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THE EFFECTS OF OESTRADIOL-17β, PROVERA AND TAMOXIFEN ON GLUCOSE METABOLISM IN MAMMARY GLAND, LIVER AND MUSCLE OF THE RAT

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SUMMARY

The activities of seven enzymes associated with glucose metabolism were measured in muscle, liver and mammary glands of intact, castrated and castrated rats treated with progesterone, oestradiol-17β or Tamoxifen. The following differences were observed: 1. Castration significantly reduces the activities of G6PDH and PGM in the mammary glands whereas it increases total protein content of the tissue. Administration of progesterone or oestradiol failed to alter these castration-induced changes. Tamoxifen treatment reduces the castration induced rise in total protein and PGM activity; 2. Castration induces a significant reduction in the activity of 6PGDH and an increase in the activity of PHI in liver. Administration of oestradiol to castrated animals increases the activity of 6PGDH to the levels found in intact animals. Tamoxifen treatment showed a significant decrease in the activities of G6PDH, 6PGDH and PHI: 3. Neither castration nor any of the subsequent treatments affected glucose metabolism in muscle.

INTRODUCTION

It is generally recognized that administration of hormones will induce rapid and diverse changes in a variety of tissues and that in target tissues a series of events take place at a molecular level which result in increased protein synthesis [1–3]. Oestrogens accelerate the growth and cornification of vaginal epithelium and, in combination with other adrenal and pituitary hormones, increase the growth of mammary tissues [4–8]. Similarly, administration of progesterone in sufficiently high doses to castrate animals produces ductal and lobular-alveolar development in the mammary gland [9]. Since an increased rate of growth necessitates an increased energy supply and since a major proportion of the cell's energy is generated by glucose metabolism, measurement of the effects of hormone deprivation or administration on the activities of some of the key enzymes associated with glycolysis may give information on the foci of hormone action.

Tamoxifen (ICI 46474) is regarded as an anti-oestrogenic compound in some species [10] and this property of the drug is considered to be due to competition with oestradiol for binding at target sites [11, 12]. Therefore it was decided to see whether oestradiol regulates the activities of a series of enzymes and if so, whether Tamoxifen could inhibit oestrogen-induced alterations. In this paper, the effect of castration, progesterone, oestradiol and Tamoxifen treatments on the activities of enzymes associated with glucose metabolism are investigated in rat mammary glands and are compared with non-target tissues such as muscle and liver.

MATERIALS AND METHODS

Chemicals

All the chemicals used in the study were purchased from British Drug Houses Ltd and were of analytical grade. α-GPDH, TPI, G6PDH, aldolase, NADH, $NADP^+$, F-6-P, G-6-P, 6PG and pyruvate were purchased from Boehringer Mannheim. DHAP was prepared from the dicyclohexylammonium salt of the dimethylketal according to the manufacturer's (Boehringer Mannheim) instructions. G-1-P was purchased from Koch-Light Ltd.

Animals

Randomly bred albino Sprague–Dawley female rats weighing between 245 and 285 grams, six to eight weeks old at the time of death were used in these experiments and were housed four to a cage. One group of four rats was left intact (I) and the animals were killed without regard for their position in the oestrous cycle. The remaining sixteen were ovariectomized two weeks before the start of hormonal treatments. The operation was performed via a dorsal

The following abbreviations are used in this paper: *Enzymes*. α-GPDH, α-glycerolphosphate dehydrogenase: TPI, triosephosphate isomerase: G6PDH, glucose-6-phosphate dehydrogenase: PFK, phosphofructokinase: 6PGDH,6-phosphogluconate dehydrogenase: PGM, phosphoglucomutase: LDH, lactate dehydrogenase: PHI, phosphohexose isomerase. *Co-enzymes*. NADH, reduced nicotinamide adenine dinucleotide: $NADP^+$ nicotinamide adenine dinucleotide phosphate: ATP, adenosine triphosphate. *Substrates*. F-6-P, fructose-6-phosphate: G-6-P, glucose-6-phosphate: 6PG, 6-phosphogluconate: G-1-P, glucose-1-phosphate: DHAP, dihydroxyacetone phosphate.

incision through the skin and body wall under fluothane anaesthesia. Four of these animals received no further treatment and were used as castrate controls (C). A second group of four rats received 800 ng/ml of oestradiol-17β (E) in their drinking water fourteen days before autopsy, fresh water containing oestrogen and 2.5% ethanol being provided on the Monday, Wednesday and Friday of each week. A third group (P) received subcutaneous injections of 5 mg of medroxyprogesterone acetate (Depo-Provera, Upjohn) suspension in 0.5 ml saline on the 14th and 7th days before death: doses of this order are physiological in that they support pregnancy in ovariectomized rodents [13]. The last group of four animals (T) received subcutaneous injections of 5 mg of Tamoxifen (ICI 46474) in 0.5 ml of arachis oil on the 14th and 13th days before autopsy. This dose of Tamoxifen was chosen because it is known to exert prolonged effects and to inhibit the growth of DMBA-induced rat mammary carcinomas [14].

At the end of the treatment period, the animals were killed by an overdose of Nembutal, muscle and liver samples were removed from the thigh and median lobe respectively. The animals were then skinned and the skins bearing the left abdominal mammary glands 4 and 5 were removed. The glands, with their fat pads, were separated from the skin by blunt dissection and frozen on solid carbon dioxide. They were then transferred to a liquid nitrogen container and remained there until further processing.

PROCEDURE FOR THE ESTIMATION OF ENZYMES, DNA AND TOTAL PROTEIN

The tissues were taken out of liquid nitrogen and partially thawed. The semi-frozen tissues were then weighed and homogenized in a silverson homogenizer according to the procedure of Shonk and Boxer [15]. A small aliquot was taken for the estimation of DNA and total protein and the remainder was centrifuged (800 *g* for 15 min) at 4°C. The supernatant was decanted off and used as the source of enzymes. The breasts with their fat pads were weighed and homogenized in 25 ml of homogenization medium, whereas a 5% solution for liver and muscle was used in the estimation of enzymes. The enzyme estimations were performed according to the procedure of Shonk and Boxer [15] as modified by Deshpande, Mitchell and Millis [16]. DNA was estimated according to the procedure of Burton [17] and proteins by the method of Lowry *et al.* [18]. The enzyme activities were estimated on the basis of units per gram wet weight for liver or muscle and per mg of DNA for the breasts. A unit is defined as that amount of enzyme which will catalyse the transformation of one μm of substrate per min. The results are expressed as mean $\pm$S.D. Each group consists of values obtained on tissues from four animals. The means were tested for significance by a Student's *t*-test.

RESULTS

The activities of PFK, G6PDH, 6PGDH, PGM, LDH, α-GPDH and PHI were estimated in muscle, liver and breast tissues. Table 1 shows the values obtained from muscle.

There were no statistically significant differences in DNA and protein values or enzyme activities amongst any of the groups. Table 2 shows the results obtained from liver.

There were no significant differences in DNA and protein values or in the activities of PFK, PGM, LDH and α-GPDH between various groups. Castration significantly reduced the activity of 6PGDH which was restored after oestrogen treatment. Higher values were observed for PHI in castrated animals. G6PDH, 6PGDH and PHI activities were significantly reduced after Tamoxifen treatment.

In Table 3 the results on breast tissues are presented. The results are expressed in terms of milligram of DNA as the breast tissues could not be separated

Table 1. Enzyme activities (μmol of pyridine nucleotide reduced or oxidized/min/g tissue) and DNA and protein (mg/g tissue) in muscle

	I	C	P	E	T
DNA	0.210 ± 0.090	0.250 ± 0.080	0.240 ± 0.040	0.270 ± 0.020	0.350 ± 0.140
Protein	182.630 ± 34.690	191.400 ± 38.000	172.970 ± 15.560	184.930 ± 45.590	194.860 ± 34.570
PFK	6.690 ± 2.470	7.430 ± 3.600	5.740 ± 2.790	7.780 ± 2.390	7.600 ± 3.290
G6PDH	0.034 ± 0.017	0.033 ± 0.017	0.034 ± 0.008	0.037 ± 0.008	0.041 ± 0.027
6PGDH	0.059 ± 0.011	0.091 ± 0.028	0.058 ± 0.012	0.076 ± 0.035	0.070 ± 0.017
PGM	81.320 ± 15.580	57.890 ± 32.700	76.480 ± 32.960	84.910 ± 6.140	96.580 ± 10.650
LDH	185.840 ± 55.450	162.800 ± 6.300	175.080 ± 37.330	146.840 ± 25.360	156.800 ± 18.640
α-GPDH	13.090 ± 2.700	13.650 ± 2.050	12.140 ± 2.220	12.060 ± 2.010	12.480 ± 0.750
PHI	86.440 ± 10.870	89.960 ± 3.450	83.640 ± 8.940	86.000 ± 4.760	83.520 ± 8.350

The results are expressed as mean $\pm$S.D. I: intact animals, C: castrated animals, P: castrated animals treated with medroxyprogesterone acetate, E: castrated animals given oestradiol-17β in drinking water, T: castrated animals treated with Tamoxifen. For details of treatments see Materials and Methods section. There were no significant differences between the enzyme activities in any of the groups.

Table 2. Enzyme activities (μmol of pyridine nucleotide reduced or oxidized/min/g tissue), DNA and protein (mg/g tissue) in liver

	I	C	P	E	T
DNA	2.740 ± 2.238	1.972 ± 0.622	1.750 ± 0.847	3.357 ± 1.448	1.977 ± 1.217
Protein	156.500 ± 25.587	191.150 ± 30.600	159.090 ± 14.120	170.250 ± 18.040	160.970 ± 19.580
PFK	0.470 ± 0.020	0.480 ± 0.020	0.460 ± 0.070	0.470 ± 0.050	0.390 ± 0.060
G6PDH	1.120 ± 0.098	1.170 ± 0.394	1.020 ± 0.683	1.060 ± 0.294	0.182 ± 0.057*
6PGDH	2.050 ± 0.200†	1.365 ± 0.385	1.145 ± 0.272	2.432 ± 0.218*	0.565 ± 0.195*
PGM	30.420 ± 3.550	35.410 ± 6.170	34.040 ± 3.360	32.530 ± 2.700	28.330 ± 1.190
LDH	72.450 ± 10.010	72.200 ± 19.430	84.300 ± 18.320	70.800 ± 15.750	78.720 ± 34.800
α-GPDH	20.890 ± 2.970	27.490 ± 6.590	23.430 ± 3.460	21.730 ± 2.770	20.800 ± 2.410
PHI	20.520 ± 1.100†	26.680 ± 2.920	24.100 ± 4.190	31.280 ± 2.080	19.200 ± 2.510†

The animals were treated as described in the text. Abbreviations as in Table 1.
* Significant differences between C and another group $P < 0.001$.
† Significant differences between C and another group $0.02 < P < 0.01$.

from their fat pads and therefore both had to be homogenized together.

We were unable to estimate the activity of PFK in these tissues. There were no significant differences in the activities of 6PGDH, LDH, α-GPDH or PHI between any of the groups. Total protein content increased after castration and could not be returned to normal levels with progesterone or oestradiol: but Tamoxifen was effective in this regard. Castration reduced G6PDH activity: none of the three hormonal treatments restored the activity to a normal level. PGM activity was reduced on castration and there was a further reduction of the activity in tissues from Tamoxifen treated animals.

DISCUSSION

Of the three tissues investigated in this study, only the enzyme activities in muscle were unaffected by castration, oestrogen, Tamoxifen and progesterone treatments. This suggests that ovarian hormones do not play a major part in energy generation in this tissue and therefore differences in the activities observed in liver or breast may be a specific and not a general effect.

In liver, castration induces highly significant changes in the activities of 6PGDH and PHI. 6PGDH activities were significantly low in castrated animals and furthermore oestradiol administration restored the enzyme levels to that found in intact animals. Therefore, it appears that this oestrogen may be involved in the regulation of enzymes associated with the pentose-shunt. Castration induced significant increases in the activity of PHI and subsequent administration of oestradiol or progesterone to castrated animals failed to alter the rise. This suggests that the increase in PHI activities in castrated animals was not directly associated with the absence of ovarian hormones. It is also difficult to interpret the finding on biochemical grounds in that activities of other enzymes in the glycolytic pathway show no alterations and therefore the fate of the extra fructose-6-phosphate produced remains unclear. Administration of Tamoxifen to castrated animals resulted in significant decreases in the activities of G6PDH and 6PGDH. Since oestradiol appears to have a regulatory effect on 6PGDH, reduction in its activity after Tamoxifen treatment indicates that the drug might be acting as an anti-oestrogen. However, neither castration nor hormone treatments induced any changes in the activity of G6PDH, the reduction observed after Tamoxifen treatment might be due to a property of the drug other than its anti-oestrogenic action. The livers from Tamoxifen treated animals also showed a significant decrease in the activities of PHI. It appears that the drug reduces the activity of this

Table 3. Enzyme activities (μmol of pyridine nucleotide reduced or oxidized/min/mg DNA) and protein (mg/mg DNA) in mammary gland (rat breast tissue)

	I	C	P	E	T
Protein	30.580 ± 5.610†	49.270 ± 10.060	50.990 ± 12.630	47.890 ± 12.010	35.220 ± 3.740*
PFK	—	—	—	—	—
G6PDH	0.067 ± 0.030*	0.020 ± 0.012	0.017 ± 0.009	0.015 ± 0.011	0.007 ± 0.002
6PGDH	0.050 ± 0.027	0.030 ± 0.017	0.023 ± 0.016	0.016 ± 0.011	0.011 ± 0.003
PGM	0.838 ± 0.324*	0.350 ± 0.061	0.413 ± 0.178	0.513 ± 0.227	0.195 ± 0.032**
LDH	2.620 ± 0.900	2.020 ± 0.717	2.231 ± 0.876	2.133 ± 0.357	1.166 ± 0.305
α-GPDH	1.285 ± 0.493	1.088 ± 0.780	0.766 ± 0.499	0.474 ± 0.228	0.251 ± 0.102
PHI	2.000 ± 0.586	1.589 ± 0.387	1.570 ± 0.580	1.665 ± 0.241	1.046 ± 0.223

Abbreviations as in Table 1. For details of treatments see text.
† Significant differences between C and another group $0.02 < P < 0.01$.
* Significant differences between C and another group $0.05 < P < 0.02$.
** Significant differences between C and another group $0.01 < P < 0.001$.

enzyme to the levels found in intact animals and since oestradiol treatment has no effect on enzyme levels, the reduction observed after Tamoxifen treatment might be due to its direct action on glycolysis.

The activities of most of the enzymes estimated in this study are lower than those found by others [15] but are similar to the values reported for human liver [19]. Therefore it is possible that the alterations in enzyme activities following various treatments in this study can be extrapolated to man. A significant number of patients with advanced breast cancer are treated by large doses of Tamoxifen [20] and long term treatment with the drug might significantly reduce the activities of enzymes of the hexose-monophosphate pathway. This could result in decreased NADPH levels and availability of substrates for nucleic acid synthesis. A decrease in NADPH levels usually results in alterations in steroid hormone metabolism, particularly in those reactions where the pyridine nucleotide is an obligatory or rate limiting factor. At this stage it is not known whether Tamoxifen treatment results in changes in the excretion of hormone metabolites.

Most of the published work on glycolysis in breast tissues relates to either lactating mammary glands [21–25] or carcinogen-induced tumours [26, 27]. Korsrud and Baldwin [23] studied the effects of hypophysectomy, adrenalectomy, ovariectomy and hormone replacement therapies on the enzyme activities in rat mammary glands and found that ovariectomy did not appreciably alter enzyme patterns but that cortisol and prolactin are involved in the regulation of the hexose-monophosphate pathway. Bartley, Abraham and Chaikoff [21] found higher mammary gland G6PDH activities in lactating mice than in virgin or pregnant animals. Similar results were recorded on the mammary glands of rats bearing carcinogen induced tumours [27]. Our results on non-pregnant, normal rat mammary glands show that castration significantly reduces the activities of G6PDH and PGM and increases the total protein content of the mammary glands. However, unlike lactating mammary glands or tumours, this castration-induced decrease in enzyme activities is not restored by treatment with oestradiol or progesterone. Neither did the increase in total protein in the glands after castration show any changes after treatment with the hormones. This indicates that the castration-induced differences observed in the non-lactating mammary glands may not be due to lack of ovarian hormones.

The effects of Tamoxifen treatment on glucose metabolism in normal breast tissue have not been reported before. The results presented here show that administration of the drug to castrated animals further significantly reduces the activity of PGM, G6PDH, 6PGDH and α-GPDH activities in the treated group were also lower than in castrated controls but level of significance was not reached. Since oestradiol treatment failed to induce changes in the activities of any of these enzymes, the effects observed after Tamoxifen administration may by due to either its direct action on the breast or alteration in the endocrine environment. Tamoxifen also reversed the castration-induced rise in total protein content of mammary glands however the mechanism by which this reversal takes place remains to be explored. In conclusion, although castration induces changes in the activities of certain enzymes in rat mammary glands, these alterations cannot be directly attributed to the absence of oestrogens or progesterone as administration of these hormones to castrated rats failed to increase enzyme activities. However oestradiol does appear to regulate the activity of 6PGDH in the liver.

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REFERENCES

1. Mueller G. E.: Estrogen action: a study of the influence of steroid hormones on genetic expression. *Biochem. Soc. Symp.* **32** (1971) 1–29.
2. Means A. R. and O'Malley B. W.: Mechanism of estrogen action: early transcriptional and translational events. *Metabolism* **21** (1972) 357–370.
3. Chan L. and O'Malley B. W.: Mechanism of action of the sex steroid hormones. *New Engl. J. Med.* **24** (1976) 1322–1328.
4. Bigger J. D. and Claringbold P. J.: Criteria of vaginal response to oestrogens. *J. Endocr.* **11** (1954) 277–284.
5. Ladinsky J. L., Grunchow H. W. and Peckham B. M.: Cellular behaviour of the vaginal epithelium treated with testosterone propionate alone and in combination with diethylstilboestrol. *J. Endocr.* **41** (1968) 161–170.
6. Lyons W. R., Li C. H. and Johnson R. E.: The hormonal control of mammary growth and lactation. *Rec. Prog. Horm. Res.* **14** (1958) 219–254.
7. Turkington R. W.: Hormone-dependent differentiation of mammary gland *in vitro*. In *Current Topics in Developmental Biology* **3** (1968) 199–218.
8. Hilf R., Michel I. and Bell C.: Biochemical and morphological responses of normal and neoplastic tissue to hormonal treatment. *Rec. Prog. Horm. Res.* **23** (1967) 229–295.
9. Benson G. K., Cowie A. T., Cox C. P. and Goldzweig S. A.: Effects of oestrone and progesterone on mammary development in the guinea-pig. *J. Endocr.* **15** (1957) 126–144.
10. Harper M. J. K. and Walpole A. L.: Contrasting endocrine activities of *cis* and *trans* isomers in a series of substituted triphenylethylenes. *Nature* **21** (1966) 87.
11. Lipman M.: Hormone-responsive human breast cancer in continuous tissue culture. In *Breast Cancer: Trends in Research and Treatment* (Edited by J. C. Heuson, W. H. Mattheim and M. Rozencweiz). Raven Press, New York (1976) 111–140.
12. Nicholson R. I., Golder M. P., Davies P. and Griffiths K.: Effects of oestradiol-17β and tamoxifen on total and accessible cytoplasmic oestradiol-17β receptors in DMBA-induced rat mammary tumours. *Eur. J. Cancer* **12** (1976) 711–717.
13. Martin L.: Decidualization and ovum implantation in spayed mice treated with oestriol. *J. Endocr.* **72** (1977) 181–185.
14. Jordan V. C.: Effects of tamoxifen (ICI 46474) on initiation and growth of DMBA induced rat mammary carcinoma. *Eur. J. Cancer* **12** (1976) 419–424.

15. Shonk C. E. and Boxer G. E.: Enzyme patterns in human tissues. I. Methods for the determination of glycolytic enzymes. *Cancer Res.* **24** (1964) 709–721.
16. Deshpande N., Mitchell I. and Millis R.: Enzyme studies in human breast tumours. *Eur. J. Cancer* **13** (1977) 1261–1267.
17. Burton K.: A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62** (1956) 315–323.
18. Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
19. Shonk C. E., Koven B. J., Majima H. and Boxer G. E.: Enzyme patterns in human tissues II. Glycolytic enzyme patterns in non-malignant human tissues. *Cancer Res.* **24** (1964) 722–731.
20. Willis K. J., London D. R., Ward H. W. C., Butt W. R., Lynch S. S. and Rudd B. T.: Recurrent breast cancer treated with the anti-oestrogen tamoxifen: correlation between hormonal changes and clinical course. *Br. Med. J.* **1** (1977) 425–428.
21. Bartley J. C., Abraham S. and Chaikoff I. L.: Activity patterns of several enzymes of liver, adipose tissue and mammary gland of virgin, pregnant and lactating mice. *Proc. Soc. exptl. Biol. Med.* **123** (1966) 670–675.
22. Walters E. and McLean P.: Effect of thyroidectomy on pathways of glucose metabolism in lactating rat mammary gland. *Biochem J.* **105** (1967) 615–623.
23. Korsrud G. O. and Baldwin R. L. Effects of endocrinectomy and hormone replacement therapies upon enzyme activities in lactating rat mammary glands. *Biol. Reprod.* **1** (1969) 21–30.
24. Field B. and Coore H. G.: Control of rat mammary gland pyruvate dehydrogenase by insulin and prolactin. *Biochem. J.* **156** (1976) 333–337.
25. Kopelovich L., Abraham S., McGrath H., De Ome K. B. and Chaikoff I. L.: Metabolic characteristics of a naturally occurring preneoplastic tissue. I Glycolytic enzyme activities of hyperplastic alveolar nodule outgrowths and adenocarcinomas of mouse mammary gland. *Cancer Res.* **26** (1966) 1534–1546.
26. Rees E. D. and Huggins C.: Steroid influences on respiration, glycolysis and levels of pyridine nucleotide-linked dehydrogenases of experimental mammary cancers. *Cancer Res.* **20** (1960) 963–971.
27. Hilf R., Goldenberg H., Michel I., Carrington M. J., Bell C., Gruenstein M., Meranze D. R. and Shimkin M. B.: Biochemical characteristics of mammary glands and mammary tumours of rats induced by 3-methylcholanthrene and 7,12-dimethylbenz(a)anthracene. *Cancer Res.* **29** (1969) 977–988.