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METABOLIC HYPOXIA: ACCUMULATION OF TYROSINE METABOLITES IN HEPATOCYTES AT LOW pO_2

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SUMMARY

The concentrations of p-hydroxyphenylpyruvate and homogentisate were measured in suspensions of isolated hepatocytes incubated in several fixed O_2 concentrations (1.4-210 μM). Metabolite concentrations, estimated by both optical spectroscopic assays and by gas-liquid chromatography, were found to be dependent upon O_2 concentration below 70 μM.

INTRODUCTION

Tyrosine is degraded in mammals primarily by a single multienzyme pathway involving tyrosine amino transferase (EC 2.6.1.5), p-hydroxyphenylpyruvate oxidase (EC 1.13.11.27) and homogentisate oxidase (EC 1.13.1.5). The products of the first two reactions, p-hydroxyphenylpyruvate and homogentisate, may accumulate in principle during hypoxia, due to dependence of p-hydroxyphenylpyruvate oxidase and homogentisate oxidase activities on $[O_2]$. These enzymes have relatively high $K_{m_{O_2}}$ values, 10^{-4} M (1) and 10^{-3} M (2) respectively. Product accumulations may interfere with metabolism (3-5). Accordingly, we examined the O_2 dependence of their cellular concentrations using isolated hepatocytes (6) to insure exposure of all cells to the same $[O_2]$ while retaining metabolic integrity.

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Abbreviations: HG, homogentisate; HGO, homogentisate oxidase; pHPP, p-hydroxyphenylpyruvate; pHPPO, p-hydroxyphenylpyruvate oxidase; TAT, tyrosine amino transferase; 2-OG, 2-oxoglutarate; TYR, tyrosine; GLU, glutamate.

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METHODS AND MATERIALS

Isolated hepatocytes were prepared from male Sprague-Dawley rats (200-300 gm, fed ad libitum) by the method of Berry and Friend (6) with minor modifications (7). Cellular integrity (8) was established (7) by trypan blue exclusion, electron microscopy, lactic dehydrogenase content, adenylate concentrations, gluconeogenesis rate, O_2 consumption rate, and lack of respiratory stimulation by ADP. Preparations were begun routinely between 9 and 10 AM with ether anesthesia. Incubations were performed at 37°C in Ca^{++}, glucose-free Hank's bicarbonate buffer in an apparatus which maintains constant $[O_2]$ during incubations (9). Incubations were equilibrated to 5% CO_2 regardless of the $[O_2]$ and stopped by injection of 0.15 ml 2N H_2SO_4/ml. Protein was removed by centrifugation and phenolic acids were extracted 3 times with 2 volumes ethyl acetate. p-Hydroxyphenylpyruvate was assayed by the method of Diamondstone (10), and homogentisate was assayed by the colorimetric method of Fellman et al. (1). Results from these methods were confirmed by gas-liquid chromatography (11) as follows. The solvent containing extracted phenolics was dried with sodium sulphate and evaporated under N_2. Silylation was performed by addition of 100 µl bis(trimethylsilyl) trifluoracetamide directly to the residue and heating for 30 min at 60°C. Suitable aliquots were chromatographed on an F & M model 810 DR-12 instrument equipped with a 12 ft SE 30 column and a hydrogen flame detector. Column temperature was programmed to increase 4°/min from 140° to 210°C. Quantitation was done relative to known standards and corrected for losses during extraction by addition of p-hydroxyphenylacetic acid as an internal standard.

Tyrosine amino transferase activity was measured by the method of Lin and Knox (12). p-Hydroxyphenylpyruvate oxidase activity was measured by two methods of Fellman et al. (1) with 1.6 mM ascorbic acid as activator. Homogentisate oxidase activity was measured by following the production of maleylacetoacetate at 330 nm upon addition of homogentisic acid (13 mM), using $\varepsilon = 1.4 \times 10^4\ M^{-1}cm^{-1}$ (13). Tyrosine was measured by the fluorometric method of Waalkes and Udenfriend (14). Protein was estimated by the method of Lowry et al. (15) with Labtrol (Dade) as standard.

Collagenase (type I), hyaluronidase (type I), homogentisic acid (grade 2), p-hydroxyphenylpyruvic acid, p-hydroxyphenylacetic acid, tyrosine HCl, and keto-enol tautomerase were obtained from Sigma. p-Hydroxyphenyl-[carboxy-^{14}C] pyruvate was kindly provided by Dr. J. H. Fellman. Distilled and deionized water was used throughout.

RESULTS

Total suspension p-hydroxyphenylpyruvate and homogentisate concentrations as a function of incubation $[O_2]$ are given in Figure 1. Half-maximal change in p-hydroxyphenylpyruvate concentration occurred at approximately 10 µM O_2, whereas the corresponding value for homogentisate concentration was about 20 µM. Detectable increases in both were apparent at 70 µM O_2 and below.

Estimation of intracellular concentrations were made by measuring the partitioning of metabolites between suspension medium and cell pellet following centrifugation (Table I). These measurements could not be made readily at fixed $[O_2]$ because of the time required for centrifugation; however,

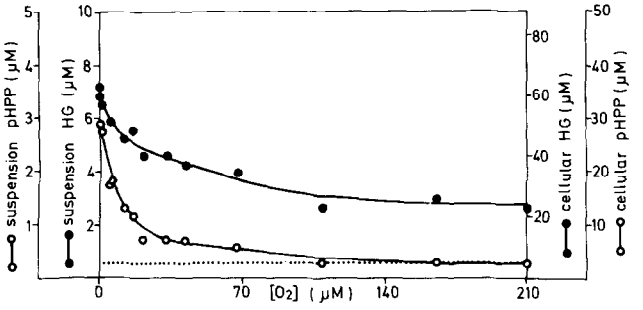

Figure 1. p-Hydroxyphenylpyruvate (pHPP) and homogentisate (HG) concentrations in suspensions of isolated hepatocytes as functions of $[O_2]$. Hepatocytes (3 x 10^6/ml) were incubated in 6 ml modified Hank's medium containing 0.22 mM tyrosine at 37°C for 10 min at indicated $[O_2]$. Estimates of total suspension concentrations (left margin) were used with partitioning data (Table I), and the equation below to estimate the cellular concentrations (right margin): $C_{cells} \times V_{cells} = C_{pellet} \times V_{pellet} - C_{supernatant} \times V_{packing}$ where C is concentration and V is volume. The value used for hepatocyte volume was 7.25 μl/10^6 cells (8). The dotted line represents the minimum detectable pHPP.

Table I. Partitioning of metabolites between pellet and suspending medium. Cells were incubated 30 min under 20% O_2, 5% CO_2 at 37°C, centrifuged 2 min at 600 rpm (50 x g), supernatant decanted and both fractions deproteinated with H_2SO_4 and centrifugation.

	Average Pellet	Average Supernatent	% of Total as Pellet
HG	15.5 ± 4.2 μM	1.79 ± 0.84 μM	88.2 ± 2.0
pHPP	0.101 ± 0.060 mM	0.013 ± 0.007 mM	91.6 ± 1.9
Tyrosine	0.410 ± 0.08 mM	0.220 ± 0.01 mM	62.2 ± 1.6

the partitioning of metabolites presented in Table I is consistent with metabolite levels found in the suspending medium at low $[O_2]$ following rapid removal of cells by filtration through glass wool (16).

Tyrosine amino transferase, p-hydroxyphenylpyruvate oxidase, and homogentisate oxidase activities of homogenized hepatocytes compare well with those of whole liver homogenates (Table II). Net tyrosine loss in incubations

Table II. Specific activities of enzymes involved in tyrosine degradation in isolated cells and intact liver. Values (± 1 S.D.) are expressed in μmoles product formed/mg protein/hr. All assays were run at 37°C under conditions assumed to give maximal activity. The number of animals is given in parentheses.

Enzyme	Cells	Liver
TAT	0.501 ± 0.130 (6)	0.406 ± 0.043 (4)
pHPPO	0.232 ± 0.021 (6)	0.256 ± 0.011 (3)
HGO	1.960 ± 0.30 (6)	2.090 ± 0.26 (3)

above 100 μM O_2 with an initial 0.22 mM tyrosine concentration was 0.030 μmole/mg protein/hr.

DISCUSSION

Our results show that at low [O_2] values p-hydroxyphenylpyruvate and homogentisate accumulate in hepatocytes. Whether such accumulations occur *in vivo* and whether they are enough, quantitatively, to perturb hepatic or extra-hepatic metabolism is unclear. The concentrations never exceeded 0.1 mM, far below the p-hydroxyphenylpyruvate concentration that inhibited sterol synthesis (3). Moreover, since *in vivo* hepatic [O_2] ranges from about 1.4-90 μM (17), it is possible that such accumulations are physiologically normal. In any case, these data indicate that in intact cells both p-hydroxyphenylpyruvate oxidase and homogentisate oxidase have high apparent $K_{m_{O_2}}$ values, *i.e.* >10 μM, in agreement with reported values for these enzymes (1,2,20). This is of particular interest with regard to the observation of Sies (18) that in hemoglobin-free perfused liver, the O_2 dependencies of cytochrome oxidase and urate oxidase are functionally coupled even though the $K_{m_{O_2}}$ values for the isolated enzymes differ by 2 orders of magnitude. The present data, when compared to apparent $K_{m_{O_2}}$ values for

mitochondrial respiration and cytochrome P-450 function in isolated hepatocytes (8,19), indicate that in isolated hepatocytes the oxidases are not functionally coupled. Thus, one may distinguish between two types of hypoxias based upon their enzymic origin. Metabolic hypoxias occur when pathomolecular changes take place due to $[O_2]$ values insufficient to maintain normal function (i.e., of oxygenases, mixed function oxidases, etc. (21)). Bioenergetic hypoxia occurs when pathomolecular changes are due to $[O_2]$ values insufficient to maintain normal bioenergetic functions of oxygen (i.e., of cytochrome oxidase). Indirect determination of level of metabolic oxidase function, for example, urinalysis for homogentisate or other oxidase substrates, may provide useful tools for clinical assessment of hypoxia.

The specific activities of the three enzymes in hepatocytes (Table II) are similar to reported values (1,2,12,22,23) with the exception that transaminase activity is apparently higher than p-hydroxyphenylpyruvate oxidase activity. This difference may be due to methodological difficulties in activity determinations in crude homogenates since the transaminase is subject to product inhibition (22), feedback inhibition (5), and activity induction (12), and the oxidase is subject to substrate inhibition from both substrates (1,24), product inhibition (25), and requires activation for maximal activity (24). Use of ascorbate rather than GSH and 2,6-dichloroindophenol for activation in the oxidase activity determinations resulted in an underestimate of these activities (24).

The possible relationship to the overall tyrosine degradative pathway of the p-hydroxyphenylpyruvate and homogentisate accumulations during hypoxia is presented schematically in Figure 2. The very low net tyrosine loss relative to the measured maximal activities for the enzymes indicates that the pathway is highly regulated. The phenolic acid accumulations are probably due to the O_2 dependencies of the oxidases rather than to indirect hypoxic effects since oxidation-reduction changes do not occur until below 30 μM O_2 (8,19). If the rate of tyrosine degradation is kinetically controlled by the tyrosine

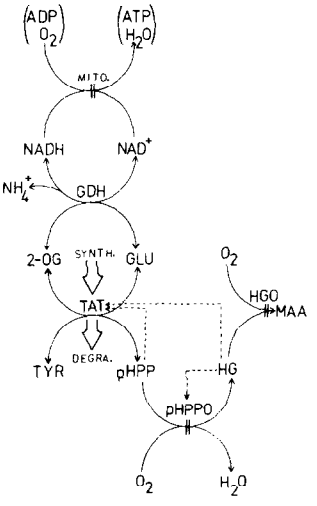

Figure 2. Metabolic regulation of tyrosine degradation. Double vertical lines indicate sights of anoxic blocks. Broken lines indicate possible feedback inhibition. 1. Under conditions where tyrosine amino transferase (TAT) is rate determining, the relative rates of synthesis and degradation of this enzyme are important in metabolic control. 2. Substrate level control of TAT may be exerted by both 2-oxoglutarate (2-OG) and tyrosine (TYR) since both have *in vivo* concentrations below that required for saturation of the TAT reaction. 3. p-Hydroxyphenylpyruvate (pHPP) and glutamate (GLU) concentrations may affect the TAT reaction by product inhibition. 4. If the TAT reaction is at equilibrium, the pHPP concentration will be defined by K_{eq} = [pHPP][GLU]/[TYR][2-OG], and the overall rate will be determined by the p-hydroxyphenylpyruvate oxidase (pHPPO) activity. 5. Oxygen concentration may directly or indirectly affect the rate by: (a) decreased pHPPO activity due to O_2 limitation decreasing the overall rate if the TAT reaction is at equilibrium. Alternatively, pHPP accumulation could give product inhibition on the TAT reaction; (b) decreased homogentisate oxidase (HGO) activity resulting in increased [HG] and feedback inhibition on the TAT reaction (5) or the pHPPO reaction (21); or (c) inhibition of mitochondrial respiration altering the redox state and shifting the glutamic dehydrogenase equilibrium. Altered [2-OG] or [2-OG]/[GLU] could subsequently affect the TAT reaction.

amino transferase reaction, the flux may be altered by product inhibition due to p-hydroxyphenylpyruvate accumulation or by irreversible inhibition due to homogentisate accumulation (5). Below 30 μM, changes in 2-oxoglutarate concentration in response to cellular oxidation-reduction change may also alter the rate. Alternatively, if the tyrosine amino transferase reaction is at equilibrium, as is suggested by the high maximal activity relative to the apparent total flux through the pathway, then the regulation of tyrosine

degradation lies in the activity of the second enzyme in the pathway, p-hydroxyphenylpyruvate oxidase. The ratio, 2-oxoglutarate:glutamate, and the tyrosine concentration would determine the p-hydroxyphenylpyruvate concentration. The p-hydroxyphenylpyruvate concentration, along with $[O_2]$, may then be important in determining the overall metabolic flux.

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