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## Role of adrenochrome in aryl hydrocarbon hydroxylase induction by epinephrine in rat liver cell culture

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THE MICROSOMAL "mixed function oxidases" of rat liver are centred on cytochrome P-450. This family of enzymes metabolise drugs such as phenobarbitone, polycyclic hydrocarbon carcinogens, e.g. 3,4-benzo(α) pyrene (BP) and 1,2-benzo(α)anthracene (BA) in addition to many normal body constituents. Aryl hydrocarbon hydroxylase (AHH) is a member of this group of enzymes and prior treatment of the rat with BP induces hepatic AHH to higher levels of activity.

Similarly AHH activity can be found and induced to higher activities by BA in cell-cultures derived from hamster, rat, mouse and chick embryonic tissues, in addition to a few established cell-lines.[2] In cultures of foetal rat hepatocytes Geilen and Nebert[3] observed that very high, unphysiological, concentrations of catecholamines such as epinephrine increased AHH activity by a process similar to BA induction. On repeating these experiments in the course of other observations, we noticed that cell-growth medium containing high epinephrine concentrations became dark brown during the induction period.

Abbreviations: AHH—Aryl hydrocarbon hydroxylase; BP—3,4-Benzo(α)pyrene; BA—1,2-Benzo(α)anthracene; RL—Rat-liver epithelial cell line; DMSO—Dimethylsulphoxide.

Since it is well-established that epinephrine is very readily auto-oxidisable, *in vitro*, to a complex mixture of transformation products,[4,5] experiments to determine whether epinephrine or one of its transformation products was the inducing species, were undertaken in a rat-liver epithelial cell line (RL).

## MATERIALS AND METHODS

RL cells, previously described by Bomford and Weinstein,[6] and given by Dr. I. A. MacPherson, Imperial Cancer Research Fund, London, were cultured in Eagles Basal Medium supplemented with 5% calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells, when just confluent, were exposed to the compounds under investigation by directly dissolving hydrophilic substances in the growth medium. The water insoluble compounds were first dissolved in small amounts of dimethylsulphoxide (DMSO) or acetone, before their addition to the growth medium.

Neither DMSO or Acetone at a final concentration of 0·30 per cent, had any effect on cell-growth or the basal level of AHH over the 18 hr induction period. The cells were harvested by scraping the monolayer from 25 $cm^2$ falcon bottles into 0·15 M KCl in which they were homogenized with 30 strokes of a Dounce type homogenizer.

AHH activity was assayed over 30 min as previously described,[7] using 0·4 to 1·0 mg cell protein in 1 ml incubation mixture; cell protein being determined by the method of Lowry *et al.*[8]

The conditions for the induction of AHH in RL cells by BA were found to be similar to those reported by Gelboin *et al*,[2] for other cell-culture systems. DL-Epinephrine, L-epinephrine bitartrate and adrenochrome were purchased from Sigma Chemical Co., London (U.K.), whilst 3,5-dihydroxy- and 3,5,6-trihydroxy-1-methylindole were synthesised from DL-epinephrine as described by Harley-Mason.[5]

## RESULTS

Table 1 shows the dose response curve to L-epinephrine, concentrations above 1 mM were cytotoxic. After 18 hr, the growth medium containing L-epinephrine produced dark brown solutions suggesting some breakdown had occurred. L-Ascorbic acid has been shown to prevent the oxidation of epinephrine by molecular oxygen[9] and when 0·25 mg/ml L-ascorbic acid was incorporated into the growth medium, the darkening of epinephrine solutions together with the induction of AHH by epinephrine (but not induction by BA) could be completely prevented (Table 2). Other anti-oxidants such as butylated hydroxytoluene or butylated hydroxyanisole when incorporated into the growth medium at a concentration of 100 μg/ml did not prevent the darkening of epinephrine solutions.

TABLE 1. DOSE RESPONSE RELATIONSHIP FOR THE INCREASE IN AHH ACTIVITY IN RL CELLS BY L-EPINEPHRINE AND ADRENOCHROME

| Experiment 1 | | Experiment 2 | |
|---|---|---|---|
| Epinephrine concn (μM) | AHH activity (pmole/30 min/mg protein) | Adrenochrome concn (μM) | AHH activity (pmole/30 min/mg protein) |
| 0 | 28 | 0 | 33 |
| 250 | 39 | 0·05 | 36 |
| 500 | 55 | 0·10 | 55 |
| 750 | 58 | 1·00 | 56 |
| 1000 | 93 | 10·00 | 78 |
| | | 25·00 | 99 |
| | | 50·00 | 127 |
| | | 75·00 | 156 |

The results above are the averages of values obtained by assaying AHH activity (expressed as pmoles 3-hydroxy-BP formed) in duplicate flasks of RL cells which had been treated with the respective concentrations of L-epinephrine or adrenochrome for 18 hr. The variation in each experiment, between duplicate flasks of RL cells derived from the same subculture, did not exceed 10 per cent. Concentrations above 1 mM L-epinephrine and 75 μM adrenochrome produced cytotoxic effects.

Exposure to 17·5 μM BA in different subcultures of RL cells induces AHH to a sp. act. of 192 ± 60 (n = 9). In experiment 1 and experiment 2, exposure to 17·5 μM BA for 18 hr produced specific activities of 172 and 213 respectively. Repetition of these experiments produced similar results.

Table 2 indicates that one of the oxidation products of epinephrine rather than epinephrine itself is responsible for AHH induction. The incorporation of the first oxidation product of epinephrine, adrenochrome, into the growth medium induced AHH at less than one-tenth of the concentration required for induction by epinephrine (Table 1). Adrenochrome itself is unstable, breaking down to 5,6-dihydroxy- and 3,5,6-trihydroxy-1-methylindole.[5] However, when these latter compounds were added to the growth medium they were without effect singly or together in doses just below those producing cytotoxicity which were found to be 50 $\mu$M and 200 $\mu$M respectively.

## DISCUSSION

This communication describes experiments which demonstrate that the increase in AHH activity previously reported[3] to be due to epinephrine, is in fact due to its oxidation product, adrenochrome. An analogous observation that adrenochrome formation rather than epinephrine was responsible for increasing oxygen consumption by a reconstituted lactic and malic dehydrogenase systems from heart muscle, has been reported.[4] These authors also demonstrated that the increased oxygen consumption could be prevented by inhibiting epinephrine autoxidation by ascorbate.

TABLE 2. EFFECT OF L-ASCORBIC ACID ON EPINEPHRINE AND BA INDUCED AHH ACTIVITIES.

| Treatment | AHH activity in RL cells expressed as (pmole/30 min/mg protein) | |
|---|---|---|
| | Medium with 0·25 mg/ml L-ascorbic acid | Medium without L-ascorbic acid |
| Control | 16·0 | 22·0 |
| 1 mM Epinephrine | 23·0 | 142·0 |
| – BA | 108·0 | 179·0 |

The results above are the average of values obtained by assaying AHH activity (expressed as pmoles 3-hydroxy-BP) from duplicate flasks of RL cells which had been treated with 1mM L-epinephrine or 17·5 $\mu$M BA for 18 hr. The variation between duplicate flasks was not greater than 10 per cent. Repetition of this experiment produced similar results.

Aminochromes can be readily obtained by the oxidation of catecholamines by molecular oxygen in neutral aqueous solutions; in addition these oxidations are catalysed by traces of transition metal ions or by copper containing plasma proteins such as ceruloplasmin[9,10] which no doubt are present in a cell-culture medium supplemented with calf-serum. Under such conditions epinephrine, norepinephrine, *N*-isopropylnorepinephrine, dopamine and dopa readily form the corresponding aminochrome.[10] From these considerations it is most likely that the induction of AHH by other catecholamines reported by Geilen and Nebert[3] was in fact due to the formation of the corresponding aminochrome, by oxidation, as this report shows to be the case for epinephrine.

Adrenochrome formation from epinephrine *in vivo* has been reported to be a minor pathway which is unlikely to contribute to the overall metabolism of epinephrine[11], and hence the physiological significance of the increase in AHH activity by adrenochrome in RL cells is difficult to ascertain. When rats received 100 mg/kg adrenochrome (i.p.) for 3 days, hepatic AHH was found to be lowered rather than raised. However, due to the hydrophilic nature of adrenochrome and hence its rapid excretion by the rat[12,13] it may be that adrenochrome concentrations high enough to induce AHH are not produced inside cells by injection of adrenochrome.

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## Inhibition of median raphe neurone metabolism by cerebrospinal fluid (CSF) containing 5-hydroxytryptamine and melatonin

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BRAIN 5-hydroxytryptamine is synthesized by many neurones of the raphe nucleus in the midbrain. For several hours after monoamine oxidase inhibition the rate of 5-hydroxytryptamine synthesis declines as the level of brain 5-hydroxytryptamine rises.[1,2] In the present study the nucleolar component of the metabolic changes in median raphe neurones was observed (a) during a prolonged (9 day) elevation of 5-hydroxytryptamine levels in cerebrospinal fluid (CSF), (b) during a similar elevation in CSF levels of other monoamines, (c) after depletion of brain catecholamines by 6-hydroxydopamine (6-OHDA),[3] and (d) after bilateral cervical sympathectomy.

The cerebral ventricles of one group of male albino rats (aged 3 months and weighing approximately 300 g) were cannulated and continuously infused during 7–9 days, at a rate (1 $\mu$l/min) roughly half that of CSF formation.[4] The infusate was artificial CSF alone, or with "low-dose" 5-hydroxytryptamine, melatonin, noradrenaline, histamine (all at 50 $\mu$g/ml), tryptophan (1 mg/ml), or "high-dose" 5-hydroxytryptamine (500 $\mu$g/ml). The rats were killed after 7–9 days of infusion.

In a second group of rats, 6-OHDA (250 $\mu$g in 0·05 ml artificial CSF) was given by a single injection into a lateral cerebral ventricle; controls received artificial CSF alone. The animals were killed at intervals during the following 16 days.

In a third group, bilateral cervical sympathectomy was performed and the rats killed 7–9 days thereafter.

From all rats, neuronal nuclei were isolated from the median raphe region of the brain.[5] The dry mass of the nucleoli in these nuclei was measured using the interference microscope,[6] and the mean values are shown in Fig. 1 and Table 1.

Although the drug-induced behaviour of the rats within each group was similar, differences between the groups were seen. Two days after commencing infusion with melatonin or with "low-dose" 5-hydroxytryptamine the rats became very drowsy and showed a reduction both in spontaneous motor activity and in reactivity to simple tactile and auditory stimuli. During infusion with "high-dose" serotonin the rats were drowsy in the first day only, subsequently becoming hyperactive and hyperreactive with a coarse tremor and titubation. Rats with noradrenaline or tryptophan in the infusate showed increased spontaneous movements and hyperreactivity starting on day 2 but not preceded by drowsiness. For 1 hr after 6-OHDA injection the rats were hyperactive and hyperreactive; the subsequent 12-hr phase of extreme immobility and unresponsiveness disappeared gradually, behavioural normality returning during day 2. Those rats with cervical sympathectomy and those receiving histamine solution or artificial CSF by itself appeared normal. Assiduous attempts have been made by many workers to attribute such drug-induced changes in behaviour to concurrent changes in brain biochemistry.[7,8] It is hoped that such speculations might be assisted by this study in which accompanying changes in neuronal metabolism have been noted by means of a different technique.