Sex Steroid Hormone Modulation of NADPH Pathways in MCF-7 Cells

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ABSTRACT

Hormonal modulation of glucose-6-phosphate dehydrogenase (G6PD) and of utilization pathways of NADPH generated by G6PD was studied in the MCF-7 human breast cancer cell line, using a quantitative cytchemical method. Our results show that G6PD is increased by 17β-estradiol (estradiol) and synthetic progestin (promegestone R5020). The synthetic antiestrogen tamoxifen has no effect on G6PD activity. When it is present in the medium with estradiol, tamoxifen can oppose the stimulatory effect of estradiol on G6PD activity. Mifepristone (RU 38486) has no effect on G6PD activity, but it inhibits the R5020 stimulation of G6PD activity. After MCF-7 pretreatment with estradiol, there is a much stronger stimulation of G6PD activity by R5020. When we studied the effect of the steroid on the two utilization pathways of NADPH generated by G6PD activity, we observed that, in the cells treated with estradiol, there is an increase in reducing equivalents generated by G6PD activity which only affects the NADPH2 pathway, and that there is cell growth stimulation. When tamoxifen is present in the medium, we found no effect on the NADPH utilization pathways, nor on cell growth. In the presence of R5020, the NADPH2 pathway activity is increased but, under our experimental conditions, there was no effect on cell growth. On the other hand, even though RU 38486 is without effect on total G6PD activity, it does cause a modification in the distribution of reducing equivalents: the NADPH2 pathway activity is decreased, while the NADPH1 pathway is stimulated.

INTRODUCTION

Neoplastic tissues are characterized by increases in the level of glucose metabolism as well as in the level of a number of associated enzymes including G6PD (1, 2). G6PD is the first enzyme in the hexose monophosphate cycle. This particular pathway is one of the principal sources of cytoplasmic NADPH. The NADPH generated by G6PD activity participates in two major metabolic pathways; either directly (NADPH2) in reductive biosynthesis as a source of equivalent reducing; or indirectly via the electron transport chain, associated with mixed-function oxidation and with the P450 cytochromes for various hydroxylation (steroid hormone synthesis) and detoxification (medicines, drugs, and toxins) reactions (NADPH1). The quantity of NADPH engaged in each pathway is variable and is linked to cell physiology. Moreover, there are existing mechanisms that adjust the level of G6PD activity according to cellular requirements. The balance between NADPH1 and NADPH2 is thus specific to the activity of different tissues (3-5).

DIFFERENT AUTHORS HAVE ATTEMPTED TO CORRELATE G6PD ACTIVITY IN MAMMARY TUMORS WITH THE PRESENCE OF ER, OFTEN WITH CONFUSING RESULTS, ESSENTIALLY BECAUSE OF CELLULAR HETEROGENEITY AND THE METHODOLOGY USED (6-9). WE HAVE THEREFORE SET UP AN EXPERIMENTAL MODEL THAT TAKES THESE DIFFICULTIES INTO ACCOUNT. WE USED THE HUMAN BREAST CANCER CELL LINE MCF-7, WHICH PERMITTED THE CONTROL OF THE HORMONAL ENVIRONMENT, AND USED A QUANTITATIVE CYTOCHEMICAL METHOD FOR THE DIRECT MEASUREMENT OF G6PD ACTIVITY AT THE CELLULAR LEVEL.

USING THIS TECHNIQUE, WE SHOWED IN A PREVIOUS WORK (10) THAT 17β-ESTRADIOL (ESTRADIOL) AND THE SYNTHETIC PROGESTIN R5020 CAUSED AN INCREASE IN G6PD ACTIVITY IN MCF-7 CELLS. IN THE PRESENT STUDY, WE DETERMINED THE EFFECTS OF THESE PRODUCTS ON THE PATHWAYS OF NADPH PRODUCED BY G6PD ACTIVITY TOGETHER WITH THE EFFECTS OF THEIR RESPECTIVE ANTAGONISTS, TAMOXIFEN AND RU 38486. WE ALSO LOOKED FOR A POSSIBLE CORRELATION BETWEEN THESE EFFECTS AND CELL GROWTH.

MATERIALS AND METHODS

Estradiol, R5020, and RU 38486 were gifts from Roussel-Uclaf (Romainville, France). Tamoxifen was gift from ICI (Macclesfield, Great Britain). Culture media and FCS were purchased from Boehringer (Mannheim, Federal Republic of Germany). Trypsin was obtained from Worthington. G6P, NADP, PMS, and NT were purchased from Sigma Chemical Co. (St. Louis, MO). NT was extracted with chloroform before use. All reagents were of analytical grade.

Cell Culture. MCF-7 breast cancer cells (11) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 4 mM l-glutamine, 0.6 μg/ml of insulin, 24 IU/ml of penicillin-streptomycin solution, and 0.6% amphotericin B. Cells were grown at 37°C in a humidified atmosphere containing 5% CO2 in air and were passed weekly using 0.25% trypsin-1 mM EDTA in Ca2+, Mg2+-free phosphate-buffered saline.

Steroid Treatment of the Cells. In order to deprive cells of endogenous steroids, the medium was replaced by Dulbecco’s modified medium containing 2.5% FCS/CDS as previously described (10, 12). Three days later, cells were trypsinized, and the cell suspension was plated (1500 cells/chamber) on Lab-Tek Chamber Tissue Culture slides (Miles, Naperville, IL) with eight chambers per slide in 250 μl of 2.5% FCS/CDS medium, without insulin. Three days after plating to the bottom of the chamber, i.e., during the growth phase, media were removed and replaced by insulin-free, 2.5% FCS/CDS medium containing steroids in ethanolic solution with a final concentration of less than 0.1%. The control medium contained an equivalent volume of ethanol.

Cell Growth Measurements. After trypsinization, for the cell growth study, cells were plated at a density of 104 cells/cm² in 4 ml of insulin-free 2.5% FCS/CDS medium and treated as described above for steroid treatment. The medium was changed every day. Each day from Day 3, cells were collected by trypsinization and counted in a Model ZF Coulter Counter (Coultronics, France), using 4 flasks for each measurement.

Enzyme Incubation Procedure. G6PD activity in cultured MCF-7 cells grown as monolayers on microscope slides was measured by quantitative cytchemical determination of generated NADPH as previously described (10, 13), using the PMS-NT procedure (14, 15).

The NADPH generated by G6PD activity can be used by two pathways which can be measured by this quantitative cytchemical method (Fig. 1). (a) The reduction of NT in the presence of G6P and NADP, in the absence of PMS, depends not only on G6PD activity in the cells, but also on the transport chain which, in the endoplasmic...
antagonists of 30 cells in a single chamber. All assays are performed in duplicate. The absolute and the G6PD activity remains constant and not significantly different from the basal value. (b) The presence of a strong hydrogen acceptor, such as PMS, permits the direct transfer of reducing equivalents from NADPH to NT (for the formation of formazan and, thus, to measure total NADPH). NAPDH2 which is used directly in a number of reductive biosyntheses is calculated as the difference between total NADPH and NAPDH1.

In practical terms, at the time of enzyme measurement, the cells were rinsed and G6PD activity was determined by incubation of the cells in a medium containing 1.5 mM G6P, 1 mM NADP, and 7.5 mM NT in the presence of 0.1 mM PMS (total NADPH) or in the absence of PMS (NADPH1) for 20 min at 37°C as described previously. At the end of the incubation period, the medium was aspirated off, and the chambers were removed from the slides and discarded, leaving microscope slides with eight distinct areas of colored cells. The slides were rinsed with distilled water and dried at 37°C.

The amount of precipitated formazan was measured in single cells by scanning and integrating microdensitometry, using a microdensitometer (Vickers Model M50) at the isobestic wavelength of 585 nm with a x40 objective and a scanning spot of 0.5 μm as previously described (10). Absorbance was normalized with reference to an absolute internal calibrated filter and expressed as absorbance (A585/min/cell). The absorbance of 30 monolayer cells in each chamber was measured. One experimental unit is therefore the result of measurement of 30 cells in a single chamber. All assays are performed in duplicate.

Statistical Evaluation. The results were expressed as mean absorbance plus or minus standard error of the mean (SEM). The data are analyzed for statistical significance by Student's t test.

RESULTS

Effects of Hormones and Antihormones on G6PD Activity

G6PD activity increased following 24 h of incubation with estradiol or R5020 and remained elevated for an additional 72 h. On the other hand, regardless of incubation time (between 0 and 96 h) in the presence of RU 38486 or tamoxifen, the G6PD activity remains constant and not significantly different from control values.

Effect of Hormonal Concentrations

The effects of estradiol and R5020 are dose dependent within certain concentration thresholds, with a maximum effect at 10^{-7} M and 10^{-6} M, respectively (Fig. 2). For RU 38486 and tamoxifen, regardless of the concentration used, the effect on G6PD activity remains nonsignificant compared with nontreated cells (Fig. 2). We note, however, the toxic effect of tamoxifen on cells at concentrations of 10^{-5} M. Cells are rarely found in the chambers in the presence of such concentrations. Finally, progesterone, regardless of concentration, has no effect on G6PD.

Effects of Simultaneous Treatment by Hormones and Their Antagonists

Effect of Estradiol plus Tamoxifen. When 10^{-6} M tamoxifen is added concurrently to the incubation medium with 10^{-8} M estradiol, G6PD activity is completely inhibited as compared with the effect of adding 10^{-6} M estradiol alone (P < 0.001) (Fig. 3). Tamoxifen also inhibits the increase of G6PD activity provoked by 10^{-7} M estradiol. However, this inhibition is not absolute, and the G6PD activity remains significantly (P < 0.05) higher than in nontreated MCF-7 cells (Fig. 3).

Effects of R5020 plus RU 38486. The simultaneous presence of 10^{-5} M R5020 and 10^{-4} M RU 38486 blocks the stimulation of G6PD activity induced by R5020 when it is present alone in the incubation medium (Fig. 4). When the concentration of R5020 is at 10^{-7} M, the inhibition of the 10^{-4} M stimulation is incomplete (P < 0.05).

Effects of Estradiol Pretreatment on the Stimulation of G6PD Activity by R5020 and RU 38486

The effect of adding R5020 to MCF-7 cells pretreated with 10^{-6} M estradiol for 3 days leads to a significant increase (P < 0.001) in G6PD activity compared with the G6PD activity obtained in the presence of R5020 without estradiol pretreatment (Fig. 5). Even a nonstimulating concentration of R5020...
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Fig. 4. Effects of RU 38486 on the R5020 stimulation of G6PD activity. Enzyme activity was measured in MCF-7 cells after 24 h in the presence of increasing concentrations of R5020 with or without $10^{-8}$ M RU 38486. Columns, mean of 30 cells; bars, SE. Each experiment was performed twice. Results are expressed as $A_{585}/$min/cell. *, $P < 0.05$; **, $P < 0.001$ (versus control value). △, $P < 0.05$; △△△, $P < 0.001$ (versus R5020 alone values); NS, not significant. □, with $10^{-8}$ M RU 38486; □□, without $10^{-8}$ M RU 38486.

Fig. 5. Effects of steroid hormones on G6PD activity after a preincubation period in the presence of estradiol. Two days after plating, cells were treated with $10^{-8}$ M estradiol for 3 days. Cells were then incubated in the presence of increasing hormone concentrations. G6PD activity was measured in cells after 24 h of treatment. Columns, mean of 30 cells; bars, SE. Each experiment was performed twice. Data are expressed as $A_{585}/$min/cell. **, $P < 0.01$ (versus control values). △△, $P < 0.001$ (versus without pretreatment values); NS, not significant. □, with estradiol; □□, without estradiol pretreatment.

NADPH2). In effect, the NADPH1 pathway represents no more than 31%, while the NADPH2 pathway exceeds 69% of total NADPH, and the NADPH1/NADPH2 ratio thus equals 0.46 and is significantly different as compared with the control cells ($P < 0.001$).

For tamoxifen, which has no effect on G6PD activity, the proportion of the NADPH1 and NADPH2 pathways is identical to that found in the control cells. The ratio is therefore not modified.

Regarding R5020, which causes global increases in total NADPH, the NADPH1 pathway is unchanged compared with the control. However, it attains less than 27% of total NADPH, whereas the NADPH2 pathway rises significantly when compared with controls (73%). The ratio has a value of 0.37 which is significantly different from those obtained for control cells ($P < 0.001$).

For RU 38486, which has no effect on G6PD activity, a change in the NADPH pathways was observed. The NADPH1 pathway represents 45% of total NADPH, while NADPH2 pathway drops off to 55%. The ratio is equal to 0.83 ($P < 0.001$ versus control).

We observed that the NADPH produced by G6PD activity is used in all the conditions studied and preferentially in the NADPH2 pathway (Fig. 6).

The relations that exist between NADPH1 and steroid hormone metabolism, the mechanism of detoxification, on the one hand, and NADPH2 and cellular proliferation on the other, led us to compare the relative quantities of these two functional types of NADPH present, as well as to study their effects on cellular proliferation. As seen from Fig. 7, estradiol stimulates cellular proliferation, whereas the antiestrogen tamoxifen inhibits it. R5020 has no effect on the growth, while the antiprogestin RU 38486 inhibits it.

DISCUSSION

This study examined the effects of estradiol, the synthetic progestin R5020, the antiestrogen tamoxifen, and the antiprogestin on G6PD activity and on the utilization pathways of NADPH generated by G6PD activity in the human breast cancer cell line MCF-7. We wished to study the activity of G6PD, the first enzyme of the hexose monophosphate pathway,
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which catalyzes the conversion of G6P to 6-phospho-gluconolactone. This step is important because it is both irreversible and rate limiting. Although the preceding studies corroborate the fact that this enzyme activity is significantly higher in neoplastic mammary tissue than in normal breast tissue in humans (1, 2), the effects of steroid hormones and antihormones on G6PD activity have not been studied.

To determine enzyme activity, we used a cytochemical technique (14, 15), which we adapted for use on cell cultures (10, 13, 16). This method allows the direct measurement of enzyme activity on a small number of cells. It also allows a cell-by-cell evaluation of enzyme activity that is independent of the positive or negative effects on proliferation by hormones and their antagonists (12, 7–20).

G6PD Activity after Treatment with Hormones or Their Antagonists

As we have previously shown (10), estradiol and R5020 stimulated G6PD activity. A maximum activity was therefore obtained at an unphysiological concentration (10⁻⁶ M) of estradiol. The lack of stimulation of G6PD by progesterone is probably because of the rapid metabolism of this hormone in mammmary cells (21). The synthetic progestin R5020, which is more stable, is metabolized less rapidly and can thus stimulate G6PD. Ojasoo and Raynaud (22) showed that R5020 is 50 times more active than progesterone in the induction of proliferation of rabbit endometrial cells. Our results show that RU 38486, a recently synthesized antagonist of progesterone, has no effect on G6PD activity regardless of concentration, nor has tamoxifen, the most commonly used antiestrogen in the treatment of breast cancer.

Effects of Simultaneous Treatment by Hormones and Their Antagonists. When we treated cells simultaneously with 10⁻⁶ M tamoxifen and estradiol at different concentrations, the inhibitory effect of tamoxifen on the estradiol-based induction of G6PD was observed. Our results show that 10⁻⁷ M estradiol alone does not reverse tamoxifen inhibition. The estradiol/tamoxifen ratio must be less than 1/10 for the effect of 10⁻⁶ M tamoxifen to be attenuated. A similar effect on MCF-7 cells was found when the measured parameter is cell growth (23, 24).

When we added different concentrations of R5020 to culture media containing 10⁻⁶ M RU 38486, RU 38486 inhibition of G6PD activity stimulation provoked by 10⁻⁶ M R5020 was observed. This inhibition is, however, partially reversed with increasing R5020 concentration. To our knowledge, this effect on G6PD has not been reported in the literature. It is probable that this effect is because of competition between the two substances for PR sites, as has been shown for estradiol and tamoxifen for ER sites (23, 24). Moreover, it has been shown that RU 38486 is a strong antiglucocorticoid (25) which acts by binding to the glucocorticoid receptors. In a previous study (10) however, we also showed that dexamethasone had no effect on G6PD activity. Thus, RU 38486 probably acts as an antagonist to R5020 stimulation of G6PD activity via PR.

Effects of Estradiol Preincubation on R5020 and RU 38486 Stimulation of G6PD Activity. While 10⁻¹⁰ M R5020 has no effect on G6PD activity, this concentration becomes stimulatory after pretreatment of MCF-7 cells with estradiol. Estradiol pretreatment of cells cultured in already stimulatory concentrations of R5020 leads to significant increases in G6PD activity. We found that RU 38486 preincubation with estradiol does not affect G6PD activity compared with control cells.

Horwitz et al. (26) demonstrated estradiol regulation of the PR in the MCF-7 cell line. Estradiol pretreatment could thus either directly stimulate G6PD activity, which would be maintained during R5020 incubation, or it could stimulate de novo synthesis of PR. It is also possible that both alternatives occur simultaneously. The first hypothesis seems more plausible, however. Indeed, as we found neither RU 38486 G6PD stimulation after pretreatment with estradiol nor that it had any effect after 3 days, we are led to the conclusion that the increase in R5020 stimulation can only be due to de novo PR synthesis.

Influence of Hormones on NADPH Pathways and Growth Curves

The value of the NADPH1/NADPH2 ratio is a good indicator of cellular activity, especially for cells with highly specialized metabolism (5, 27, 28). We have shown here that the use of a quantitative cytochemical method coupled to a microcyto-metric approach allows the simple and efficient measure of the NADPH1/NADPH2 ratio at the cellular level. The importance of the relation between NADPH and cellular growth led us to measure and compare the use of these reducing equivalents in MCF-7 cells with the growth curve under different hormonal conditions.

We note that the stimulation of G6PD activity after incubation in the presence of estradiol led to increases in only the NADPH2 pathway. The increase in total NADPH may be the result of the increased need for ribose 5-phosphate for the RNA synthesis necessary for protein synthesis. Furthermore, this increase in the NADPH2 pathway in cells treated with estradiol could be related to the fact that estradiol stimulates cell growth and thus necessitates the biosynthesis of metabolites essential for such growth. The increase in growth by estradiol was approximately 20%, as has been reported by other workers (23, 29). It should be noted that this effect occurs well before estradiol-induced cellular proliferation can be detected.

Solomon and Daly (28) working on tumor biopsy samples of
both benign and malignant breast tumors were unable to show significant differences in G6PD activity between these tumor types. However, they showed that estrogens could have a direct effect on all the dehydrogenases of the hexose monophosphate pathway in certain mammary tumors. In their study, however, this effect was mainly on the distribution of the reducing equivalents rather than on the total G6PD activity itself. The differences between our respective findings are probably because of the biological model used.

It has also been shown that estradiol induces the synthesis of specific proteins secreted into the culture medium (30, 31), among these a M, 52,000 glycoprotein. This protein is able to stimulate the growth of MCF-7 cells deprived of estrogen. Thus, estradiol not only stimulates the growth of MCF-7 cells, but it also induces the synthesis and secretion of growth factors into cell media. It is possible that the reducing equivalents are used in lipogenesis or in reduction of glutathione. This hypothesis merits further investigation.

In the tamoxifen assays, we observed no modification in either the level of G6PD activity or the distribution levels of reducing equivalents in the NADPH1 and NADPH2 pathways. We have shown here that tamoxifen inhibits cellular growth after 4 days and that it also has a cytotoxic effect after 10 days of culture, an effect which has been well documented elsewhere (32, 33).

R5020 has the same effect as estrogen on reducing equivalent pathways but has no effect on MCF-7 cell growth. R5020 stimulates production of a M, 250,000 protein in MCF-7 cells after 6 h of treatment (34). At least a part of the increase in the NADPH2 pathway in our study is accounted for by the increased need for reducing equivalent for protein synthesis (in particular, the M, 250,000 protein), but above all for the synthesis of ribose 5-phosphate.

RU 38486 causes modification in the distribution of reducing equivalents without modification of total G6PD activity; the NADPH1 pathway is stimulated, while the NADPH2 pathway is inhibited. RU 38486 inhibits growth of MCF-7 cells, synthesis of a M, 250,000 protein (34), and biosynthesis linked to cell growth. As a consequence, we think that the cell reorients its metabolism toward the hydroxylation and detoxification of certain molecules, including RU 38486, via the chain linked to mixed-function oxidation under these conditions.

In conclusion, this work, as our previous studies (10, 16) underscores the fact that the study of the effects of hormones and antihormones on enzyme activity of glucose metabolism and on NADPH pathways provides a useful tool in the investigation of the sensitivity of mammary tumor tissues to the hormonal environment and to the action of anticancer drugs. Because of its sensitivity at the cellular level, quantitative cytomtery should allow one to work on microsamples available from mammary tumor biopsies and thus it adds important information to cytopathological data.

Finally, these studies raise two important questions. (a) Does G6PD activity vary as a function of the cell cycle phases? (b) What are the relations between enzyme activity, receptors for both hormones and antihormones, and growth factors? Our present line of research is attempting to address these questions.

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