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Effects of Dietary Creatine on Tissue Metabolite Contents and Uptakes in Rat Liver

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랫드 간장에서 Creatine 섭식에 의한 metabolites의 변화

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요 약 : 흰쥐 간정맥 및 간문맥에 PE10 튜브를 마취하에 이식시킨 후 5일째부터 고형사료와 10% creatine을 함유한 고형사료를 5일 동안 섭식 시켜 급속 냉동 시켰다. 냉동시킨 흰쥐 간장, 뇌 및 근육에서 creatine을 포함한 간장 metabolites을 조사하여 다음과 같은 결과를 얻었다. 또한 간장세포내의 water compartment의 변화를 동시에 실시 하였다. Creatine을 섭식시킨 실험군은 정상 대조군과 비교하여 섭식에 의한 간세포내의 water compartment에 변화를 주지 않았고 간장 세포내의 creatine농도에 있어서는 약 43배 이상 세포내 증가를 유도하였으며, 이는 세포외 및 세포내의 creatine의 gradient에 의해 영향을 받고 있슴을 알 수가 있었고, 또한 동시에 뇌, 근육 등에서는 섭식에 의해 유의성 있는 농도 변화는 보이지 않았다. 이는 세포 외액속에 함유된 creatine이 여러 수송기전에 의하여 간세포내로 흡착이 이루어진 것을 알 수 있었으나, 간세포 분리 시험법으로 Creatine Kinase 효소 활성도를 측정한 결과 non-parenchymal hepatocytes에서만 거의 100% 효소 활성도가 있음을 증명 하였다.

Key words : Creatine, Creatine kinase, Liver metabolites, Perfused hepatocyte, Rat, Water compartment

Introduction

In the normal situation, the creatine is synthesized in liver from the urea cycle intermediate arginine in a two step sequence of reaction : glycine-amidinotransferase (EC2.1.4.1) and guanidinoacetate-methyltransferase (EC2.1.1.2)[8]. It is transported through the blood and taken up by tissue with high energy demand via an active transport system. Sodium-dependent creatine transport into isolated myoblast in a protein synthesis requiring process while the K_m remained unaltered at about 40 to 60 μM[13]. While the K_m for creatine uptake in macrophages has been estimated to be about 30 μM.[6], about the same as for muscular tissue, the V_{max} was only 0.18 nmol/min/g cells. or about 1000 times less than that found in muscle tissue. About 3% of intracellular creatine leaves cultured myoblasts/hour[8] which is similar to the approximately 2%

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of total body creatine which is non-enzymatically dehydrated to creatinine with subsequent excretion in the urine[6]. There is no information on the K_m or V_{max} of creatine transport into hepatocytes. Furthermore, creatine is subsequently transported out of liver by an unknown mechanism, where it is taken up primarily by muscle and nervous tissues[9]. While it is known that dietary creatine represses glycine amidinotransferase[27], little is known about the mechanisms controlling the extremely variable creatine content from tissue to tissue. Analogues of creatine which inhibit creatine uptake by muscle, 3-guanidinopropionate[1], and cyclocreatine[10] can also be taken up by muscle and other tissue and are substrates for the enzyme creatine kinase[18]. Because intracellular creatine is related to the intracellular creatine phosphate [PCr^{2-}], the intracellular [ATP]/[ADP] ratio, intracellular proton and free [Mg^{2+}] concentrations through the near-equilibrium brought about by creatine kinase (EC 2.7.3.2)[14,26], unlimited creatine uptake would lead to the formation of large amounts of anionic PCr^{2-}. There is a growing interest in the biological and clinical relevance of creatine feeding both in experimental models and humans. Thus various tumor cell lines implanted in rodents also show a significantly decreased growth rate upon the animals fed with creatine or creatine analogues[15]. PCr^{2-} is synthesized *in vivo* exclusively through the reversible reaction catalysed by creatine kinase, from creatine and ATP^{2}, where creatine itself originates either from the diet or via a two-step *de novo* biosynthesis pathway present in various organs[2,8,18].

Rodent liver unlike human liver lacks the first enzyme involved in creatine synthesis (glycine amidinotransferase), leaving the pancreas and kidney as major sources for endogenous creatine[3,16]. There are a few report concerning the hepatic transport of creatine, either *in vitro* or *in vivo*[11].

In the present study, we have undertaken a series of experiment with feeding model for 5 days and measured creatine phosphate and related metabolites to investigate creatine uptake system in the liver.

Materials and Methods

Animals

Rats were fed *ad libitum* either a regular chow diet or the same diet supplemented with 10% (w/w) creatine for 5 days. Liver, muscle, and brain tissues were rapidly freeze-clamped, grinded in liquid nitrogen, and neutralized with 1.5 M potassium hydroxide/potassium phosphate. Tissue powders were extracted (0.3 g approximately), measured and calculated as the methods described previously[4,17,19,23].

Surgical preparations for measuring metabolites

Five to seven days prior to the experiment, the right external carotid artery and right superior jugular vein under phenobarbital anesthesia (40 mg/kg body weight) were exposed and PE 10(Becton Dickenson, Parsippany, NJ, USA) cannulae placed in the external carotid and through the jugular and right atrium until lodged in the hepatic vein[12]. The position of hepatic vein cannulae was verified upon the completion of experiments and sacrifice of the animals. The abdomen was then opened, a branch of the hepatopancreatic duodenal vein was surrounded by a ligature and a PE10 cannula placed within the vein and threaded up into the portal vein. The cannula was then secured by a ligature to the abdominal wall and threaded through a subcutaneous tunnel made by a blunt dissection to exit the skin in the intrascapular region. The abdomen was closed. The rats were then placed in a rodent jacket (Alice King Chantam Medical Arts, Los Angeles, CA, USA) to which was attached a spring wire guide allowing all cannulae to exit the individual cages in which the animals were housed. The cannulae were filled with 1:1 heparin (solution) and checked for patentancy twice daily by withdrawal of blood into the visible portion of the cannulae until the time of the experiment.

Space measurements for calculating intracellular metabolites

Each rat was injected with 10 μCi of ^{3}H-H_2O plus 100 μCi of ^{14}C-carboxyl-inulin through the implanted jugular vein cannula 20 min prior to sacrifice. The cannula was rinsed immediately with 200 U of heparin (1000 U/ml) to deliver. After 20 min, whole blood (0.5 ml) was withdrawn from hepatic vein cannulae, centrifuged for 30 seconds at 15,000 g

force in an Eppendorf bench top centrifuge to sediment cellular components and 50 μl of plasma transfered to 10 ml of hydrofluor (National Diagnostics, USA) for counting solutions for determination of ^{3}H and ^{14}C activity in a Beckman LS6100IC liquid scintillation counter (21). A second aliquot was then transfered to a capillary tube for determination of hematocrit. For determination of vascular space, heparinized whole blood was collected from a donor rat, centrifuged, washed 4 times with phosphate buffered saline, and then the cells were incubated with 340 μCi of $Na_2{}^{51}CrO_4$ for 40 min at 38°C[25]. The cells were then washed 3 times, resuspended, and 0.2 ml of labeled cells given intravenously to each rat. After 20 min, the animals injected with labeled red cells were sacrificed, 0.5 ml of whole blood withdrawn along with 1 g of ground freeze-clamped liver and counted in an LKB/Wallc 1282 CompuGamma counter to determine the red cell volume in liver.

Metabolites measurements

Freeze-clamped liver, brain and muscle were extracted with a 2 M ice-cold perchloric acid for measurement of organic metabolites and nitric acid digestion for metal analyses[20]. Organic metabolites including creatine kinase activity, creatine, phosphocreatine, intracellular hepatic creatine were measured enzymatically using either a Zeiss PMQIII spectrophotometer or an optical Technology Devices Inc. ratio fluorimeter (Elmsford, NY, USA)[4,17,19]. Perkin-Elmer model 3030 atomic absorption spectrophotometer (Norwalk, CT, USA) was used for metal determination. Adenine nucleotides metabolites were measured using C18 column on a Waters/Millipore HPLC (Milford, MA, USA)[24].

Enzymatic liver perfusion for measuring creatine kinase activity

The procedure for the separation of parenchymal and nonparenchymal liver cells was performed according to the methods previously described[23]. The hepatic portal vein in anesthetized normal adults rats were cannulated with a 16-guage intravenously cannula, and perfused with oxygenated Hank's balanced salt solution (HBSS, pH 7.2) without calcium and magnesium at a flow rate of 30 ml/min at 37°C. After 5 to 7 min, 0.05% collagenase (Sigma Chemicals Co., St. Louis, MO, USA) in serum free medium was started to recirculate through the liver at a 15 ml/min. Perfused liver cells were collected by gently agitating whole liver. A single cell suspension was produced by filtering the crude cell suspension through 100 mesh stainless steel screen using 30 ml of phosphate bufferd saline (PBS, pH 7.2). Parenchymal and nonparenchymal hepatocytes were isolated by differential centrifugation of the cell suspension and finally fractioned through electroelutor centrifuge.

Results

Body weight

The changes of body weight did not show any significantly during feeding creatine diet.

Creatine uptake

The ratio of the concentration of creatine in blood to that in tissue was increased by feeding creatine in liver, but remained essentially unchanged in muscle and brain (Table 1) so that the energy of gradient of [creatine] o/i increased about 3 fold in liver from -1. 59 to 6.04 kJ/mol but decreased 2-3 fold from -7.29 and -9.06 kJ/mol to -2.64 and -4.18 kJ/mol in brain and muscle. As shown in Table 1, feeding creatine increased [creatine content] which was 5.38±0.09 and 6.85±0.30 in brain (n=5) and 10.67 and 12.4 μmoles/g wet weight in muscle (n=5) in control and

Table 1. Hepatic vein plasma and tissue creatine content

	Plasma	Liver(7)	△G o/i	Brain(5)	△G o/i	Muscle(5)	△G o/i
Control	0.321±0.009	0.59±0.045	-1.59	5.38±0.09	-7.29	10.67±0.21	-9.06
Creatine fed	2.47±0.29*	25.5±0.73*	-6.04	6.85±0.30	-2.64	12.42±0.56	-4.18

Results are given μmoles/intracellular ml H_2O (mM)±standard error for liver creatine and μmoles/g wet weight±standard error for muscle and brain. *indicates a significant difference, assessed by using the Mann-Whitney U test at $P<0.05$.

Table 2. Hepatic tissue levels of metabolites

Metabolites	Control rat (n=6)	Creatine fed rat (n=6)
Creatine	0.38±0.03	13.6±0.321*
Creatine phospahte	0.1±0.003	0.51±0.05*
Pyruvate	0.14±0.023	0.077±0.015
Lactate	0.72±0.111	0.68±0.05
ATP	2.36±0.14	2.26±0.14
ADP	0.97±0.05	1.01±0.034

Results are in μmoles/g wet weight and expressed as mean±SEM for the number of livers in parentheses. *indicates a significant difference, assessed by using the Mann-Whitney U test at P<0.05.

creatine fed animals, respectively. Taken together, these results suggest an active uptake mechanism for creatine in hepatocytes.

Hepatic water space

Total intracellular water and combination of the vascular volume did not change in creatine fed group. Total hepatic water was 0.735 ml/g wet weight liver, while extracellular water was 0.19 ml/g wet weight and total intracellular water was 0.545 ml/g wet weight. These data were used for calculating intracellular concentration of hepatic metabolites[20].

The activity of creatine kinase (EC 2.7.3.2) in hepatocytes isolated from the liver of normal fed rats was 8 μmol/min/g wet weight at 38°C, about 500 times less than the enzyme activity of the enzyme in liver from transgenic mice (data not shown). Nevertheless, the measured hepatic content of phosphocreatine increased on feeding a creatine diet in freeze-clamped rat liver (Table 2).

Even though the cellular pools of ATP are little, no significant change in overall ATP levels can be detected, because ATP levels is continuously replenished from the large pool(s) of PCr through the reaction catalyzed by creatine kinase (EC2.7.3.2).

Creatine reversibly catalyzes the transfer of a high energy phosphate bond from creatine phosphate, serving as a reservoir of high energy phosphate. Creatine kinase activity was found in isolated rat hepatocytes as a 8 μmoles/min/g protein. However, 4% non-parenchymal cells contaminated could be account for observed creatine kinase activity. If parenchymal cells

Table 3. Hepatic calculated cytosolic metabolites. * indicates a significant difference, assessed by using the Mann-Whitney U test at P<0.05

Metabolites	Control rat (n=6)	Creatine fed rat (n=6)
[NAD+]/[NADH]c	1126±274.3	639±117*
[ΣATP]/[ΣADP][ΣPi]c	11490±2843	3357±731*
△G ATP kJ/mol	-57.15±0.781	-54.9±0.59
KCK	131	94
[ATP]/[ADP]c	16.9±0.91	3.66±0.36*
△G Creatine o/i(kJ/mol)	-1.6	-6.04*
Calculated free cytosolic[ADP]	0.118	0.766*

Table 4. Creatine kinase activity on freeze-clamped rat liver and perfused rat hepatocytes

Control rat liver(freeze-clamped liver)	Creatine fed rat liver (freeze-clamped liver)	Perfused hepatocytes (normal)	Calculated perfused non-hepatocytes (normal)
33.3	30.6	8.0	140

Values are given μmoles/min/g protein (n=5).

have no creatine kinase activity, non-parenchymal cells would represent 10-15% of total hepatic cellular masses, the calculated total hepatic creatine kinase activity would be 28 μmoles/min/g protein which is similar to our observed total hepatic creatine kinase activity of 33.3 μmoles/min/g protein (Table 3). These results were well matched to the same data that published previously suggesting no creatine kinase activity in parenchymal cells from a normal mouse as shown by immunoblotting and immunohistochemical studies.

Discussion

The creatine kinase enzyme is crucial evident to provide important functions in a complex regulated network of energy generation and utilization in organ system. Creatine kinase (EC 2.7.3.2) is often described as an energy buffering system in tissues of high energy demand, such as motility organs[1]. Creatine kinase(CK) reversely catalyzes the transfer of a high energy phosphate bond from creatine phosphate (PCr^{2-}), as a reservoir of high energy phosphate. The major consumers of phosphocreatine can

not synthesize creatine, so that must obtain phosphocreatine or its immediate precursor, guanidoacetic acid[6], from extracellular compartment. Creatine levels themselves have been reported to play a role in tumor growth[21]. Creatine is either derived from the diet or synthesized from amino acids in a two-step process in several organs. In the first step, guanidinoacetate is formed from arginine and glycine in a reaction catalysed by an amidinotransferase (EC2.1.4.1)[16]. In human, the pancreas appears to be the most active organ for creatine biosynthesis, more so than liver and kidney[16]. In rodents, the liver lacks the amidinotransferase which leave the pancreas and kidneys as the main sources of guanidinoacetate[22]. In normal rat, the feeding of 10% creatine in the diet resulted in a 25- to 35-fold increase in the creatine content of liver according to these results. The [PCr^{2-}] content resulting from increasing intracellular [creatine] was increased only 5-fold in the rat. These data suggest that the enhanced activity of creatine kinase did not alter significantly the ratios of the metabolites of the creatine kinase reaction. Three fold decrease in the energy of the gradient of [creatine]o/i between the extra- and intracellular phase of liver on feeding creatine. Hepatic tissue content of creatine was 0.59 ± 0.05 μmole/ml intracellular H_2O in normal fed animal, but 25.5 ± 0.73 μmole/ml intracellular H_2O in rats fed creatine. The intracellular/hepatic vein [creatine] was 1.8 in normal rats but increased to 10.3 in rats fed creatine suggesting the creatine uptake system in creatine fed rat liver. We found 0.074 μmoles of phosphocreatine/ml intracellular water *in vivo* in normal rats, but all concentration of phosphocreatine measured were confined to non-hepatocyte *in vivo* and non-hepatocyte represent 10-15% total hepatic mass, then, the [PCr^{2-}] in non-hepatocyte water would be on the order of 7-10 times higher than calculated or 0.5-0.7 μmoles/ml non-parenchymal hepatocyte water. For creatine fed rats, the calculated hepatic intracellular [PCr^{2-}] was 0.99 μmoles/ml intracellular water. If all [PCr^{2-}] confined to non-hepatocyte water, the [PCr^{2-}] in those cells would be on the order of 7-10 μmoles/ml non-hepatocyte water. This values are near found in perfused working rat heart[12,20]. In addition to the expression of creatine kinase in normal, differentiated tissue, it is overexpressed in many tumor type and cancer patients[5,22].

Concluding from these data, there are at least two metabolic consequences: (i)creatine kinase is highly expressed at the non-parenchymal hepatocytes, and (ii)prevents the inactivation of ATPases which related to the role ADP and inorganic phosphate in the regulation of oxidative phosphorylation and other metabolic process.

Conclusion

Metabolite contents of brain, muscle and liver were measured in freeze-clamped livers of 250-300 g male Wistar rats 5 days after implanting cannulae in hepatic and pancreato-duodenal vein and feeding a diet of either chow or the same diet supplemented 10% (w/w) with creatine. In another set of animals, total liver [^3H_2O] water was determined to be 0.735 ml H_2O/g wet weight of liver: 0.029 ml/g. wet weight [^{51}Cr] red blood cell H_2O, 0.055 ml of g wet weight plasma H_2O, 0.134 ml/g wet weight interstitial [^{14}C]-inulin accessible H_2O, and 0.545 ml/g wet weight of intracellular H_2O, respectively. The portal vein creatine was 0.294 ± 0.019 and hepatic vein 0.321 ± 0.009 μmoles/ml in control rat fed a normal diet and 2.89 ± 0.27 and 2.47 ± 0.29 μmoles/ml in creatine fed animals. Hepatic creatine content was 0.59 ± 0.045 μmoles/ml intracellular H_2O in normal fed animals, but 25.5 ± 0.73 μmoles/ml intracellular H_2O in rats fed creatine. The intracellular/hepatic vein[creatine] was 1.8 in normal rats, but increased to 10.3 in rats fed creatine suggesting the creatine uptake system in liver. The $\triangle G[\text{creatine}\pm]$ outside/inside increased from -1.59 kJ/mol in the control to -6.04 kJ/mol in the creatine fed animals. In contrast, feeding creatine did increase [creatine$\pm$] which was 5.38 ± 0.09 and 6.85 ± 0.30 in brain and 10.67 and 12.4 μmoles/g wet weight in muscle in control and creatine fed animals, respectively. The measured activity of creatine kinase (EC 2.7.3.2) in hepatocytes isolated from perfused rat livers was 8 μmoles/min/g wet weight of hepatocytes. These results seem to explain uptake of rat liver when creatine is accumulated in extracellular compartment.

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