Ornithine decarboxylase, transglutaminase, diamine oxidase and total diamines and polyamines in maternal liver and kidney throughout rat pregnancy

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1. Ornithine decarboxylase (ODC; EC 4.1.1.17), transglutaminase (EC 2.3.2.13), diamine oxidase (DAO; EC 1.4.3.6) and total di- and poly-amines were studied in rat liver and kidney cortex throughout pregnancy. 2. In liver, ODC activity exhibited two major peaks (4.5–5 times the control activities) on days 15 and 17. Also putrescine and spermidine increased biphasically (3–4-fold), but no variation in spermine content was observed. Transglutaminase activity showed slight variations only near the end of gestation. 3. In kidney, ODC activity did not fluctuate significantly during pregnancy, whereas both transglutaminase activity and putrescine content showed three major increases, in very early, middle and late pregnancy. No significant variations in spermidine and spermine were observed. 4. In both organs, DAO activity, very low or undetectable until day 10, dramatically increased (10- and 20-fold in kidney and liver respectively) in the second half of pregnancy, reaching maxima on days 16–17 and 19. 5. The results obtained for transglutaminase, ODC and total di- and poly-amines are interpreted on the basis of hyperplastic and hypertrophic events in the liver and kidney respectively. The behaviour of DAO suggests that the enzyme plays an important role in the control of intracellular diamine concentration.

INTRODUCTION

The diamine putrescine and the polyamines spermidine and spermine are widely distributed in living organisms. Increased di- and poly-amine concentrations during cell proliferation have been well established; accordingly, the enzymes involved in their biosynthesis, in particular ODC, have been found to correlate positively to growth stimuli (Heby, 1981; Tabor & Tabor, 1984). Although a large literature suggests that these compounds can exert effects over a wide range of cellular activities, their biological role is still poorly understood (Heby, 1981; Pegg & McCann, 1982). It has been shown that diamines and polyamines are also present in mammalian tissues and body fluids covalently linked to glutamine residues of proteins (Williams-Ashman & Canellakis, 1980; Chan *et al.*, 1981; Beninati *et al.*, 1985). The polyamine-glutamine linkage is catalysed by transglutaminase, a family of Ca^{2+} -dependent enzymes widely distributed in mammalian cells and in their biological fluids (Folk, 1980).

Pregnancy in mammals, bringing about large morphological and functional modifications of the maternal organs, may be a suitable model to investigate *in vivo* how different cell kinetics can be associated with different polyamine patterns. Increased diamine and polyamine urinary excretion and concentration in the maternal plasma and in the organs of the reproductive system, elevated ODC and DAO activities in these organs, together with enhanced plasma contents of the latter enzyme, have been reported during pregnancy (Kobayashi,

1964; Guha & Jänne, 1975; Andersson & Henningsson, 1981; Porta & Della Pietra, 1981). Gestation determines relevant anatomical and biochemical modifications also in maternal liver and kidney; however, little information is available on their diamine and polyamine metabolism (Campbell *et al.*, 1974; Andersson & Henningsson, 1981). The two organs exhibit a different cellular response to pregnancy: in liver, increases in both nuclear volume and DNA concentration, followed by a 2-fold enhancement of mitotic index near delivery, suggest marked cell proliferation (Campbell *et al.*, 1974); in kidney, the observed weight gain with elongation of proximal convoluted tubules may be related to cell hypertrophy (Matthews, 1977; Garland *et al.*, 1978; Atherton & Pirie, 1981; Atherton & Green, 1983).

We have investigated total putrescine, spermidine and spermine contents (including also acid-insoluble amines) and related enzymic activities (ODC, DAO and transglutaminase) in maternal liver and kidney cortex during rat pregnancy, to obtain further insight into the role *in vivo* of diamines and polyamines in hyperplasia and hypertrophy.

EXPERIMENTAL

Chemicals

[1,4- ^{14}C]Putrescine dihydrochloride (109 mCi/mmol), [1,4(n)- ^3H]putrescine dihydrochloride (26.3 Ci/mmol) and DL-[1- ^{14}C]ornithine (57.3 mCi/mmol) were from Amersham International (Amersham, Bucks., U.K.).

Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); DAO, diamine oxidase (EC 1.4.3.6).

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Toluene and Instagel II were from Packard (Zurich, Switzerland). Chloral hydrate, aminoguanidine hemisulphate, pyridoxal phosphate, dithiothreitol, putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrahydrochloride, Hyamine, *NN'*-dimethylcasein, bovine serum albumin and L-ornithine were purchased from Fluka (Buchs, Switzerland). All other chemicals were of reagent grade and used without further purification.

Animals

Wistar female rats, weighing 250–300 g, were used. The animals were kept at 20–22 °C with 12 h light and 12 h darkness and fed *ad libitum* with a standard diet. On the day of oestrus, rats were transferred in separate cages for mating; the following day, a post-coital test was performed, and the presence of a vaginal plug was considered as positive evidence of pregnancy.

All rats were killed between 09:00 and 10:00 h. Rats were anaesthetized with sodium thiopental (Farmotal, Farmitalia; 100 mg/kg body wt.). Livers were perfused *in situ* by injecting cold 0.25 M-sucrose through the portal vein. The homogenate (25%, w/v) was prepared either in 0.2 M-sodium/potassium phosphate buffer, pH 7.6, for DAO determination, or in 0.25 M-sucrose for diamine and polyamine determinations and for preparing the cytosolic fraction. The latter was obtained by centrifuging the postmitochondrial supernatant at 120000 *g* for 2 h in a 50Ti rotor (L5-50 Beckman ultracentrifuge). Kidneys were perfused with cold 0.25 M-sucrose through the renal artery, and the cortex tissue was obtained by dissecting transverse sections of decapsulated kidneys; homogenates were prepared as described for liver. All manipulations were carried out at 0–4 °C.

Enzyme assays and chemical determinations

DAO activity was determined by the method of Okuyama & Kobayashi (1961), which measures the [¹⁴C]Δ¹-pyrroline formation from [¹⁴C]putrescine. The incubation mixture contained 140 mM-sodium/potassium phosphate buffer, pH 7.6, tissue homogenate (about 10 mg of protein), 2.5 mM-putrescine containing 1 μCi of [1,4-¹⁴C]putrescine for kidney or 1.5 mM-putrescine containing 0.6 μCi of [1,4-¹⁴C]putrescine for liver; the medium also contained 10 mM-chloral hydrate to exclude the interference of aldehyde-metabolizing enzymes with Δ¹-pyrroline formation (Deitrich & Erwin, 1975). Blanks contained 0.1 mM-aminoguanidine hemisulphate. The final volume was 2 ml. The reaction was allowed to proceed for 2 h at 37 °C in a shaking bath and was stopped by adding 200 mg of NaHCO₃ and 10 ml of toluene. Aminoguanidine hemisulphate (final concn. 0.1 mM) was also added to test tubes. The mixture was shaken for 3 min, centrifuged at 3000 *g* for 10 min and left at –20 °C until the lower aqueous phase was frozen. The upper layers were transferred into counting vials and assayed in a Packard liquid-scintillation spectrometer model C2425. One enzyme unit is defined as the amount of enzyme that converts 1 nmol of substrate into product/h.

ODC activity was determined by the method of Russell & Snyder (1968) by measuring the liberation of ¹⁴CO₂ from [¹⁴C]ornithine. The reaction mixture contained 100 mM-glycylglycine buffer, pH 7.2, 0.2 mM-pyridoxal phosphate, 5 mM-dithiothreitol, 1 mM-L-ornithine plus

1 μCi of DL-[1-¹⁴C]ornithine and liver or kidney cytosolic fraction (10–15 mg of protein) in a final volume of 1.5 ml. The incubation was carried out in a shaking water bath for 60 min at 37 °C in glass flasks equipped with an internal reservoir containing 0.5 ml of Hyamine, closed by an air-tight rubber cap. The reaction was stopped by injecting 1.5 ml of 40% (w/v) trichloroacetic acid, and after 20 min of additional shaking the Hyamine was transferred into vials containing 5 ml of Instagel and assayed for radioactivity. One enzyme unit is defined as the amount of enzyme that converts 1 pmol of substrate into reaction product/h.

Transglutaminase activity was measured by the method of Folk & Cole (1966), with some modifications, by detecting the incorporation of [³H]putrescine into *NN'*-dimethylcasein. The incubation mixture contained 50 mM-Tris/HCl buffer, pH 8.3, 5 mM-CaCl₂, 10 mM-dithiothreitol, 30 mM-NaCl, 2.5 mg of *NN'*-dimethylcasein/ml, 0.2 mM-putrescine containing 1 μCi of [³H]-putrescine, and 0.5–1 mg of protein of either liver or kidney cytosolic fraction, in a final volume of 0.3 ml. After 20 min of incubation at 37 °C in a shaking water bath, 50 μl of samples were spotted on Whatman 3MM filter paper moistened with 20% trichloroacetic acid. Free [³H]putrescine was eliminated by washing with large volumes of cold 5% trichloroacetic acid containing 0.2 M-KCl, and filters were transferred into counting vials containing 5 ml of Instagel. One enzyme unit is defined as the amount of enzyme that binds 1 nmol of putrescine to *NN'*-dimethylcasein/h. Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Total putrescine, spermidine and spermine were determined as dansyl (5-dimethylaminonaphthalene-1-sulphonyl) derivatives in 2 ml samples of either liver or kidney total homogenate after hydrolysis in 6 M-HCl for 16 h at 110 °C. This procedure allows the detection of all diamines and polyamines of the tissue, including those covalently and tightly bound to cellular components, that are undetectable when using, for analysis, the soluble phase obtained by acid precipitation of tissue homogenate (Beninati *et al.*, 1985). The dansylation procedure was essentially that described by Seiler (1970), with 1,6-diaminohexane as internal standard and toluene instead of benzene to extract the derivatives from the reaction mixture. The chromatography was performed as described by Van Rooijen *et al.* (1984) on a 4.6 mm × 150 mm column packed with Hypersil ODS (5 μm particle diameter), with a Beckman h.p.l.c. apparatus equipped with a 450 data system/controller.

RESULTS

All data reported in this paper refer to perfused organs, since blood polyamine concentrations fluctuate in mammals as a function of either the reproductive cycle (Lundgren *et al.*, 1976) or the gestational age (Henningsson *et al.*, 1983); moreover, a drastic increase in plasma DAO during pregnancy has been described (Kobayashi, 1964; Porta & Della Pietra, 1981). The absence of blood from the homogenate also excludes the action of plasma transglutaminase (Factor XIIIa) (Folk, 1980).

Fig. 1 shows the patterns of ODC, transglutaminase and DAO activities in rat liver during pregnancy; values

Fig. 1. Liver ornithine decarboxylase, transglutaminase and diamine oxidase activities throughout pregnancy in rats

ODC and transglutaminase activities were assayed on liver cytosolic fraction; DAO activity was determined on total homogenate (see the Experimental section). Values are expressed as percentages of activities in non-pregnant animals, which are: ODC, 26.1 ± 8.0 pmol/h per mg of protein (Δ); transglutaminase, 4.3 ± 0.4 nmol/h per mg of protein ($\square$); DAO, 0.225 ± 0.05 nmol/h per mg of protein ($\bullet$). Values are means of duplicate determinations performed on four to six animals for each age of pregnancy and for the controls. Bars represent S.E.M.: *significantly different from control values ($P < 0.05$); **significantly different from peak values on days 16 and 19 ($P < 0.05$).

Fig. 2. Liver total putrescine, spermidine and spermine contents throughout pregnancy in rats

Di- and poly-amines were assayed on 2 ml samples of total homogenate, after hydrolysis in 6 M-HCl at 110°C for 16 h (see the Experimental section). Values are expressed as percentages of those detected in non-pregnant animals, which are: putrescine, 0.15 ± 0.013 nmol/mg of protein ($\bullet$); spermidine, 1.69 ± 0.37 nmol/mg of protein ($\blacktriangle$); spermine, 3.45 ± 0.34 nmol/mg of protein ($\circ$). Values are means of duplicate determinations performed on two or three animals for each age of pregnancy and for the controls. Bars represent S.E.M.: *significantly different from control values ($P < 0.05$).

are expressed as percentages of those found in the liver of non-pregnant rats. From at least day 4 of gestation ODC activity is doubled in the liver of pregnant rats; marked increases are detected on days 15 and 17; a

decrease to values of non-pregnant animals at the end of gestation is observed. Slight variations of liver transglutaminase activity are present only near delivery. Very low DAO activity is detected until days 10–11; thereafter a rapid and remarkable increase (20-fold) occurs between days 14 and 17, followed by a partial fall on day 18 and then by a new increase to values 10 times those detectable in non-pregnant-rat liver. Fig. 2 shows the total di- and poly-amine contents in liver during pregnancy. Values are expressed as percentages of those of non-pregnant rats. Putrescine content shows a biphasic pattern, with peaks on days 13 and 19; at the end of gestation, values are still twice controls. A rather similar behaviour is shown by spermidine, with broad increases on days 13–14 and 19–21, but no significant modification of spermine concentration is observed throughout pregnancy. It is noteworthy that the increase in putrescine content observed between days 4 and 13 parallels that of ODC activity, when DAO activity is very low, just beginning to increase on day 13. Between days 14 and 17 putrescine decreases to about control values in the presence of the highest DAO activity. Corresponding to the subsequent decrease of DAO and to the second peak of ODC, a new increase in putrescine is observed. Moreover, it is noteworthy that the spermidine peaks parallel those of putrescine.

Fig. 3 reports the enzymic pattern of rat kidney cortex during pregnancy (values are expressed as in Fig. 1). An early increase in ODC activity on day 4 is observed; the activity progressively decreases, reaching control values around day 13 and remaining practically unchanged until

Fig. 3. Kidney ornithine decarboxylase, transglutaminase and diamine oxidase activities throughout pregnancy in rats

ODC and transglutaminase activities were assayed on kidney cytosolic fraction; DAO activity was determined on total homogenate. Values are expressed as percentages of activities in non-pregnant animals, which are: ODC activity, 17.9 ± 2.3 pmol/h per mg of protein; transglutaminase activity, 0.4 ± 0.1 nmol/h per mg of protein; DAO activity, 0.09 ± 0.06 nmol/h per mg of protein. Values are means of duplicate determinations performed on four to six animals for each age of pregnancy and for the controls. Bars represent S.E.M.: *significantly different from control values ($P < 0.05$); **significantly different from peak values on days 14 and 19 ($P < 0.05$). For symbols see Fig. 1.

Fig. 4. Kidney total putrescine, spermidine and spermine contents throughout pregnancy in rats

Di- and poly-amines were assayed on 2 ml samples of total homogenate, after hydrolysis in 6 M-HCl at 110°C for 16 h. Values are expressed as percentages of those detected in non-pregnant animals, which are: putrescine, 0.26 ± 0.08 nmol/mg of protein; spermidine, 1.11 ± 0.11 nmol/mg of protein; spermine, 3.30 ± 0.07 nmol/mg of protein. Values are means of duplicate determinations performed on two or three different animals for each age of pregnancy and for the controls. Bars represent S.E.M.: *significantly different from control values ($P < 0.05$). For symbols see Fig. 2.

term. The same pattern of ODC activity was obtained in rat kidney cortex by Brandt *et al.* (1972) after unilateral nephrectomy. DAO activity shows a biphasic pattern, with the major peaks on days 16–17 and 19 of pregnancy, followed by a partial decrease in activity (5 times control values). Large fluctuations of transglutaminase are also observed: the activity is already enhanced at 4 days after mating; a second broad increase is observed between days 13 and 16; on day 18 a marked and significant fall is detected, followed by a sharp peak (4–5-fold increase) on day 19. Although the reported transglutaminase activity was assayed on the cytosolic fraction, the relative percentages of soluble transglutaminase with respect to the total homogenate activity do not vary throughout pregnancy, in both liver and kidney ($79 \pm 13.6\%$ and $52 \pm 10.6\%$ of total, respectively; results not shown). Fig. 4 shows the total putrescine, spermidine and spermine contents in rat kidney cortex during pregnancy (values are expressed as in Fig. 2). Although spermidine and spermine contents show no significant variation throughout gestation, that of putrescine exhibits wide fluctuations: three major peaks are observed, at days 4, 13 and 18 and values twice the control are present in all other stages of pregnancy; only at term does putrescine concentration approach control values. In kidney a reverse correlation may be noted between putrescine content and DAO activity; in fact, a decrease in putrescine concentration is observed whenever DAO activity increases, and vice versa. Transglutaminase peaks follow those of putrescine, but no such correlation between ODC activity and di- and poly-amine contents is found.

DISCUSSION

The data in the present paper demonstrate that pregnancy induces large modifications of total di- and poly-amine contents and some related enzyme activities (ODC, DAO and transglutaminase) in both rat liver and kidney. The results obtained for the liver may be explained on the basis of cell proliferation; in fact, it is well known that in rat pregnancy relevant modifications of cell turnover, leading to hyperplasia, take place in liver (Campbell *et al.*, 1974; Mayel-Afshar & Grimbale, 1983). Extension of S and/or G2 phases between days 12 and 18 of gestation, with increases in liver weight, total RNA and DNA content and a parallel increase in hepatocyte nuclear volume, have been demonstrated. From day 18 until delivery an elevated mitotic index is associated with a rapid fall in nuclear volume (Campbell *et al.*, 1974). It is noteworthy that the major peaks of liver ODC activity are temporally correlated with the above-mentioned premitotic stages. Moreover, the biphasic increase in ODC activity strictly resembles that described in dividing cell cultures as well as in rat liver regeneration (Heby, 1981; Pegg & McCann, 1982; Tabor & Tabor, 1984). Further support for the hypothesis that ODC fluctuations are related to parenchymal-cell proliferation comes from the behaviour of total content of diamines and polyamines in liver of pregnant rats. In fact, the biphasic increase in both total putrescine and spermidine practically parallels that of ODC, depicting a situation generally present in proliferating eukaryotic cells (Heby, 1981; Sunkara & Rao, 1981; Pegg & McCann, 1982). The present study shows that the requirement for increased putrescine and spermidine for adequate cell renewal can be extended also to the liver during pregnancy. Moreover,

the high polyamine contents detected in liver on days 12–19, coinciding with the pre-replicative phase of parenchymal cells (mitotic index reaches a maximum on days 20–21 of gestation), confirm *in vivo* the important role played by diamines and polyamines in DNA replication and in the progression through the cell cycle (Heby, 1981; Pegg & McCann, 1982; Luk & Baylin, 1984; Tabor & Tabor, 1984).

In kidney the cellular response to pregnancy is mainly of hypertrophic type: no cell proliferation or DNA synthesis has been reported, whereas RNA and protein syntheses are enhanced (Atherton & Pirie, 1981); the only anatomical change reported in kidney of the pregnant rat, i.e. the elongation of proximal convoluted tubules, is already completed at 5–6 days of gestation (Matthews, 1977; Atherton & Pirie, 1981). An increasing number of reports show a positive relationship between the enhancement of transglutaminase activity and differentiation of cultured cell lines (Rice & Green, 1977; Birckbichler *et al.*, 1982; Scott *et al.*, 1982; Lee-Hsu & Friedman, 1984). Moreover, the process of differentiation is often associated with unchanged or decreased ODC activity, often in the presence of increased putrescine (Nakos-Canellakis & Bondy, 1983; Ewton *et al.*, 1984; Jetten & Shirley, 1985). Although kidney hypertrophy during pregnancy cannot be considered a differentiative process, it is doubtless characterized by synthesis of specific mRNAs and proteins required, at the cellular level, for the enhancement of renal function (Brandt *et al.*, 1972). Thus the similarity of the enzyme and amine patterns in kidney during pregnancy to those observed during cell differentiation suggests that an enhanced transglutaminase activity, in the presence of high putrescine concentrations, may be related to the post-translational modification of specific proteins synthesized to satisfy the increased functional requirements.

Although much information is available on the biochemistry of tissue transglutaminase, little is known about its physiological role. It has been hypothesized that the enzyme participates in receptor-mediated endocytosis; in particular, Fesus *et al.* (1981) reported that, as a consequence of exposure to immuno-complexes, macrophages show an increased transglutaminase activity, with incorporation of amine into membrane proteins. Also, during pregnancy an incorporation of putrescine into kidney proteins may be hypothesized. In fact Andersson & Henningsson (1981) reported that, in rat kidney on day 19 of pregnancy, the acid-soluble putrescine concentration is 6-fold lower than the values obtained in the present study for the total diamine. Such a difference cannot be explained only on the basis of the analytical method used, since all other putrescine values in liver and in kidney of non-pregnant animals are similar. Thus it is tempting to speculate that the high transglutaminase activity observed just after mating until term of gestation can determine the formation of polyamine-protein complexes in the kidney during the processes leading to the described anatomical and functional hypertrophy. Studies are required to test this hypothesis.

Unlike ODC and transglutaminase, DAO exhibits essentially the same pattern in liver and kidney during pregnancy, suggesting that the enzyme is regulated by factors different from those that regulate the other enzymes studied. The observed correlation between DAO and putrescine, in agreement with that obtained in regenerating rat liver and in kidney hypertrophy (Perin

et al., 1981), seems to support the suggested role of DAO mainly dealing with the control of the putrescine concentration inside the cell (Argento-Cerù & Autuori, 1985). However, the complementary behaviour of DAO and putrescine is not always present (for instance, in the liver on day 19 both are increased). On the other hand, ODC activity is at all times less than that of DAO, both in percentage increase and in absolute value. These findings lead us to hypothesize that, during pregnancy, the increased DAO activity may also be related to the high extracellular amine concentrations of fetal origin detected in both maternal plasma and urine in late pregnancy (Kobayashi, 1964; Andersson & Henningsson, 1981). Thus the intracellular kidney and liver DAO could represent a defence against the circulating amines, as previously suggested for the placental DAO; in this context it is noteworthy that both the time course and the magnitude of the DAO increase are similar to those reported in the uterus and maternal placenta during rat pregnancy (Guha & Jänne, 1975).

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