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The Stimulation of Purine Nucleotide Production by Pyrroline-5-Carboxylic Acid in Human Erythrocytes

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SUMMARY: We previously reported that pyrroline-5-carboxylate (PC), the intermediate in the interconversions of proline, ornithine and glutamate markedly stimulates hexosemonophosphate-pentose pathway activity in human erythrocytes. The stimulation is mediated by pyrroline-5-carboxylate reductase which generates $NADP^+$ accompanying the conversion of pyrroline-5-carboxylate to proline. We now report that the previously demonstrated effect of pyrroline-5-carboxylate on glucose oxidation through the hexose-monophosphate-pentose pathway is accompanied by increased phosphoribosyl-pyrophosphate production and increased formation of nucleotides via the salvage pathway. The demonstrated effect of pyrroline-5-carboxylate on purine processing may provide a regulatory link between amino acid and nucleotide metabolism.

The formation of phosphoribosyl pyrophosphate (PRPP) depends on the activity of PRPP synthetase and on the availability of ribose-5-phosphate, an intermediate which can be generated by the hexosemonophosphate-pentose (HMP) pathway. We recently reported that pyrroline-5-carboxylate (PC), a physiologic intermediate in the interconversions of proline, ornithine and glutamate, markedly stimulated the HMP pathway (1). The stimulation is mediated by PC reductase (EC 1.5.1.2.) which oxidizes NADPH concomitant to the conversion of PC to proline (1). We now report that the effect of PC is not limited to the HMP pathway but that PC initiates a sequence of events resulting in increased production of PRPP and increased nucleotide formation by the salvage pathway.

We used intact human erythrocytes to demonstrate this effect because 1) they exhibit rapid turnover of certain purine nucleotides (2), 2) lacking

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the pathways for de novo synthesis of purines, they incorporate precursors into nucleotides only by the salvage pathway (3). 3) they have a high level of PC reductase activity (1) and 4) PC markedly stimulates the HMP pathway in erythrocytes (1).

MATERIALS AND METHODS:

Erythrocyte preparation: We obtained human venous blood by venipuncture from nonfasting normal adults, anticoagulated it with heparin (1000 U/ml, 0.1 ml/5 ml blood), and separated erythrocytes from plasma and leukocytes by standard techniques (4). Erythrocytes were used for the experiments within two hours of cell isolation.

HMP measurement: Erythrocytes from three male and three female normal volunteers were used for measurement of HMP activity by a previously published method (5). The concentration of glucose was 2.5 mM and 2 μCi of [1-^{14}C]glucose (Amersham) was used per assay. L-PC, enzymatically synthesized and purified by a previously published method (6), was present in some of the flasks at 0.5 mM concentration. The reaction started with the addition of 50 μl of erythrocytes.

Incubation of Erythrocytes for PRPP Production: We used Human erythrocytes from the same aforementioned subjects to assess the effects of PC on PRPP formation. Erythrocytes (50 μl) preincubated for 1 hr at 37° without glucose or PC in medium containing: NaCl 145 mM, Tris-HCl 10 mM pH 7.4, sodium phosphate 25 mM pH 7.4, $MgCl_2$ 12 mM in a total volume of 1 ml. After preincubation, glucose (2.5 mM) and PC (0.5 mM), where indicated, were added to the medium and the preparations were incubated for an additional period of 20 min at 37°. We stopped the reaction by chilling the sample in an ice water bath and subsequently processed them by boiling in a H_2O bath for 45 sec. Samples were centrifuged at 9000 x g for 10 min and supernatants stored at -20° for PRPP determination on the following day.

PRPP assay: We measured 5-phosphoribosyl-1-pyrophosphate (PRPP) by a sensitive enzyme-coupled radioisotopic methods (7). The assay is based on the PRPP-dependent release of $^{14}CO_2$ from [^{14}C-carboxyl]orotic acid (New England Nuclear) in the presence of a mixture of orotate phosphoribosyltransferase (OPRT; EC 2.4.2.10) and orotidine-5'-monophosphate decarboxylase (ODC; EC 4.1.1.23) (Sigma).

Purine nucleotides formation: The incubation conditions were similar to those described for PRPP production except that [8-^{14}C]adenine, [8-^{14}C]-guanine, [8-^{14}C]hypoxanthine or [8-^{14}C]adenosine (New England Nuclear) at 10 μM were added following preincubation and incubated for 20 min at 37°. The reaction was stopped with 1 ml of 12% TCA and supernatants saved for nucleotide determination by high pressure liquid chromatography (HPLC). Prior to application samples were extracted with H_2O saturated diethyl ether to remove TCA (8).

Nucleotide determination by HPLC: The procedure for nucleotide separation was based on that described by Brown et. al. (9). We used a Waters Associates HPLC model 6000 A solvent delivery system, model 440 absorbance detector, model 720 system controller and data module and a Whatman Partisil-10 SAX anion exchange column. The low-concentration eluent was 0.007 M KH_2PO_4 in 0.007 M KCl (PH 4.0) and the high-concentration eluent was 0.25 M KH_2PO_4 in 0.5 M KCl (PH 5.0). All eluents were filtered on Millipore membrane filters (HA; Millipore, Bedford, MA) and degassed prior to use. An automatic injector, WISP model 710A applied the sample (300 μl) to the column and a model 660 solvent programmer monitored a #8 convex gradient with a flow rate of 1 ml/min. The gradient time was 30 min from 100% low-concentration eluent to 100% high-concentration eluent.

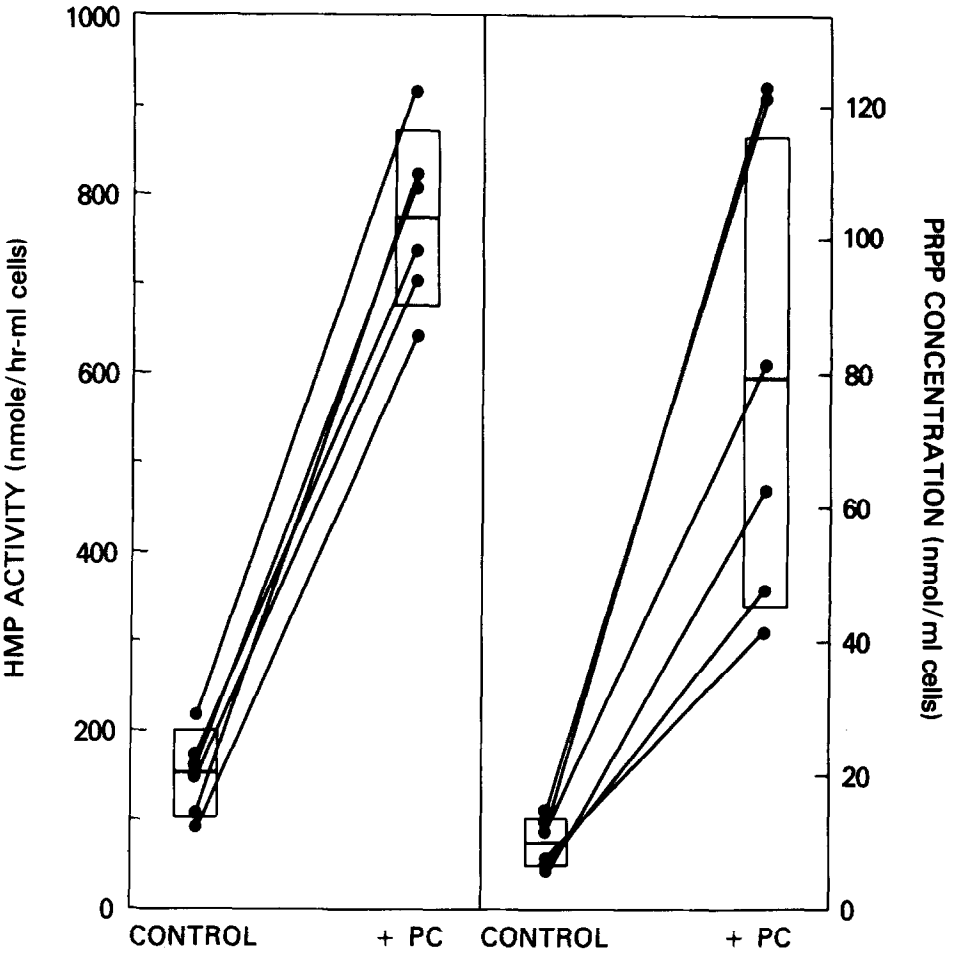

Fig. 1 The effect of PC on HMP activity and PRPP production in erythrocytes. Erythrocytes from 6 normal volunteers (3 male, 3 female, age 26-42) were used for measurements of both HMP activity and PRPP concentration. The conditions for the incubation are as described under methods. The concentration of PC, where indicated, was 0.5 mM. Each point represents the average of duplicate determinations and the box shows mean values ± 1 standard deviation.

After completion of the gradient, the column was eluted for an additional 40 min with 100% high-concentration eluent. The optical density was obtained at 254 nm and peaks were identified by use of standards. The incorporation of ^{14}C into the identified peaks was quantitated by using a LKB (2111 Multirac) fraction collector at 1 fraction/min and radioactivity in the samples were determined by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

In agreement with our previous findings (1) we found that PC markedly stimulated the HMP pathway in erythrocytes. PC, at a concentration of 0.5 mM, produced a mean increase of 408% in HMP activity (Figure 1). In our normal adult population (3 male and 3 female, age range 26 to 42), the production of $^{14}CO_2$ from [1-^{14}C]glucose by the HMP pathway was 152 $\pm$ 18.8 (mean $\pm$ SE) and 772 $\pm$ 40.6 nmole/hr-ml cells in control and PC-treated cells, respectively.

The oxidative arm of the HMP pathway is an important source of ribose-5-phosphate, the precursor of PRPP. Therefore we measured PRPP concentrations in PC-treated erythrocytes. We found that the level of PRPP was 80 $\pm$ 14.5 nmole/ml cells in PC-treated cells as compared to 10 $\pm$ 1.4 nmole/ml cells in similarly incubated controls. The level of PRPP in freshly isolated erythrocytes was 5.2 nmole/ml cells decreasing modestly to 2.1 nmole/ml cells with preincubation (see Methods). Thus, the treatment with PC following preincubation markedly increased PRPP levels in erythrocytes.

Since PRPP is the necessary substrate for the incorporation of purines into nucleotides by the salvage pathway, we examined the effect of PC on nucleotide formation. With PC at 0.5 mM the incorporation of [8-^{14}C]adenine, [8-^{14}C]guanine, [8-^{14}C]hypoxanthine and [8-^{14}C]adenosine into their corresponding nucleotides were 300%, 220%, 250% and 145% of control values, respectively. Expectedly, incorporation of purines into their respective monophosphates showed the highest percent increase since this step is directly affected by the availability of PRPP. Increased incorporation into di- and tri-phosphates probably was due to increases in specific radioactivity of the respective precursors. With either [8-^{14}C]hypoxanthine or [8-^{14}C]adenosine, the effect of PC was limited to an increase in incorporation into IMP. The effect on [8-^{14}C]adenosine incorporation is of special interest because this ribonucleoside is incorporated into nucleotides by two pathways. PC affected only the salvage pathway which is sequentially catalyzed by adenosine deaminase, purine nucleoside phosphorylase, and hypoxanthine-guanine phosphoribosyl transferase.

The Effect of PC on Incorporation of Precursors into Purine Nucleotides in Erythrocytes

		Incorporation into nucleotides (nmole/20 min-ml cells)	
PRECURSOR	PRODUCT	CONTROL	PC(0.5 mM)
Adenine			
	AMP	0.6	7.0
	ADP	4.0	21.2
	ATP	22.6	55.2
Guanine			
	GMP	3.0	15.6
	GDP	8.6	29.8
	GTP	40.8	72.4
Hypoxanthine			
	IMP	47.8	118.4
Adenosine			
	AMP	0.4	1.2
	ADP	5.2	7.2
	ATP	39.0	32.4
	IMP	53.6	101.6

Erythrocytes were incubated with one of the indicated ^{14}C-labeled precursors at a concentration of 10 μM in the presence and absence of 0.5 mM PC. The duration of incubation with precursor was 20 min. Following incubation, nucleotides were separated by high-pressure liquid chromatography. Incorporation of labeled precursor was significant only for those nucleotides shown. We quantitated the incorporation by fractionating the effluent from HPLC and determining the radioactivity in the fractions by liquid scintillation spectrometry.

The specific step affected by PC is most likely the PRPP-dependent step, i.e. that mediated by hypoxanthine-guanine phosphoribosyl transferase. In contrast, the conversion of adenosine to adenine nucleotides by adenosine kinase was unaffected by PC although adenine nucleotides accounted for almost 50% of the adenosine incorporated into nucleotides (Table I).

These studies show that the previously demonstrated effect of PC on glucose oxidation through the HMP pathway is accompanied by increased PRPP production

and increased formation of nucleotides via the salvage pathway. The initiating event is the oxidation of NADPH concomitant with the conversion of PC to proline by PC reductase. This enzyme in erythrocytes is present at high activity levels, oxidizes NADPH preferentially over NADH and is inhibited by $NADP^+$ but not by proline (10), characteristics which suggest that a function of PC reductase in erythrocytes is linked to the oxidative arm of the HMP pathway. By stimulating the conversion of glucose to ribose-5-phosphate, PC increases the availability of PRPP and the formation of nucleotides.

Although PC produced a marked effect on erythrocyte nucleotide metabolism in vitro, the physiologic importance of this stimulation remains to be established. Nevertheless, the augmentation of nucleotide synthesis by this physiologic intermediate may provide a regulatory link between amino acid and nucleotide metabolism. However, the end point of such regulation may be other than to maintain nucleotide levels in erythrocytes per se. In fact, it has been shown that purines and ribonucleosides can be transferred from liver to erythrocytes and subsequently delivered by erythrocytes to other tissues (11-13). In this context, PC also can be supplied to the erythrocyte from hepatocytes, at least in vitro (14). Thus, PC released from hepatocytes could stimulate the production of PRPP required for processing of preformed purines and ribonucleosides for delivery to other tissues.

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