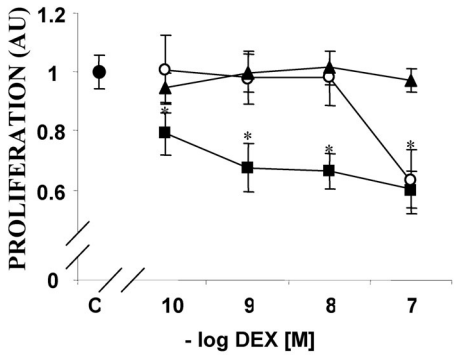

Figure 5. The effect of dexamethasone on the proliferation of MCF-7 cells in the presence of TPA or forskolin.

Cells were grown in culture, and when 30% confluence was reached test substances were added, cells were incubated for 48 h and cell proliferation was determined by the MTT test. C, control, (black circle); dexamethasone (open circles); dexamethasone and tetradecanoyl-phorbol acetate (black squares); dexamethasone and forskolin (black triangles). AU, arbitrary unit. The results are mean ± S.E.M. for at least three experiments. *Statistically significant relative to control ($P < 0.05$).

that the PKC pathway affects proliferation of MCF-7 cells indirectly, probably by inhibiting *HSD11B2* expression.

Regulation of *HSD11B2* by the two signal transduction pathways might constitute an important factor in the proliferation of breast cancer cells, because the induction of *HSD11B2* expression by the activator of the PKA pathway forskolin was accompanied by a substantial decrease in the antiproliferative effect of dexamethasone.

It has not been established, however, which hormone(s) regulate *HSD11B2* expression in MCF-7 cells. Peptides acting through receptors coupled to adenylyl cyclase and the PKA signal transduction pathway should be taken into account. These peptides might reduce the antiproliferative action of glucocorticoids on these cells. On the other hand, deficiency of these peptides might markedly reduce the level of *HSD11B2* expression resulting in an increased antiproliferative effect of glucocorticoids.

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