INDUCTION OF ARYL HYDROCARBON HYDROXYLASE BY A LIGHT-DRIVEN SUPEROXIDE GENERATING SYSTEM IN LIVER CELL CULTURE

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Received April 10, 1974

Summary

Aryl Hydrocarbon Hydroxylase activity can be increased in rat liver cell cultures by the generation of the superoxide ion (O_2^-) in the growth medium. This can be achieved by mild illumination of the cells in the presence of riboflavin and methionine. The increased activity that results can be prevented by Cycloheximide and appears to be a typical induction. It is suggested that superoxide generation within cells may be a common factor linking different microsomal enzyme inducers.

The synthesis of the microsomal Cytochrome P450 linked family of mixed-function oxidases, which includes Aryl Hydrocarbon (3,4 Benzo(α)pyrene) Hydroxylase (AHH), can be induced in vivo and in cell culture by various substrates. These inducing substrates have been divided into 2 main classes, one of which comprises the polycyclic hydrocarbons such as 1,2 Benzanthracene (BA) whilst the second group is typified by drugs such as Phenobarbital (1-3).

Our studies on the induction of AHH in rat liver (RL) cell culture lead us to propose that another type of inducer exists which may show an underlying unity between the many structurally diverse compounds that act as inducers.

This proposal is based on our findings that AHH activity can be increased in RL cells in tissue culture by systems that generate the superoxide (O_2^-) ion. The superoxide generating processes studied are first, epinephrine autoxidation (4,5) and now the production of O_2^- during the illumination of

Abbreviations: AHH - Aryl Hydrocarbon Hydroxylase
BA - 1,2 Benzanthracene
RL - Rat Liver epithelial cell line
O_2^- - Superoxide ion
DMEM - Dulbecco's modification of Eagle's Medium

riboflavin solutions (5). We report here that the increase in AHH activity by illumination of RL cells in a growth medium containing riboflavin requires protein synthesis and seems to be a typical induction (2).

Methods

RL cells (4) were grown in Dulbecco's modification of Eagle's Medium (DMEM) supplemented with 5% Calf serum. The cells, when just confluent, were illuminated with or without Riboflavin (British Drug Houses, Poole, Dorset, U.K.) as described in Table 1. AHH activity and cell protein were assayed as previously described (4).

The incorporation of L-[1 - C^{14}] Leucine (Radiochemical Centre, Amersham, Bucks, U.K.) into cellular protein was measured by adding 50 µl of an L-[1-C^{14}] Leucine solution in 0.15M NaCl (specific activity = 10µC/0.16µmole/ml) to the growth medium 1 hour prior to harvesting the cells.

A 0.2 ml. aliquot of the cell homogenate, made as previously described (4), was added to 0.1 ml. of a 'cold carrier' solution composed of 5 mg/ml. L-Leucine plus 1% Bovine Serum Albumin. This was then diluted to 2 ml. with 0.15M Saline and the protein precipitated with 2 ml. 10% Trichloro acetic acid. 1 hour later the precipitate was collected by centrifugation, dissolved in 1M NaOH and counted in a liquid scintillation counter. The d.p.m. values were corrected for quenching by using internal standards and automatic external standardization.

Results

The increase in AHH activity by illuminating RL cells in a medium containing 15 µM Riboflavin for 24 hours is shown in Table 1. Treatment of RL cells with Riboflavin without illumination or light without Riboflavin gave no increase in AHH activity; whereas mild illumination of DMEM containing 15 µM Riboflavin was as effective as a maximal inducing dose of BA. This same large increase in activity was found whether illumination with Riboflavin was for 1 hour followed by 23 hours in a dark incubator or continuously for 24 hours.

Table 1 Effect of Illuminating RL cells in a Medium With and Without 15 μM Riboflavin for 24 hours

	AHH activity (pMoles 3-Hydroxy Benzo(α)pyrene formed/ 30 mins/mg. Protein)	
	Without Riboflavin	+ 15μM Riboflavin
Not illuminated	30	35
Illuminated	23	198

RL cells in DMEM with and without Riboflavin were illuminated by a 60 watt household tungsten lamp, 17 cms. from the culture flasks, for 24 hours. The cells were maintained at 37°C in a water bath throughout the illumination. A 24 hour exposure to 17.5 μM BA in the dark produced a specific activity of 186.

We have found that O_2^- generation by the Riboflavin system ceases when illumination stops, and maximal induction by one hour's illumination would suggest that either O_2^- has activated the inducing mechanism or else an inducer has been formed in the medium. Replacement of the growth medium after an hour's illumination with non-illuminated medium caused induction of AHH to be found only at 6 hours, while by 24 hours this increase had died away suggesting that the continual presence of the inducer is required for maximal induction to occur at 24 hours.

The inclusion of 3.5 μM Cycloheximide into the growth medium decreased the induction by O_2^- found at 24 hours by 75% (Table 2).

Discussion

The induction of the same enzyme system in cultured cells by varied compounds such as BA, Phenobarbital in addition to epinephrine and other amines (6), presents a major problem when mechanisms of induction are considered. Our findings that O_2^- generation in the growth medium during epinephrine autoxidation or else by illumination in the presence of Riboflavin suggests

Table 2 Effect of Cycloheximide on Induction of AHH by O_2^- generation or BA

Treatment	3.5 μM Cycloheximide	AHH activity pMoles/30 mins/mg. Protein	^{14}C Leucine incorporation dpm/hour/μg. Protein
Without Riboflavin, + Light	-	22	5.50
Without Riboflavin, + Light	+	27	0.65
15μM Riboflavin, + Light	-	186	6.50
15μM Riboflavin, + Light	+	46	0.72
17.5μM BA not illuminated	-	141	6.00
17.5μM BA not illuminated	+	67	0.69

Cycloheximide was added simultaneously with the inducer; then RL cells with and without Riboflavin ± Cycloheximide, were illuminated as described in Table 1, for 1 hour followed by 23 hours in a dark incubator. RL cells with BA ± Cycloheximide were not illuminated during the 24 hour induction period. AHH activity is expressed as pmoles 3-hydroxy Benzo(α)pyrene formed. 14 C Leucine incorporation was measured as described in the section on Methods.

that O_2^- or possibly other active species, such as the hydroxyl radical (OH•), formed may be the common factor in induction.

We believe that the induction of AHH by O_2^- generation is of special significance because both microsomes and purified microsomal enzymes have been shown to generate O_2^- during electron transfer (7,8). This evidence suggests that O_2^-, or similar active radicals, can be generated inside cells during electron flow and substrate oxidation in the Endoplasmic Reticulum.

Such a working hypothesis could provide a common mechanism for all the induction systems. Thus Phenobarbital and BA could generate O_2^- inside the cell during oxidation, while the non-substrates such as epinephrine or Riboflavin plus light, in the in vitro model system, generate O_2^- outside the cell; in this way, the same basic sequence of events can be initiated inside the cell by either means.

Acknowledgements

This work has been supported by the Cancer Research Campaign, Medical Research Council and the British Nutrition Foundation.

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