INTRODUCTION

Creatine acts as an energy storage mechanism and transports/shuttles high-energy phosphate in tissues with high metabolic demands such as the heart, brain, retina, testes and skeletal muscle. Deficiencies in the enzymes involved in creatine synthesis, guanidinoacetate-methyltransferase (GAMT) and L-arginine:glycine amidinotransferase (AGAT), and in the 2Na+/1Cl-/creatine transporter (CRT) (2) caused myopathies, mental retardation and other diseases (for reviews see 3-5). These observations strengthened the physiological relevance of creatine.

Plasma creatine levels depend on creatine biosynthesis, intestinal creatine absorption and renal reabsorption of filtered creatine.

Creatine is synthesized from arginine and glycine in two steps. The first reaction is catalyzed by AGAT and the second by GAMT. AGAT activity is under nutritional and hormonal control (6-12). Creatine represses AGAT mRNA expression and activity, thus regulating its own biosynthesis (7, 9, 12). The biosynthesis of creatine appears to be an inter-organ process, which mainly involves the pancreas, liver and kidneys. The synthesized creatine is exported to the blood and is taken up by creatine-requiring tissues via the CRT transporter (for reviews see 3, 5).

Oral creatine supplementation is widely used by athletes to improve performance (13) and in the medical field it may be useful for the treatment of neuromuscular, cardiovascular, and mitochondrial diseases as well as Parkinson’s and Huntington’s; muscle rehabilitation; hypoxia and energy-related brain pathologies; as an antitumoral, antiviral, neuro-protective agent and in organ transplantation (3, 14, 15).

Human and animal individuals have been used to investigate the benefits of oral creatine supplementation. CRT mediates the first step of small intestinal creatine absorption (16, 17) and ontogeny regulates intestinal CRT activity (18). However, information on whether: i) the intestine contributes to creatine synthesis, ii) the intestine transports creatine or iii) ontogeny regulates intestinal creatine metabolism is not available. The current study investigates creatine transport activity in the colon and the intestinal abundance of CRT, AGAT and GAMT mRNAs and that of CRT protein in E20 foetuses, newborn, suckling, weaning and adult rats.

The results revealed that in the early stages of life the colon transports creatine by a specific NaCl-dependent process; that the intestine expresses AGAT and GAMT mRNAs and that both, creatine transport and synthesis are ontogenically regulated.
MATERIALS AND METHODS

Substances

[14C]-creatin was purchased from American Radiolabeled Chemicals Inc. and [α32P]-UTP from GE Healthcare Europe GmbH. The other reagents used in the current study were obtained from Sigma Chemical Co, Spain.

Animals

20 day gestation foetuses (E-20), newborn (less than 1 day-postpartum), suckling (12 and 15 day-old), weaning (30 day-old) and adult (2 and 5 month-old) Wistar rats were used. Rats were humanely handled and sacrificed in accordance with the European Council legislation 86/609/EEC concerning the protection of experimental animals. Mothers and adult rats were fed with a rat chow diet (Panlab 04) ad libitum and had free access to water. The experiments were performed in accordance with national/local ethical guidelines.

Creatine uptake experiments

The whole colon was removed, opened longitudinally and rinsed clean with ice-cold saline solution (0.9% NaCl). 2 cm pieces of the whole colon were incubated at 37°C in a thermostatic bath, with continuous shaking, in Ringer’s solution containing of, in mM: 140 NaCl, 1.2 CaCl2, 2.4 KH2PO4, 0.4 KH3PO4, 1.2 MgCl2, pH 7.4, and 0.012 [14C]-creatin, and it was continuously bubbled with 95%O2/ 5%CO2. When required, NaCl was isosmotically substituted by mannitol. At the end of the incubation time period the tissues were washed, weighed wet and extracted with Ba(OH)2 and ZnSO4. Samples were taken from the bathing solution and from the tissues extracts for radioactivity counting.

RNA preparation and Northern blotting

Total RNA was extracted as described (19). Northern hybridisation was carried out using Poly(A)+RNA obtained from duodenum, jejunum, ileum and colon of 30 day-old rats, as reported (20). [α32P]-UTP-labelled antisense riboprobe of CRT was generated from a rat cDNA fragment as described (16). Relative quantification of mRNA was determined with a phosphoimager system (Fuji Photo Film Co., LTD) using the BetaDat program (Raytest GmbH). The other reagents used in the current study were obtained from Sigma Chemical Co, Spain.

In situ hybridisation

Floating 30 µm-sections of colon obtained from 15 day-old rats were hybridised with either antisense or sense digoxigenin-UTP-labelled riboprobes, generated from rat cDNA fragments as described (16). In situ hybridisation was performed as described (21), except that the sections were washed to high stringency conditions at 56°C. The sections were photographed with a Zeiss Axioskop 40 microscope.

Immunohistochemistry

Immunohistochemistry was carried out on 10 µm-sections of colon obtained from 15 day-old rats as described (16). The primary antibody used was a polyclonal anti-CRT from Alpha Diagnostic. Controls were carried out without primary antibody. The sections were photographed with a Leica TCS-SP2 confocal fluorescence microscope.

Western blotting

Protein samples of small intestinal mucosa homogenate and enterocyte apical membranes were used. Brush-border membranes were isolated as described (22). SDS-PAGE was performed according to Laemmli (23) as described (16). The blots were probed with a polyclonal anti-CRT antibody from Alpha Diagnostic and subsequently incubated with an anti-β-actin antibody to normalize CRT density values.

Relative quantification of real-time PCR

Total RNA was extracted from rat small and large intestine of different ages using RNeasy® kit (Qiagen). Real-time PCR was performed as described (24). Primers for the genes tested are given in Table 1. β-actin served as reference gene for samples normalisation.

Analysis of data

Data are presented as mean± SEM for (n) separated animals. In the figures, the vertical bars that represent the SEM are absent when they are less the symbol height. Comparison between different experimental groups was evaluated by the two-tailed Student’s t-test. Two-way ANOVA followed by Newman-Keuls’ test (p< 0.05) was used for multiple comparisons in Figs 1, 5 and 7 (GraphPad Prism Program).

RESULTS

Creatine uptake into the colon

30 min (14C)-creatin uptake into the colon isolated from newborn, suckling and weaning rats was measured in the presence and nominal absence of NaCl in the incubation buffer. Fig. 1 A shows that NaCl-dependent creatine uptake was maximal at birth. Thereafter it decreased and it was no longer measurable in 30 day-old rats. NaCl-independent creatine uptake was not affected by age.

Creatine uptake into the colon isolated from newborn and suckling rats was inhibited by cold creatine and by guanidinopropionic acid (GPA), a high-affinity substrate of CRT (Fig. 1B).

Northern blots and in situ hybridisation

CRT mRNA expression in the colon of 30 day-old rats was examined by Northern assays using poly (A') RNA. The

Table 1. Oligonucleotides used in relative quantification of real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Antisense (5'…3')</th>
<th>Sense (5'…3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRT</td>
<td>NM017348</td>
<td>TTTATATACCTGTGCAAGTCCT</td>
<td>GCTCAAGACTTTGTGTCTCC</td>
</tr>
<tr>
<td>AGAT</td>
<td>NM031031</td>
<td>CCTGCTCTCTCTAACAAGC</td>
<td>TCTATGATTTGGGTCCTTCACC</td>
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<tr>
<td>GAMT</td>
<td>NM012793</td>
<td>GGCAGTCACAGTTCGACAC</td>
<td>CATCTGTTGCAATACATGAC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM031144</td>
<td>AACCACACGTGGCCCATCTA</td>
<td>CGGAACGCCATGCCTGGC</td>
</tr>
</tbody>
</table>

Oligonucleotides were chosen according to the rat cDNA sequences entered in Genbank and designed using PerlPrimer program.
antisense riboprobe hybridised to two transcripts of 2.7-kb and 4.2-kb (Fig. 2). For comparison, the expression of CRT mRNA in the small intestine is given. The abundance of the two transcripts was greater in the small intestine than in the large intestine. In the former, the 2.7-kb transcript was significantly more abundant than that of 4.2-kb. In the colon the abundance of the two transcripts was of similar magnitude.

Northern blots do not inform on the type of cells expressing CRT mRNA within the epithelia as do the in situ hybridisation. The latter studies revealed that CRT mRNA expression was restricted to the cells lining the surface of the colonic mucosa (Fig. 3, antisense panel). The background labelling with the sense riboprobe on adjacent sections of the tissue was low (Fig. 3, sense panel).

Immunohistochemistry

The localisation by immunofluorescence of CRT protein in rat colon at the confocal microscopy level is given in Fig. 4. There was a strong specific signal at the apical membrane of cells lining the colonic mucosa. No immunofluorescence signal was detected when the intestine was incubated without the primary antibody (data not shown).
animals in each experimental condition. Two-way ANOVA showed an effect of age (p<0.0001). Newman-Keuls test: “a”, as compared with adult rats (5 months old). “b”, distal colon vs proximal colon. “c” jejunum and ileum vs. colon.

**CRT mRNA expression versus age**

The effect of ontogeny on CRT mRNA abundance in small and large intestine was examined by the real-time PCR technique. Fig. 5 shows that in both small and large intestine CRT mRNA abundance increases from the foetus stage to adulthood. At all the ages tested, CRT mRNA abundance was significantly higher in the small intestine in comparison to the large intestine.

**Small intestinal CRT protein expression versus age**

The antibody used detected 57-, 65-, 80- and 116-kDa polypeptides in the apical membrane of the enterocytes (Fig. 6). The intensity of all the bands was very faint in the homogenate (H) but highly enriched in the apical membranes (AM). Maturation decreased the intensity of the 65- and 116-kDa bands and that of 80-kDa was undetectable in 2 month-old rats. The 57-kDa polypeptide was only observed in the apical membrane of 2 month-old rats.

**Intestinal mRNA levels of AGAT and GAMT versus age**

The expression of AGAT and GAMT mRNAs in the small and large intestine was examined by the real-time PCR technique. The results (Fig. 7) revealed that at all the ages tested, AGAT mRNA abundance was higher in the colon than in the small intestine. In both intestinal regions the maximal abundance occurred in the foetuses and it decreased with age. In the foetuses, AGAT mRNA levels were significantly higher in the proximal colon than in the distal, and higher in the jejunum than in the ileum.

Compared to AGAT, intestinal GAMT mRNA abundance remains at a low level and it was affected by neither intestinal region nor development.

**DISCUSSION**

Body creatine homeostasis depends on endogenous synthesis, absorption from diet and renal handling. In rats, the first step of renal (25) and intestinal (16, 17) transepithelial creatine transport occurs via an apical Na⁺/Cl⁻/creatine transporter (CRT).

Traditionally, the upper part of the gut is the focus of studies on nutrient transport because it is the place where most nutrients are digested and absorbed. Here for the first time, we give evidence that in the early stages of life, the colon transports creatine via a Na⁺/Cl⁻/creatine transporter; creatine uptake into rat colon was NaCl-dependent and inhibited by either GPA or cold creatine.

The molecular studies indicate that an apical-membrane CRT mediates the NaCl/creatine transport activity measured in the colon. Thus, the rat colon expresses the same two CRT mRNA transcripts (ca 4.2- and 2.7-kb) previously observed in the rat small intestine (16). The in situ hybridisation studies corroborated the presence of CRT mRNA in the colon and revealed that this expression is restricted to the cells lining the surface of the colon mucosa. The crypt cells do not express CRT mRNA. Finally, the immunohistochemistry experiments show that fluorescence mostly appears along the apical membrane of

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**Fig. 5.** Expression of rat CRT mRNA in the small and large intestine during development. CTR mRNA expression was evaluated by real-time PCR analysis. Total RNA was isolated from small and large intestine of rats of different ages. The mRNA relative expression values were normalised in each sample using the β-actin as reference gene. The mRNA levels measured in the distal colon of rat foetuses were set at 1. Means ± SEM of 4 animals in each experimental condition. Two-way ANOVA showed an effect of maturation (p<0.0001). Newman-Keuls test: “a”, as compared with adult rats (5 months old). “b”, distal colon vs proximal colon. “c” jejunum and ileum vs. colon.

**Fig. 6.** Western blots of small intestine homogenate (H) and apical (AM) membranes. 70 µg protein were loaded to each lane. The blots were probed with the polyclonal anti-N-terminal anti-CRT antibody. Histograms represent the relative abundance of CRT protein in apical (AM) membrane. Means ± SEM, n=3. *p<0.001, as compared with newborn. #p<0.001, as compared with suckling.
the cells lining the surface area of the colon mucosa, which agree with the in situ hybridisation results.

As observed in the small intestine (18), colonic NaCl/creatine transport activity is regulated by ontogeny, showing its highest value at birth and being no longer evident after weaning.

The Western assays detected four polypeptides in the apical membrane of the intestine but the results do not allow us to determine which corresponds to the CRT transporter. The 57-kDa polypeptide could be dismissed since it was only detected in 2 month-old rats, when CRT activity is not longer measurable. The 80-kDa polypeptide decreased with age and it could be hypothesised that the 65-kDa polypeptide represents the plasma membrane CRT and that of 116-kDa corresponds to a dimer form of CRT.

Studies on the size of CRT protein have yielded conflicting results. Estimates of its molecular weight vary from 40-kDa to 210-kDa. Immunoreactive bands of >100-kDa may represent aggregates of CRT (26). Tran et al. (27) concluded that the 55-kDa polypeptide is the plasma membrane CRT. West et al. (26) purified bovine CRT and the glycosilated transporter run as an approx 70-kDa band. Speer et al. (28) considered that the CRT polypeptide of 60–65 kDa (depending on the tissue and the species) very likely constitutes the CRT protein. They concluded that the conflicting results on the molecular size of CRT may arise from the cross-reaction of the anti-CRT antibodies with non-CRT polypeptides. More recently, Straumann et al. (29) suggest that the plasma membrane CRT has an apparent monomer molecular mass of 58-kDa.

Whereas brush-border membrane CRT abundance and activity decreased with maturation, CRT mRNA levels increased with age. This opposite developmental pattern suggests that post-transcriptional mechanisms may regulate CRT protein synthesis and/or insertion in the apical membrane. The CRT protein presents consensus sequences for phosphorylation that may be potentially phosphorylated by cAMP-dependent protein kinase A and by protein kinase C, affecting CRT transport activity (see 4, for a review). Our own unpublished observations revealed that in the chickens, intestinal NaCl/creatine transport activity is increased by phorbol 12-myristate 13-acetate, a PKC activator, and by forskolin, an adenylate cyclase activator. Other reports, however, showed that phorbol esters reduced CRT activity in cultured muscle cells and that PKA activation did not affect creatine transport rates (30, 31).

As previously observed (32), rat embryos express AGAT and GAMT mRNAs, the two enzymes involved in creatine synthesis. AGAT mRNA abundance was higher in the colon than in the small intestine and in both tissues it was down-regulated by ontogeny. The abundance of GAMT mRNA was not affected by intestinal region or by development. These observations agree with the view that the reaction catalyzed by AGAT is a regulated step in the biosynthesis of creatine (for reviews see 3, 5).
The presence of AGAT and GAMT mRNAs in the intestine suggests that this organ may have the capacity to synthesize creatine. In the early stages of life, the colon might contribute significantly to the biosynthesis of creatine since AGAT mRNA abundance is 25% of that in the renal cortex. Later on, the contribution of the kidney to creatine synthesis is much larger than that of the intestine, since AGAT mRNA abundance is around 3,000 times higher than that in the colon.

In conclusion, the results reveal that the rat colon takes up creatine via the CRT transporter. Ontogeny regulates colonic creatine transport activity, intestinal expression of CRT and AGAT, and renal AGAT expression. In the late gestation period and early stages of life the colon may significantly contribute to creatine homeostasis.

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