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Involvement of Erythrocyte Calpain in Glycine- and Carnitine-Treated Isovaleric Acidemia

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ABSTRACT

When a 12-y-old girl suffering from isovaleric acidemia was treated with L-carnitine, there was a considerable increase in her blood and urine concentration of isovalerylcarnitine. When later the patient received an infusion of glycine in place of carnitine, isovalerylcarnitine reverted toward the low levels found in a normal subject. At the end of either treatment, erythrocyte calpain was measured and found to be decreased after carnitine therapy (140 *versus* 96 U/mg Hb with glycine or carnitine, respectively). Because we have previously shown that the activity of calpain isolated from erythrocytes was markedly modified by isovalerylcarnitine, the present results might be seen as the consequence of the chronic exposure of the patient's red blood cells to high levels of isovalerylcarnitine. The lowered calpain activity was also proved by an increase in erythrocyte band 3 phosphorylation together with an increased erythrocyte fragility after calcium loading in the presence of the ionophore A-23187. Calpastatin, the natural inhibitor of calpain, was only slightly modified. (***Pediatr Res* 36: 182–186, 1994**)

Abbreviations

IVC, isovalerylcarnitine

The intracellular nonlysosomal calcium-activated cysteine proteinases, commonly referred as calpains or calcium-activated proteinases, are present in virtually every eukaryotic cell type. On the basis of their sensitivity for Ca^{2+}, calpains have been subdivided into μ-calpain and m-calpain, requiring 5–50 μM Ca^{2+} and 0.2–0.6 mM Ca^{2+}, respectively, for half maximum activity (1–5).

We have recently found that IVC, a product of leucine catabolism, is a potent activator of the m-calpains, but not the μ-calpains, isolated from various rat tissues (6, 7). This activation comprises a 10-fold increase in the Km of calpain for Ca^{2+} together with an increase in the V_{max} above the values observed with the native enzyme at saturating Ca^{2+} concentrations. It is additional to the activation produced by adding phospholipid vesicles, showing that IVC could be acting as a highly selective activator of calpain, whether cytosolic or membrane bound. These effects were highly specific for the L-isomer of IVC. The D-isomer, other branched-chain acylcarnitines, and palmitoylcarnitine (6) as well as acetylcarnitine and propionylcarnitine, short-chain acyl-CoA, and fatty acids (Pontremoli S, Melloni E, Michetti M, Sparatore B, Salamino F, Siliprandi N, Horecker BL, unpublished experiments) were not effective.

It should be outlined again that these results have been obtained *in vitro* on calpains purified from tissues or cells. Attempts to confirm whether this action could be seen in a more organized system have failed. For instance, no action on calpain has been observed by incubating human neutrophils in the presence of 2 mM IVC for 15 min at 30°C. One reason for this failure could be the impossibility of obtaining *in vitro* the sufficient length of exposure, a condition that might be conveniently achieved *in vivo*.

Because under physiologic conditions IVC is almost undetectable in both plasma and urine, the problem of a possible activation of calpain *in vivo* by this carnitine ester does not exist.

A condition implying a significant accumulation of IVC is isovaleric acidemia, especially when the patient is treated with a large amount of carnitine (8, 9). In this condition, blood and tissue cells are chronically exposed to abnormally high concentrations of IVC, as is the case for the patient described in the present paper.

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In a girl affected by isovaleric acidemia, the activity of erythrocyte calpain has been comparatively evaluated either after a 6-mo period of carnitine treatment or after the same period of glycine treatment. We found that carnitine treatment actually decreases erythrocyte calpain activity, providing evidence that the decrease in calpain activity is in fact caused by its initial activation by IVC. Once activated, calpain undergoes autodigestion with a progressive decrease in its activity (10). The observed decrease could therefore be caused by the initial activation of erythrocyte calpain activity by the increased IVC (6).

METHODS

Clinical description and study. C.S., now a 12-y-old female, came to our attention 3 y ago after a short history of persisting vomiting. She presented with muscular atrophy and lethargy. Routine laboratory tests revealed hyperammonemia (83 μmol/L), hyperuricemia (0.62 μmol/L), and metabolic acidosis. The diagnosis of isovaleric acidemia was based on the urinary excretion of isovalerylglycine and 3-hydroxyisovaleric acid. Her blood carnitine concentration was extremely low (<5 μmol/L).

Despite oral L-carnitine therapy (100 mg/kg/d) and a controlled protein intake (40 g/d), her plasma total carnitine concentration remained low (15 μmol/L) and was normalized after increasing the dosage to 200 mg/kg/d. No additional episodes of vomiting and lethargy were observed.

Blood and urine samples were first collected, then the carnitine was stopped and immediately replaced with glycine (250 mg/kg/d). After 6 mo of glycine treatment, blood and urine samples were collected again. No major clinical differences were observed as a result of exchanging carnitine for glycine. Control blood and urine samples were obtained from untreated healthy subjects of the same sex and age.

Metabolite assays. Free carnitine was determined by a radioenzymatic method (11) in plasma (after perchloric acid deproteinization) and in urine (after chloroform extraction). Total carnitine was assayed after alkaline hydrolysis. The acid-soluble fraction was also assayed radioenzymatically for acetylcarnitine (12) and for short-chain acyl esters by HPLC (13). Briefly, radioactive carnitine was exchanged to isotopic equilibrium into the acylcarnitine pool of the sample (0.5–1.0 nmol of total carnitine), and individual acylcarnitines were detected and quantitated by reverse-phase HPLC coupled to an instream continuous flow through a beta counter (Flo-One, Radiomatic, Canberra-Packard, Tampa, FL). A pretreatment of the samples by means of solid-phase extraction was used to avoid the underestimation of the esterified fractions due to the presence of high concentrations of free carnitine (14). Samples were applied to Sep-Pak C_{18} cartridges (Waters, Millipore Corp., Milford, MA) equilibrated with 5 mM acetic acid. Free carnitine, acetylcarnitine, and propionylcarnitine were eluted with 8 mL of 5 mM acetic acid. Longer chain (>C4) acylcarnitines were eluted by methanol. The recovery of carnitine esters in the eluate was complete. Isovalerylglycine and 3-hydroxyisovaleric acid were measured by gas chromatography–mass spectrometry by following common procedures of extraction and derivatization (15).

Calpain and calpastatin activities in erythrocytes. Blood samples, immediately after collection from an antecubital vein, were washed four times with an isotonic solution (pH 7.4) containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.14 M NaCl, 5 mM KCl, and 5 mM glucose. Leukocytes and platelets were removed by using a modification (10) of the filtration procedure reported by Beutler *et al.* (16). Packed erythrocytes (2 mL) were lysed in five volumes of 1 mM EDTA, pH 7.4, and centrifuged at 24 000 × *g* for 10 min. The membrane fraction was discarded, and the clear supernatant (cytosolic fraction) was collected and dialyzed against 50 mM Na acetate buffer, at pH 6.4, containing 1 mM EDTA and 0.5 mM 2-mercaptoethanol. A sample containing 200 mg of Hb was submitted to diethylaminoethyl ion exchange chromatography on a column (1 × 10 cm) of diethyaminoethyl-cellulose 50 equilibrated in the above dialysis medium, and the absorbed proteins were eluted by a linear gradient of NaCl (from 0 to 0.3 M). Aliquots (50 mL) of the eluted fractions were heated at 90°C for 3 min, then assayed for calpain and calpastatin. These substances were eluted in separate peaks (17, 18), and from the areas of these peaks their individual concentrations were computed.

Calpain activity was assayed as previously described (19). One unit is defined as the amount of enzyme releasing 1 nmol of free amino groups per hour under the assay conditions.

Calpastatin inhibition was measured as previously reported (19). One unit of calpastatin activity is defined as the amount that inhibits one unit of calpain activity.

Phosphorylation of erythrocyte inside-out vesicle proteins. Erythrocyte inside-out vesicles (0.1 mg protein), prepared as previously described (20), were incubated at 37°C in 0.25 mL of 50 mM sodium borate, pH 7.5, containing 5 μM $MgCl_2$ and 10 μM labeled ATP (1 Ci/mmol). At the times indicated, aliquots (0.05 mL) were withdrawn and the reaction was stopped by addition of 0.05 mL of 20 mM Tris-HCl, pH 8.0, containing 2% SDS, 40% glycerol, 2 mM EDTA, and 80 mM 2-mercaptoethanol, followed by heating at 90°C for 3 min. Samples were then submitted to polyacrylamide slab gel electrophoresis (21). The gels were stained with Coomassie brilliant blue, destained with 7% acetic acid containing 10% ethanol, and dried. The labeled proteins were identified by autoradiography of dried gels.

Loading of human erythrocyte with Ca^{2+} and measurement of erythrocyte fragility. To erythrocytes (3 mL of packed cells), prepared as described above, were added 3 mL of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-

sulfonic acid, pH 7.4, containing 5 mM KCl, 140 mM NaCl, and 5 mM glucose. Aliquots (1 mL) were incubated at 37°C with 5 μM A23187 in the absence of Ca^{2+} or in the presence of increasing concentrations of Ca^{2+}. The cells were incubated for 30 min and then collected by centrifugation at 800 × *g* for 10 min. The Hb content in the clear supernatant was determined by the absorbance at 540 nm (22).

RESULTS

The amount of the carnitine fraction in blood plasma and in urine of the patient at the end of carnitine and glycine treatments is reported in Table 1. Despite the large amount of carnitine given (200 mg/kg/d), the free carnitine concentration in the plasma (13 μmol/L) was lower than that found in untreated normal subjects (31 μmol/L), although the esterified carnitine concentration was significantly higher (21 *versus* 7 μmol/L).

These results are in agreement with those previously obtained in other cases of isovaleric acidemia (8, 9). We have, in addition, established that the dominant carnitine ester in blood and urine after carnitine treatment is indeed IVC. This was still true after glycine treatment, although the levels were lower, especially in the urine. In contrast, IVC was undetectable in the control. Because IVC readily diffuses across cell membranes, erythrocyte calpain must be exposed to the same IVC concentration as plasma.

The ratio of free to esterified carnitine in plasma was also much lower after carnitine or glycine treatment than in the controls. This is partly because, after 6 mo of glycine treatment, the free carnitine concentration in plasma was well below that of controls, perhaps because endogenous carnitine is used in preference to glycine for removing excess isovaleryl groups. This would be concordant with the different published values for the affinity of isovalerylCoA toward carnitine acetyl transferase (Km < 0.05 mM) (23) and toward glycine-N-acylase (Km > 0.5 mM) (24).

The elimination of carnitine with urine (Table 1) at the end of the carnitine supplementation period shows that IVC represents the most abundant fraction, accounting for 85.1% of total carnitine and 97.9% of esterified carnitine. After the glycine supplementation period, urinary excretion of carnitine was greatly reduced even in comparison with that of normal subjects. However, even in this condition, IVC represented the most abundant fraction. These data indicate the severe carnitine deficiency in the patient in the absence of carnitine treatment and show that administered carnitine was almost entirely used to drain the intramitochondrial accumulated isovaleryls.

Table 2 shows the activity of calpain of the red blood cells collected from the patient after 6 mo of a daily supplementation of glycine. The activity of the enzyme was significantly lower than that in erythrocytes of a normal subject of the same age (control) as well as lower than that in the erythrocytes of the same patient 6 mo after ending carnitine supplementation and replacing it with glycine supplementation.

Carnitine treatment did not greatly affect the activity of calpastatin, which was similar to that found in the control.

After L-carnitine treatment, the rates of erythrocyte band-3 phosphorylation were about twice those obtained after glycine treatment or in the control (Fig. 1). This increased phosphorylation, which is concomitant with calpain autodigestion, is attributed to the unmasking of new phosphorylation sites (6, 25). Our results are therefore additional evidence for activation of calpain by IVC *in vivo*.

An increase in erythrocyte band-3 phosphorylation increases the lytic effect of Ca^{2+} in the presence of the Ca ionophore A-23187 (6) (Fig. 2). Indeed, after carnitine treatment, the cells lysed with increasing Ca^{2+} concentration much more readily than in the control or after glycine treatment.

DISCUSSION

The metabolic defect producing isovaleric acidemia (26) is a defiency of isovaleryl-CoA dehydrogenase (27). This results in the accumulation of isovaleryl-CoA in mitochondria with a consequent deficiency of free CoA.

Transfer of the excess isovaleryl groups by conjugation with either glycine (28, 29) or carnitine (8, 9, 25) has been proposed as a way to restore the CoA level. The isovalerylglycine and IVC formed, unlike isovaleryl-CoA itself, readily diffuse across mitochondrial and cellular membranes to be excreted in the urine. Without treatment or after treatment with glycine, endogenous carnitine is rapidly depleted by this conjugation (30), giving a low level of free carnitine itself and increased acyl-carnitine/free carnitine ratios in plasma and urine. Carnitine synthesis is evidently inadequate to maintain a level of free

Table 1. *Concentrations of carnitine and its esters in blood plasma and urine after giving the patient carnitine or glycine*

	Free	Isovalerylester	Other esters
Plasma (μmol/L)			
After carnitine	13	19	2
After glycine	7	5	1
Control	31	ND*	7
Urine (μmol/g creatinine)			
After carnitine	382	2500	55
After glycine	18.2	45.4	9.0

* ND, not determined.

Table 2. *Calpain and calpastatin levels in erythrocytes**

	Calpain (U/mg Hb)	Calpastatin (U/mg Hb)
Control	170 ± 23	303 ± 25
Carnitine-treated patient	96	260
Glycine-treated patient	140	255

* The values of control subjects (mean ± SD) were obtained by assays on five different normal subjects. The values for treated patients are the mean of four different determinations.

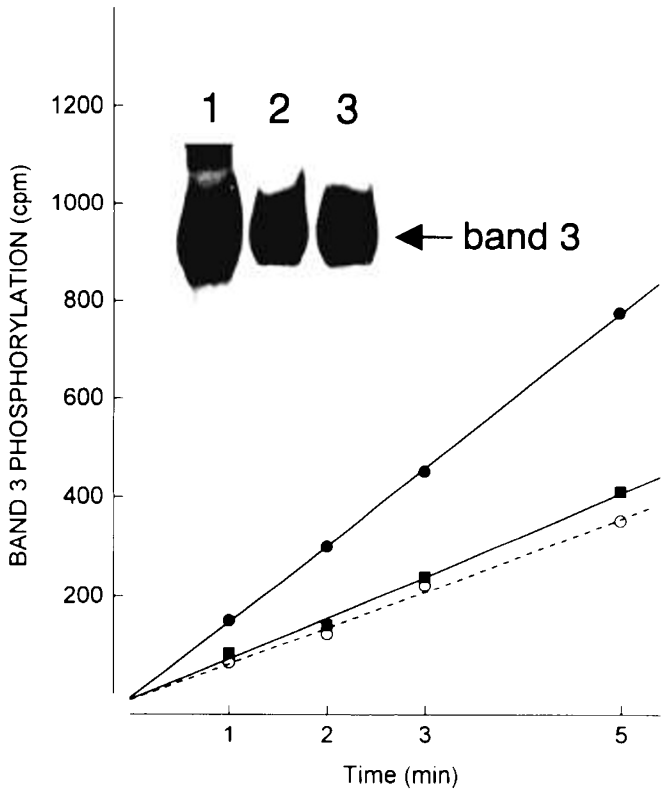

Figure 1. ^{32}P incorporation in the band-3 region of erythrocyte inside-out vesicles from control subjects (○- - -○; *lane 1* in the insert) or the patient after treatment with carnitine (●—●; *lane 2*) or glycine (■—■; *lane 3*). Erythrocyte inside-out vesicles were incubated at 37°C in the presence of 10 μM labeled ATP (1 Ci/mmol). At the times indicated, the reaction was stopped as described in Methods. After heating at 90°C for 3 min, samples were submitted to polyacrylamide slab gel electrophoresis. The labeled proteins were identified by autoradiography of dried gels as shown in the insert. The relative bands were then cut and counted by liquid scintillation, allowing the quantitation of ^{32}P incorporation, which is shown in the lower panel.

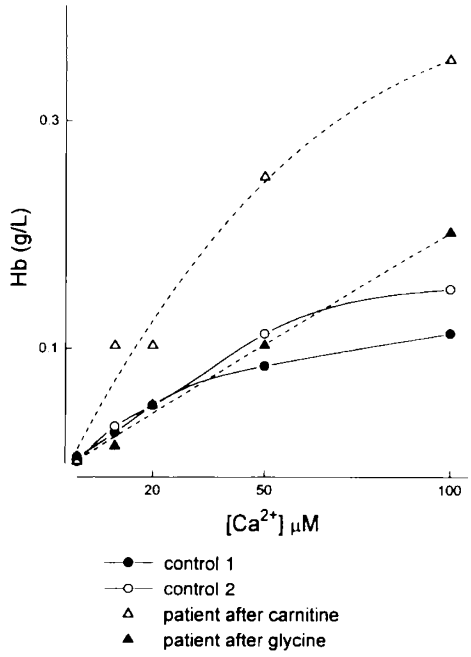

Figure 2. Effect of Ca^{2+} loading on the lysis of erythrocytes from control subjects and from the patient after treatment with carnitine or glycine. Erythrocytes were incubated with 5 μM ionophore A-23187 in the presence of the indicated concentrations of Ca^{2+}, as described in Methods.

carnitine sufficient for the conjugation of the excess isovaleryls, and this also accounts for the fact that loading with carnitine induces a remarkable increase both in the blood level of IVC and in its urinary excretion.

The continuous exposure of erythrocytes to elevated IVC levels can activate their calpain (6), principally by inducing a 10-fold increase in affinity toward Ca^{2+}, the binding of which is necessary for its activity. This activation also initiates calpain autodigestion and eventually leads to destruction of the enzyme. The relative stability of erythrocyte calpastatin, the natural inhibitor of calpain, further supports the previous results showing that *in vitro* IVC activates calpain despite the presence of calpastatin (6). In other terms, IVC releases calpain activity from the calpastatin control. This validates the suggestion of an initial activation by IVC on the "calpain-calpastatin" system.

This scenario plausibly accounts for our observation that there was a decrease of erythrocyte calpain activity in our patient after carnitine but not after glycine treatment. The decrease is also consistent with the increased rate of erythrocyte band-3 phosphorylation, found only after carnitine treatment, which we believe is probably caused by unmasking of new phosphorylation sites. Their appearance may be a prelude to the degradation of certain membrane glycoproteins (22, 25), thus accounting for the higher susceptibility of erythrocytes after carnitine treatment to lysis induced by calcium loading in the presence of a calcium ionophore.

All these effects are therefore seen as secondary to the initial calpain activation by IVC and may be significant from both a diagnostic and a prognostic point of view. The decline in calpain activity does not therefore seem to be injurious, and indeed it could be part of a complex and as yet unknown mechanism linking carnitine with metabolic improvement. Nevertheless, measurement of erythrocyte fragility can be appropriate for the follow-up of patients receiving carnitine treatment.

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