Biochemical Pharmacology, Vol. 33, No. 9, pp. 1555–1557, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
Pergamon Press Ltd.

SHORT COMMUNICATIONS

Synthesis of adenosine nucleotides from hypoxanthine by human malaria parasites (*Plasmodium falciparum*) in continuous erythrocyte culture: inhibition by hadacidin but not alanosine

(*Received* 8 *April* 1983; *accepted* 18 *October* 1983)

Human malaria parasites, like all living cells, require purines for energy metabolism and synthesis of nucleic acids. The source of purines and the pathways by which purine nucleotides are synthesized have only recently been systematically studied in human intraerythrocytic (IE) *Plasmodia* growing in continuous erythrocyte culture [1, 2] or in "isolated" parasite preparation [3]. These studies showed that the IE malaria parasite depends on salvage pathways, primarily involving hypoxanthine, for synthesis of purine nucleotides. Studies with simian and murine malaria parasites have shown that purines are not synthesized *de novo* in *Plasmodia* [4]. In contrast, host human mature erythrocytes require purines primarily for synthesis of ATP [5]. The mature erythrocyte cannot make purines *de novo* nor synthesize ATP from hypoxanthine via IMP due to the absence of an adenylosuccinate (AMPS) pathway [6].

Earlier studies in our laboratory focused on purine metabolism in the IE parasite of malignant tertian malaria, *Plasmodium falciparum*, grown in continuous human erythrocyte culture. These studies showed a unique purine pathway distinction in *P. falciparum* infected erythrocytes involving the synthesis of adenosine nucleotides from hypoxanthine via adenylosuccinate [1].

In the present study we examined the effect on the IE malaria parasite of two known inhibitors of adenylosuccinate synthetase [7]: hadacidin (*N*-formyl hydroxyaminoacetic acid) and alanosine [L-2-amino-3-(*N*-hydroxy, *N*-nitrosamino) propionic acid].

Methods

Malaria culture. The FCR-3 strain of *P. falciparum* was maintained by continuous erythrocyte culture using described methods [2, 8, 9].

Experimental assays were done as follows. Cultures were incubated (37°, in 90% N_2, 5% O_2, 5% CO_2) for 48 hr. Growth cultures were then pooled in a large sterile flask to provide a uniform parasitemia and distribution of parasitized RBC (PRBC). Aliquots were then removed to individual flasks. [^{3}H]Hypoxanthine (3.6 Ci/mmole) and inhibitor were added to these flasks, and incubation was continued for 3–4 hr. Control cultures consisted of unparasitized RBC (CRBC) and PRBC without inhibitor.

Nucleotide extracts. Cultures were centrifuged (270 *g*, 5 min, 4°) to separate the RBC and extracellular medium (ECM). The RBC and ECM were extracted using perchloric acid (PCA) as previously described [2]. Determination of radioactivity incorporated into nucleic acids was done by alkali solubilization of the acid-insoluble material formed during PCA extraction of PRBC.

Measurement of purine compounds. Purine nucleotides were assayed using a high performance liquid chromatography (HPLC) system that simultaneously measured the concentration and radioactivity of separated components. The system has been described in detail [10]. Concentrations were determined by an external standard method based on absorbance (254 nm) using solutions of purine standards of known purity. For radioactivity measurements, peak areas were machine-integrated and recorded as total counts per radioactivity peak.

Results

Purine nucleotides. Table 1 compares purine profiles for control and parasitized erythrocyte cultures following 3 hr of incubation with [^{3}H]hypoxanthine. PRBC showed a very active hypoxanthine phosphoribosyl transferase for synthesis of IMP from hypoxanthine. PRBC and CRBC synthesized guanylates by the following pathway: HYP → IMP → XMP → GMP → GDP → GTP. There was, however, no significant quantitative synthesis of guanylates (<20 counts/peak in GTP) from [^{3}H]hypoxanthine in CRBC under the study conditions. Adenylates were synthesized by PRBC by the following pathway: HYP → IMP → AMPS → AMP → ADP → ATP (Table 1). These data clearly show the synthesis of adenosine nucleotides from hypoxanthine via IMP. This pathway requires AMPS as an intermediate between IMP and AMP and the two enzymes adenylosuccinate synthetase and adenylosuccinate lyase. CRBC showed no incorporation of label from hypoxanthine into the adenylate pool.

AMPS levels were not measured in these experiments. Accumulation of adenylosuccinate would not, however, be expected, and low levels of this intermediate would be consistent with a strong forward reaction equilibrium favoring production of AMP from AMPS.

Synthesis of purine nucleotides de novo. When [^{14}C]glycine or [2-^{14}C]formate was added to malaria cultures and incubation continued for 3 hr, no label was observed in IMP, ATP and GTP. Similar experiments were done in which incubation with these labeled precursors was continued for 24 hr with no evidence for incorporation into purine nucleotides by the *de novo* pathway.

Effects of hadacidin and alanosine. Table 2 shows that hadacidin (5×10^{-5}M) but not alanosine (5×10^{-5}M) blocked (84%, $P < 0.01$; Student's *t*-test) synthesis of ATP from hypoxanthine via IMP. Radioactivity in total adenylates was likewise reduced. There was only a slight decrease in [ATP] in hadacidin-treated PRBC compared to untreated PRBC, suggesting that hadacidin interfered with newly synthesized ATP (Table 2). Incorporation of label from hypoxanthine into IMP was not affected by hadacidin. There appeared to be an increase in [IMP] and total radioactivity in hadacidin-treated PRBC compared to untreated PRBC which would be consistent with a block in IMP utilization for production of adenylates. Alanosine had no demonstrable effect on synthesis of ATP from hypoxanthine via IMP by PRBC (Table 2). The levels for IMP and total adenylates were similar with alanosine to that observed for untreated PRBC.

Figure 1 shows that alanosine had no effect on incorporation of labeled hypoxanthine into acid-precipitable material (nucleic acids) of PRBC cultures whereas hadacidin-treated PRBC showed a 72% inhibition ($P < 0.01$, $N = 3$). These data are consistent with the observations in

Table 1. Incorporation of [^{3}H]hypoxanthine into nucleotides of malaria parasitized (*P. falciparum*) and control erythrocytes *in vitro**

	ATP	ADP	AMP	GTP	GDP	GMP	IMP	XMP
Control erythrocytes (CRBC)								
Nmoles/ml RBC	1,651 ± 83	132 ± 8	4 ± 1	32 ± 2	9 ± 1		159 ± 12	
Radioactivity			(<20)				8,890 ± 428	
Specific activity‡							56	
Parasitized erythrocytes (PRBC)§								
Nmoles/ml RBC	1,802 ± 94	137 ± 7	13 ± 2	90 ± 2	21 ± 2	3 ± 1	216 ± 17	3 ± 1
Radioactivity	4,938 ± 137	417 ± 38	109 ± 8	1,045 ± 48	178 ± 11	67 ± 7	29,532 ± 642	155 ± 17
Specific activity	3	3	8	12	9	22	137	52

* Values are the mean ± S.E.M. for three cultures and are representative of results obtained in eight experiments at different parasitemias.
† Integrated radioactive counts (area) per chromatography peak.
‡ Specific activity = radioactivity per nmole.
§ Initial parasitemia (% PRBC) = 4.5%.

Table 2. Effects of adenylosuccinate synthetase inhibitors on incorporation of [^{3}H]hypoxanthine into nucleotides and nucleotide levels of control and malaria (*P. falciparum*) infected erythrocytes*

	Infected RBC	Infected RBC + hadacidin†	Infected RBC + alanosine†
Inosine monophosphate (IMP)			
Nmoles/ml RBC	218 ± 11	362 ± 21	247 ± 15
Radioactivity‡	38,008 ± 351	42,965 ± 412	33,220 ± 368
Specific activity§	174	119	135
Adenosine triphosphate (ATP)			
Nmoles/ml RBC	1,553 ± 28	1,396 ± 23	1,612 ± 20
Radioactivity	4,779 ± 126	790 ± 72	4,570 ± 137
Specific activity	3	0.6	3
Total adenylates (ΣA)			
Nmoles/ml RBC	1,780 ± 37	1,624 ± 31	1,848 ± 27
Radioactivity	5,375 ± 136	1,116 ± 94	5,307 ± 129

* Initial parasitemia (% PRBC) = 5.8%. Values are the mean ± S.E.M. for three cultures.
† Concentration: 5×10^{-5} M.
‡ Integrated radioactive counts (area) per chromatography peak.
§ Specific activity = radioactivity per nmole.

Table 2 and demonstrate the importance of hypoxanthine and the adenylosuccinate pathway to parasite metabolism and survival.

Discussion

Although both hadacidin and alanosine have been shown to inhibit AMPS synthetase of other cell types, their mechanisms of action appear to be quite distinct. Hadacidin acts directly on the enzyme AMPS synthetase by competitive inhibition [7]. Alanosine, however, appears to require activation by way of formation of an intermediate anabolite (L-alanosyl-AICOR) [11]. AICOR (5-amino-4-imidazole carboxylic acid ribonucleotide) is an intermediate in the *de novo* pathway of purine synthesis. Since neither the mature RBC nor *Plasmodia* are capable of synthesizing purines *de novo*, the presence of AICOR and L-alanosyl-AICOR in PRBC would not have been anticipated.

Inhibition of PRBC AMPS synthetase by hadacidin suggests that the adenylosuccinate pathway is essential for synthesis of parasite adenosine nucleotides through salvage of hypoxanthine. The lack of any demonstrable inhibition with alanosine underscores the critical role of purine base salvage, in distinction to an alternative *de novo* pathway, in meeting the purine metabolic requirements of the malaria parasite. We have shown recently that hypoxanthine is used by *P. falciparum in vitro* for synthesis of guanosine nucleotides using an inhibitor specific for IMP dehydrogenase [2]. Overall it appears that hypoxanthine is a critical precursor efficiently used for synthesis of purine nucleotides by malaria infected erythrocytes.

In summary, human erythrocytes parasitized with the major human malaria pathogen, *P. falciparum*, were shown to synthesize adenosine nucleotides from hypoxanthine via IMP. This purine pathway which requires adenylosuccinate as an intermediate between IMP and AMP for synthesis of adenosine nucleotides is not present in normal human mature erythrocytes. Hadacidin but not alanosine blocked the synthesis of adenylates from hypoxanthine. The presence of a parasite-specific purine pathway in the malarious erythrocyte for synthesis of adenosine nucleotides suggests

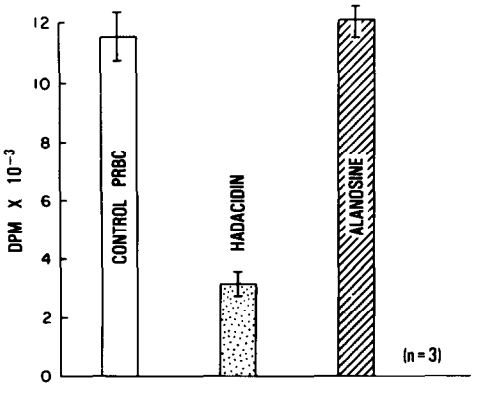

Fig. 1. Effects of adenylosuccinate synthetase inhibitors on incorporation of [^{3}H]hypoxanthine into the acid-insoluble fraction (nucleic acids) of malaria infected erythrocytes. Malaria cultures (4.8% PRBC) were incubated (3 hr) with and without inhibitors (5×10^{-5} M). Unparasitized, untreated RBC cultures served as controls. Following incubation, PCA extracts were prepared on all cultures. The acid-insoluble precipitate was recovered by centrifugation, washed three times in cold PCA, and solubilized in 1 N NaOH. Aliquots were taken for counting in a liquid scintillation counter. These data provide an estimate of incorporation of [^{3}H]hypoxanthine into parasite nucleic acids. Control RBC cultures contained 496 ± dpm, N = 3.

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Biochemical Pharmacology, Vol. 33, No. 9, pp. 1557–1559, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Substrate induction of the biliary excretion of sulfobromophthalein in rats

(*Received* 15 *February* 1983; *accepted* 16 *November* 1983)

Renal organic acid transport (as measured by *p*-aminohippurate uptake into renal cortical slices and isolated tubuli) is stimulated by penicillin pretreatment [1, 2]. Intestinal glucose absorption is also induced by prolonged infusion of glucose, which is thought to increase the number of carriers in the basolateral membranes of enterocytes [3, 4]. In rats, repeated oral administration of taurocholate [5] and cholate [6] enhanced the biliary excretion of taurocholate due to an increase in the density of bile acid carriers in the liver surface membrane [6], but did not affect the biliary excretion of two xenobiotics, phenol-3,6-dibromphthalein and ouabain [5]. All these observations suggest that it is possible to induce various transport processes by prolonged administration of their substrates, and that the increase in overall transcellular transport is due to increased carrier-protein synthesis.

The present experiments were designed to study the inducibility of the biliary excretory system for foreign cholephilic organic acids. Sulfobromophthalein (BSP), BSP-glutathione (BSP-GSH), rose bengal and eosine, which are thought to be excreted into bile by the same transport mechanism, were used as inducers [7]. BSP is known to be conjugated with glutathione (GSH) in the liver whereas rose bengal and eosine are excreted into bile unchanged. Of the four compounds, only BSP proved to be an inducer. In this paper, experimental data which show that increased biliary excretion of BSP is due to increased conjugation of BSP with GSH rather than enhancement of the hepatic transport function itself are presented.

Materials and methods. Disodium salt of BSP was purchased from Merck A.G. (Darmstadt, F.R.G.); rose bengal from Fluka A.G. (Buchs, Switzerland); water-soluble eosine (C.I. 45380) from Reanal (Budapest, Hungary); and diethyl maleate from Eastman Chemicals (Rochester). BSP-GSH was synthesized from BSP by the method of Whelan *et al.* [8].

The experiments were performed on male Sprague–Dawley rats weighing 200–250 g. The animals were pretreated with BSP (60 and 120 μmole/kg i.p.), BSP-GSH (80 μmole/kg i.p.), rose bengal (50 μmole/kg i.p.) and eosine