Br. J. Pharmac. (1979), **67**, 109–114

EFFECTS OF THE OESTROUS CYCLE AND EXOGENOUS OVARIAN STEROIDS ON METABOLISM OF β-PHENYLETHYLAMINE IN RAT LUNG

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1 Metabolism of [^{14}C]-β-phenylethylamine (PEN), a substrate for monoamine oxidase-B (MAO-B), was measured in lung homogenates and in perfused lungs during the 4 day oestrous cycle of the rat.

2 Metabolism *in vitro* was high during met-oestrus and di-oestrus and low during pro-oestrus and oestrus; this variation in activity correlated with changes in V_{max} of the enzyme without changes in K_m.

3 PEN metabolism in lung homogenates was also altered by treatment of rats with 17β-oestradiol but not by progesterone treatment.

4 Metabolism of [^{14}C]-PEN in perfused lungs was the same during either pro-oestrus or met-oestrus. Uptake of [^{14}C]-PEN in perfused lung measured directly was also the same at these two stages.

5 These results demonstrate that in lungs MAO-B activity was affected by endogenous changes in steroid level but that such changes in enzymic activity were not reflected in the metabolic properties of whole lung.

Introduction

We have already shown, using 5-hydroxytryptamine (5-HT) as the substrate, that monoamine oxidase (MAO) activity in lung homogenates varied during the 4 day oestrous cycle of the rat and that the inactivation of 5-HT in perfused lungs during met-oestrus differed from that at pro-oestrus (Bakhle & Ben-Harari, 1978a). 5-Hydroxytryptamine is a substrate for A-type MAO (MAO-A) (Bakhle & Youdim, 1979), and changes in MAO-A are not necessarily predictive of changes in MAO-B activity (Lyles & Callingham, 1974). β-Phenylethylamine (PEN) is a substrate for MAO-B (Bakhle & Youdim, 1979) and we have therefore measured the metabolism of [^{14}C]-PEN in homogenates of lung and in perfused lungs in order to assess the variation of MAO-B activity over the oestrous cycle in rats. A preliminary account of some of this work has been communicated to the Physiological Society (Bakhle & Ben-Harari, 1978b).

Methods

Experiments were carried out on sexually mature virgin female rats (Wistar strain, 150 to 200 g) kept in normal lighting conditions of our animal house (white light from 08 h 00 min to 17 h 00 min and darkness from 17 h 00 min to 08 h 00 min). The animals were allowed food and water *ad libitum* and smears were taken not earlier than 7 days after the rats' arrival in the animal house to allow for a 'settling-down' period. Smears were taken each morning at 10 h 00 min, i.e. 2 h after the onset of white light and only rats which exhibited at least 2 consecutive regular cycles of 4 day length were used.

The effects of exogenous steroids were studied by treating female rats with either progesterone (1 mg/kg body weight) or 17β-oestradiol (0.5 mg/kg) given daily by intraperitoneal injection for 8 days. The steroids were injected as a suspension in 0.9% w/v NaCl solution (saline) and control rats received injections of saline only for the 8 day period.

Preparation of lungs

Animals were killed within 30 min of taking a smear. The lungs were removed and perfused via the pulmonary artery as described previously (Alabaster & Bakhle, 1970) with Krebs solution (mM: $NaHCO_3$ 25, NaCl 120, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 5.6) at 37°C, gassed with a 95% O_2

0007-1188/79/090109-06 $01.00

and 5% CO_2 mixture at a constant flow of 8 ml/min. After 10 to 15 min of perfusion, the effluent was free of blood and the lungs were either taken for homogenization or the perfusion continued for measurement of PEN metabolism.

Preparation of homogenate and incubation

Once clear of blood, the lungs were placed in 0.1 M phosphate buffer, pH 7.4, at 0°C, and dissected free from the heart, trachea and any extraneous tissue. The lungs were chopped, followed by homogenization in 0.3 M sucrose in the phosphate buffer (tissue to buffer ratio of about 1:10) at 0°C using two 10 s bursts of a Polytron homogenizer (PCU-2). The crude homogenate was filtered through gauze to remove any remaining large fragments and the filtrate was used as the source of enzyme. Each homogenate was divided into 5 ml aliquots and stored frozen (−20°C) until required. No significant loss of activity in the frozen homogenates occurred for the duration of these experiments (2 weeks).

Metabolism of PEN by the homogenate was assayed in an incubation mixture consisting of: 0.5 ml lung filtrate (approximately 0.4 mg protein/ml); 0.5 ml [^{14}C]-PEN (10 to 200 μM) containing approximately 10^4 d/min and 4 ml of 0.1 M phosphate buffer, pH 7.4. The mixture was incubated at 37°C in a shaking water bath for 60 min. Buffer and enzyme were pre-incubated for 5 min before addition of the substrate to start the reaction: the zero time sample (1 ml) was taken immediately after adding the substrate. The 0 and 60 min samples were added to 0.5 ml of 1.5 M perchloric acid at 0°C to stop the reaction. Each sample was adjusted to pH 6 to 6.5 with 3 M potassium hydroxide and the resulting clear supernatant analysed by ion-exchange chromatography. Enzyme activity bore a linear relationship to protein concentration and time of incubation. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as the standard.

Metabolism and uptake in perfused lungs

After the initial perfusion period, PEN metabolism was measured by collecting lung effluent, for a total of 30 min, during and after a 3 min infusion (0.4 ml/min) of PEN into the pulmonary arterial cannula. The PEN infused was a mixture of [^{14}C]-PEN (10^4 d/min per infusion) and unlabelled PEN to give a final concentration of PEN from 0.15 μM to 150 μM. At the end of the 30 min collection time, radioactivity in the effluent had fallen to background levels and another infusion of PEN was given. Each lung received in all 3 infusions (at 30 min intervals) of widely different concentrations, e.g. 0.15, 1 and 10 μM; 0.5, 10 and 50 μM. Each concentration was tested in at least 3 lungs and usually in 5 lungs.

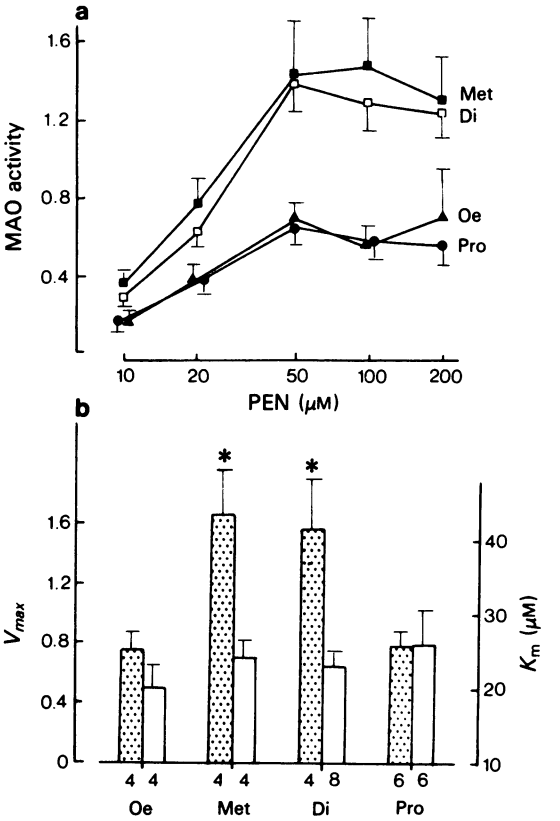

Figure 1 Metabolism of [^{14}C]-β-phenylethylamine ([^{14}C]-PEN) by homogenates of the rat lung. In (a) the variation of metabolism with substrate concentration is shown, for all four stages of the oestrous cycle (Pro = pro-oestrus; Oe = oestrus; Met = met-oestrus; Di = di-oestrus). Monoamine oxidase activity (vertical axis) is expressed as pmol.μg protein^{-1}.min^{-1} and the substrate concentration (horizontal axis) as μM PEN. Each concentration was used with lung samples from at least 4 rats, and the means are shown; vertical lines show s.e. mean. Details of the incubations given in the text. In (b) apparent K_m (open columns) and V_{max} (stippled columns) values calculated from these results are shown. Whereas K_m values were not different at different stages of the cycle, V_{max} (expressed as pmol.μg protein^{-1}, min^{-1}) at met- and di-oestrus were almost twice that at the other stages.

To measure uptake of [^{14}C]-PEN, a 3 min infusion was given through the lungs and immediately afterwards perfusion stopped and the lungs removed from the perfusion system. The lungs were dissected free of the trachea, the remainder of the heart and any

extraneous tissue and then homogenized in cold (0°C) 0.3 M perchloric acid. The homogenate was centrifuged at 1000 *g* for 20 min and samples of the resulting supernatant together with samples of lung effluent collected during the infusion were taken for chromatographic analysis. To check for breakdown of substrate during the extraction procedure, [^{14}C]-PEN was added to perfused lungs immediately before homogenization in perchloric acid and the extraction and analysis carried out as usual. Less than 5% of the added radioactivity appeared as 'metabolite' after chromatography

Ion exchange chromatography and measurement of radioactivity

Samples (0.5 ml) of the neutralized supernatant from the incubation mixtures and of the effluent collected from the lung were applied to columns of Amberlite CG-50 resin (Southgate & Collins, 1969). The metabolites of PEN were eluted from the column with water (2 ml) and radioactivity in the total eluate measured after mixing with Triton-toluene scintillation fluid (PPO 5 g, DMPOPOP 0.25 g, toluene 1 litre, Triton X-100 0.5 litre) using a liquid scintillation counter. Corrections were made for quenching using sample channels ratio.

Materials

Progesterone, 17β-oestradiol and β-phenylethylamine hydrochloride were obtained from Sigma; pentobarbitone from May and Baker and heparin from Evans. Other chemicals used were of analytical reagent grade. Radioactive PEN ([1-^{14}C]-PEN, 48 mCi/mmol) was obtained from New England Nuclear, Frankfurt.

The unpaired *t* test was used to determine the significance of differences between means and values of $P < 0.05$ were accepted as significant.

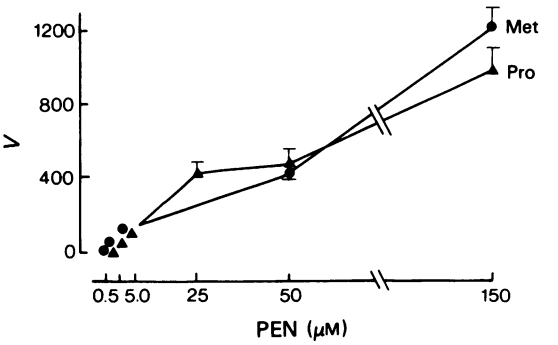

Figure 2 Metabolism of β-phenylethylamine (PEN) in perfused lungs during met- (●) and pro-oestrus (▲). The radioactivity in lung effluent derived from a 3 min infusion of [^{14}C]-PEN was collected for 30 min and analysed. The concentration of PEN (horizontal axis) has been plotted on a log scale because of the thousand-fold range used (0.15 to 150 μM), and *V* is expressed as nmol of metabolite in the 30 min effluent collected. The means of at least 4 separate assays are shown; vertical lines show s.e. mean. At no level of substrate was there a significant difference in the amount of metabolites produced by lungs during either stage of the cycle.

Results

Metabolism in lung homogenates during oestrous cycle and after treatment with steroids

At first PEN metabolism was studied at each stage of the cycle using a single concentration of 100 μM. The metabolism was greatest at met-oestrus and di-oestrus (1.47 ± 0.24 pmol.min^{-1}.μg protein^{-1}; 1.28 ± 0.14 pmol respectively) and significantly lower at oestrus and pro-oestrus (0.56 ± 0.09 pmol; 0.58 ± 0.1 pmol respectively).

Table 1 Effect of exogenous progesterone and oestradiol on β-phenylethylamine (PEN) metabolism in lung homogenates

	K_m (μM)	V_{max} (pmol.μg protein^{-1}.min^{-1})
Saline	30 ± 5 (7)	1.4 ± 0.2 (7)
17β-Oestradiol	58 ± 8 (4)*	2.2 ± 0.2 (4)*
Progesterone	16 ± 1 (4)	1.2 ± 0.1 (4)

* Significantly different from control.
Rats with regular oestrous cycles were treated with a suspension (in saline) of progesterone (0.5 mg/kg) or 17β-oestradiol (1.0 mg/kg) by i.p. injection once a day for 8 days. On the 9th day the rats were killed, lung homogenates prepared and incubations carried out as usual. The control rats were injected with saline only and therefore could be at any stage of the cycle. The values of K_m and V_{max} shown are the means ± s.e. from the number of animals shown in brackets.

The variation of metabolism over a range of concentrations (10 to 200 μM PEN) is shown in Figure 1a and the values of K_m and V_{max} calculated from these results (Figure 1b) show that K_m did not change during the cycle but that V_{max} at met- and di-oestrus was double that at oestrus and pro-oestrus. Metabolism of PEN was measured over the same range of concentrations (10 to 200 μM) in homogenates of lungs taken from rats treated with progesterone (1.0 mg/kg) or with 17β-oestradiol (0.5 mg/kg) for 8 days. The results (Table 1) show that only oestradiol treatment affected metabolism increasing both K_m and V_{max}.

Metabolism and uptake in perfused lungs

The metabolism of PEN in perfused lungs was studied during met-oestrus and pro-oestrus. These two stages were chosen because they provided the extremes of activity observed in homogenates. There was no significant difference between metabolism at these two stages of the cycle over a wide range of concentrations (Figure 2). Analysis of these results gave an apparent K_m value of 66 μM and V_{max} of 137 nmol.lung^{-1}.30 min^{-1} at either stage of the cycle.

The uptake of radioactivity following the standard 3 min infusion of [^{14}C]-PEN (100 μM) was measured in lungs from rats during met- and pro-oestrus. This concentration (100 μM) was chosen because at this point the metabolism in homogenates is about five fold greater during met-oestrus than that during pro-oestrus. However, in perfused lung, total radioactivity retained by the lung, the proportion of amine metabolite in lung and in the effluent all had the same values at either stage of the cycle (Table 2).

Discussion

Our results have demonstrated that in homogenates of rat lung, the metabolism of PEN, a substrate for MAO-B, was affected by the oestrous cycle and exogenous ovarian steroids. During the oestrous cycle the changes in PEN metabolism seemed to reflect a changed V_{max} rather than alterations in K_m. Such changes in enzyme activity could result from synthesis of more enzyme of equal affinity and this mechanism would be compatible with the duration of increased activity over two stages of the cycle, although there is no direct evidence on this point.

Treatment with exogenous steroids, using the regimen of Holzbauer & Youdim (1973) was effective only with oestradiol. This steroid increased K_m, V_{max} and activity over the whole of the concentration range studied. We used their regimen of steroid treatment so that we could more easily compare results and this comparison reveals some differences. In their experiments, 'non-target' tissues, i.e. tissues not usually associated with ovarian steroid action or production, like liver, heart and kidney, did not show changes in MAO activity measured at a single concentration of kynuramine, a substrate for both MAO-A and B. Further, where changes in activity did occur, in ovary, uterus and adrenal gland, there were decreases with oestradiol and increases with progesterone. In our experiments, MAO activity in lung, another non-target tissue, was changed and that change was an increase with oestradiol and no effect with progesterone. We cannot, at present, see the physiological implications of these differences, especially as far as PEN is concerned, but the differences do underline the point made later by these authors (Youdim & Holzbauer, 1976), that it is difficult, if not misleading, to extrapolate from results obtained with one substrate to another in terms of changes in MAO activity.

A similar difficulty in extrapolation, this time from homogenates to whole tissue, is seen in the results of PEN metabolism in perfused lung. Despite large differences in the homogenates, PEN metabolism was the same in perfused lungs from rats in met- or pro-oestrus. This lack of change we interpret as being

Table 2 Uptake of β-phenylethylamine (PEN) by perfused lung during met- and pro-oestrus

	Lung		*Effluent*
Stage of cycle	*Radioactivity retained* (% *of total infused*)	*Metabolite retained* (% *of total retained*)	*Metabolite present* (% *of total effluent*)
Met-oestrus (n = 4)	18.5 ± 3.2	39.0 ± 4.0	18.7 ± 1.9
Pro-oestrus (n = 4)	24.5 ± 2.4	38.3 ± 4.9	20.5 ± 2.0

Lungs received a 3 min infusion of [^{14}C]-PEN (100 μM) and, immediately after, were removed for analysis. Lung effluent was collected during the 3 min infusion and also analysed. The values shown are the means (±s.e. means) of results from 4 rats. There is no significant difference between the values shown for the different stages of the oestrous cycle.

due to the lack of change in the transport of PEN into the cell, thus negating the increase in intracellular metabolic capacity. Our interpretation was supported by the unchanged uptake of PEN (measured directly) at these stages of the cycle.

Uptake is here used to denote the transfer of PEN from the perfusate to the lung without any suggestion that this process is energy requiring, Na^+-dependent or fulfils any of the other criteria of amine uptake processes already described, for instance, for noradrenaline or 5-hydroxytryptamine. Indeed, PEN uptake in lung has at least two differences, its resistance to inhibition by desmethylimipramine and its high K_m (Bakhle & Youdim, 1979), from the uptake processes for the other two amines in lung (Alabaster, 1977; Gillis & Roth, 1976). It may be that with PEN we are studying a process closer to that described for imipramine (Junod, 1972a) and propranolol (Dollery & Junod, 1976).

We undertook this series of experiments to compare the effects of ovarian steroids on PEN metabolism with those already described for 5-HT (Bakhle & Ben-Harari, 1979) and this comparison shows that the metabolism of both amines was affected. Thus we must conclude that, for at least two amine substrates covering the two types of MAO so far defined, MAO activity in lung can be affected by changes in ovarian steroid levels even though lung is not a target tissue. If, as we believe (Bakhle & Vane, 1974; Bakhle, 1976), amine metabolism in lung is an important physiological control mechanism, then this effect of steroids is perhaps not so surprising.

There are nevertheless important differences between the fate of the two amines studied. During the oestrous cycle, 5-HT metabolism in homogenates is changed only for one stage, met-oestrus, whereas the increased PEN metabolism persists through met-oestrus into di-oestrus and we have already suggested a possible explanation of this. With exogenous steroids, although 5-HT metabolism responded to both oestradiol and progesterone, the metabolism of both amines was increased rather than decreased (Holzbauer & Youdim, 1973) by oestradiol.

In perfused lungs, the dominant influence of uptake on metabolism was apparent for both amines. For PEN, a substrate whose metabolism was not obviously limited by uptake in male rats (Bakhle & Youdim, 1979), uptake became limiting when the intracellular MAO activity was increased during met-oestrus. For 5-HT, a substrate whose metabolism is strongly limited by uptake (Junod, 1972b; Bakhle & Youdim, 1979), changes in uptake probably reversed the changes in metabolism expected from homogenates (Bakhle & Ben-Harari, 1979).

Our experiments, with both amines, re-emphasize the pitfalls present in attempting to predict metabolic capability in whole tissue, and probably also *in vivo*, directly from a knowledge of enzymic properties *in vitro* (Bakhle & Vane, 1974; Youdim & Woods, 1975). They also demonstrate that the activities of enzymes in lung may be unexpectedly altered by ovarian steroids and provide evidence for the role of amine metabolism in lung as a control mechanism itself susceptible to control by endogenous substances.

This work was supported by the M.R.C.

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(Received November 23, 1978.
Revised January 29, 1979.)