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Partial cloning, tissue distribution and effects of epigallocatechin gallate on hepatic 3-hydroxy-3-methylglutaryl-CoA reductase mRNA transcripts in goldfish (*Carassius auratus*)

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ABSTRACT

Epigallocatechin gallate (EGCG), the major active component of the green tea, has recently been found to inhibit 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoAR) activity in vitro and to modulate lipogenesis in vivo. In this study we have evaluated the effects of short-term in vivo exposure to EGCG (6 μg g^{-1} BW or 9 μg g^{-1} BW) on hepatic HMGCoAR gene expression of goldfish (*Carassius auratus*). We initially characterized a partial sequence of goldfish HMGCoAR suggesting that the obtained fragment shares high similarity (>92%) with other fish HMGCoAR sequences. Further, the HMGCoAR transcript was detected in all goldfish tissues (except muscle) but primarily in liver, brain and gonads; on the contrary, low expression levels were found in intestine, heart, gill, and kidney. Both EGCG doses significantly decreased hepatic HMGCoAR mRNA levels 180 min post-injection. HMGCoAR was also significantly down-regulated at 90 min after injection in fish treated with the highest dose of EGCG. Our results demonstrate that hepatic HMGCoAR gene expression is acutely responsive to short-term EGCG exposure in goldfish. This finding suggests a potential role of EGCG in transcriptional regulation of the rate-limiting enzyme in cholesterol synthesis.

1. Introduction

Catechins are polyphenolic compounds that have been suggested as responsible for most of the beneficial effects of green tea. Epigallocatechin gallate (EGCG) is the most abundant of the green tea catechins and it is considered as the major active component of the tea plant (Nagle et al., 2006; Wolfram et al., 2006) given that several studies have demonstrated EGCG involvement in antioxidative, antiinflammatory, and anticarcinogenic mechanisms (Cabrera et al., 2006; Singh et al., 2011). Most of these functions are thought to be attributable to the presence of a galloyl moiety at the C-3 position. In addition, EGCG has been shown to have positive effects on both plasma cholesterol reduction (Chan et al., 1999; Hasegawa et al., 2003; Murase et al., 2002) and liver cholesterol accumulation (Alshatwi et al., 2011; Muramatsu et al., 1986; Yang and Koo, 1997) during high fat diet. It is generally established that EGCG interferes with micellar solubilization of cholesterol thus resulting in decreased intestinal absorption, increased fecal excretion and reduced cholesterol synthesis (Ikeda et al., 1992; Koo and Noh, 2007; Raederstorff et al., 2003; Wang et al., 2006). Evidences also suggest that EGCG may inhibit lipogenesis by suppressing expression of lipogenic genes (Kao et al., 2000; Moon et al., 2007). Moreover, the low toxicity during treatments has made EGCG an ideal candidate for use as a therapeutic agent in steatotic mice (Fiorini et al., 2005). EGCG treatment has also been demonstrated to alter the expression of genes related to cholesterol metabolism in human hepatoma cells (Goto et al., 2011) and to modulate the hepatic cholesterol 7alpha-hydroxylase (CYP7A1) at transcriptional level in HepG2 cells (Lee et al., 2008). EGCG was also revealed as a potent in vitro inhibitor of squalene epoxidase (Abe et al., 2000) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoAR) (Cuccioloni et al., 2011), two key enzymes in the cholesterol biosynthetic pathway. HMGCoAR catalyzes the conversion of HMGCoA to mevalonate and is considered a rate-regulating enzyme in the cholesterol synthesis. HMGCoAR regulates several pathways within animal cells, mainly cholesterol biosynthesis. Recently, it has been demonstrated that cholesterol-independent compounds of the HMGCoAR pathway are involved in different mechanisms such as protein isoprenylation. Inhibition of HMGCoAR activity during development results in prenylation-dependent germ cell (PGC) migration disruption in zebrafish (Thorpe et al., 2004) and also in other models (Santos

Abbreviations: ABCA1, ATP-binding cassette transporter A1; BW, body weight; CYP7A1, cholesterol 7alpha-hydroxylase; EGCG, Epigallocatechin gallate; HMGCoAR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCoAR-a, 3-hydroxy-3-methylglutaryl-CoA reductase isoform A; HMGCoAR-b, 3-hydroxy-3-methylglutaryl-CoA reductase isoform B; LXR, liver X receptor; MS-222, 3-aminobenzoic acid ethyl ester; NADPH, nicotinamide adenine dinucleotide phosphate; ORF, open reading frame; PPM, part per million; RT-PCR, real-time PCR; SEM, standard error of the mean; Srebf1, Sterol regulatory element-binding transcription factor 1; Srebf2, Sterol regulatory element-binding transcription factor 2; SREBP, Sterol regulatory element binding protein; SSD, sterol-sensing domain.

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and Lehmann, 2004; Van Doren et al., 1998). Furthermore, the HMGCoAR pathway (specifically due to non-cholesterol dependent processes) is involved in developmental vascular stability; in fact inhibition of HMGCoAR induces cerebral hemorrhage in developing zebrafish (Eisa-Beygi et al., 2012). In this context, EGCG was found to potently inhibit the in vitro activity of HMGCoAR by competitively binding to the NADPH binding site of the enzyme (Cuccioloni et al., 2011). Little is known, however, about the in vivo effects of EGCG on gene expression of enzymes related to lipid metabolism (Wolfram et al., 2006; Yasui et al., 2012). In this context, it has been suggested that HMGCoAR mRNA levels may be reduced by several factors, including exposure to procaine (Xu et al., 2003), fenofibrate (Guo et al., 2001) and cerivastatin (Estey et al., 2008); on the contrary, HMGCoAR transcription can be increased by pathological conditions, such as tumors (Hentosh et al., 2001).

In order to further clarify the recently suggested functional interplay between EGCG and HMGCoAR, this work aimed to investigate the effects of short-term in vivo exposure to EGCG on hepatic HMGCoAR gene expression using goldfish (*Carassius auratus*) as a model. In this regard, evidences have more recently emerged that teleost fish can be considered as excellent models of vertebrate lipid metabolism (Anderson et al., 2011; Holtta-Vuori et al., 2010). Our studies were also undertaken to both characterize HMGCoAR transcript and identify its mRNA tissue distribution in goldfish.

2. Materials and methods

2.1. Fish and experimental design

Sexually immature goldfish (*C. auratus*), ranging in size from 7 to 9 cm in length, were provided by a commercial fish farm (COF, Bologna, Italy). Fish were acclimated for 2 weeks in 50-L glass aquaria with constant aeration and natural photoperiod (Palermo et al., 2008a). Water quality parameters were monitored every 2 days, showing the following values: pH = 7.8, O_2 = 5.5–7 ppm and temperature = 21–23 °C; the level of nitrites (NO^{2-}) and ammonia (NH_3) were undetectable. Fish were fed a commercial diet once a day during the acclimation period (Tetra Werke, Germany). Following the acclimation, 120 fish were randomly divided into 4 control- and 8 (according to the number of time points) treatment-tanks each consisting of 10 fish.

On the day of the experiment, fish were lightly anesthetized using 3-aminobenzoic acid ethyl ester (MS-222; Sigma; 0.1 g L^{-1}), individually weighted and intraperitoneally injected [i.p. 10 μL g^{-1}; (Pomatto et al., 2011)] with saline solution (0.75% NaCl) or EGCG (6 μg g^{-1} BW or 9 μg g^{-1} BW respectively) dissolved in saline solution. EGCG concentrations were chosen according to the study of Ogata et al. (2004) that demonstrated a peak plasma EGCG concentration of about 6.5 μg/mL 3 h after i.p. injection of 12.5 mg EGCG/Kg BW in goldfish. In addition, EGCG concentrations in the range of 0.3–7.5 μM were found to inhibit HMGCoAR activity in vitro (Cuccioloni et al., 2011) and to modulate lipogenesis in vivo (Hirsova et al., 2012; Kao et al., 2000). Fish were sampled at 0, 30, 90 or 180 min post-injection, and at each time point, fish (n = 10) from control and treatment groups respectively were anesthetized with MS 222 within 5 min after capture, and blood was immediately collected into freshly heparinized tubes and stored on ice until processed. After centrifugation (1500 *g* for 15 min at 4 °C), plasma was frozen on dry ice and stored at −70 °C until assay. Nine tissues (testis, kidney, hearth, muscle, intestine, gill, ovary, brain, liver) were harvested and rapidly frozen in liquid N_2 and stored at −70 °C for molecular biology analysis. Animal manipulation was performed according to the recommendations of the University Ethical Committee, to the European Union directive (2010/63/EU) for animal experiments and under the supervision of the authorized investigators.

2.2. Amplification of goldfish HMGCoAR cDNA

Total RNA was extracted from 100 mg of liver tissues using Trizol Reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. DNase digestion (2 U, 30 min, 37 °C; Ambion, Austin, TX) was performed to eliminate genomic DNA contamination. RNA concentration and purity were assessed spectrophotometrically at absorbance of 260/280 nm, and the integrity was confirmed by electrophoresis through 1% agarose gels stained with ethidium bromide. The complementary DNA (cDNA) was synthesized from 4 μg of total RNA in 20 μL of total volume reaction using random hexamers (50 ng μL^{-1}) and 200 U of SuperScript™ III RT according to the manufacturer's instruction (Invitrogen Life Technologies, Milan, Italy). A polymerase chain reaction was performed with degenerate primers, designed from a multiple alignment for highly conserved sequences of HMGCoAR [*Homo sapiens*, accession no. AAH33692; *Rattus norvegicus*; accession no. NP_037266.2; *Gallus gallus*, accession no. BAD01519; *Dicentrarchus labrax* (sea bass), accession no. AAR02862; *Danio rerio*, accession no. NP_001073446 (HMGCoAR-a) and NP_001014314 (HMGCoAR-b)] using CODEHOP (http://blocks.fhcrc.org/codehop.html). These degenerated primers (forward: 5′-CCATCTG(C/T) ATGATGTCCA-3′; reverse: 5′-GACATGCA(A/G)CCAAAGCA-3′) delimited a region of 517 bp. Two microliters of cDNA was amplified using a PCR Master mix 2× (Fermentas) in a final volume of 25 μL. Cycling parameters were as follows: 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min, with a final extension step of 72 °C for 10 min. The final PCR products were separated by electrophoresis in

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  3 aatatccggatttgttggctggactttgattgccccaaattggaggagctaatcttaagc
    N  I  R  I  C  W  L  D  F  D  C  P  K  L  E  E  L  I  L  S
 63 agtgatatcattatcctgacaatcacaagatgcatagcgatcgtctatatttactttcag
    S  D  I  I  I  L  T  I  T  R  C  I  A  I  V  Y  I  Y  F  Q
123 ttccaaaatcttcgacagttaggatctaaatacatattgggcatcgcaggacttttcaca
    F  Q  N  L  R  Q  L  G  S  K  Y  I  L  G  I  A  G  L  F  T
183 atattctccagttttgtgttcagcacagttgtgattcacttcctggacaaggagctgaca
    I  F  S  S  F  V  F  S  T  V  V  I  H  F  L  D  K  E  L  T
243 ggcctcaatgaggccttgccattctttctgctgttgattgacctctccaaagcatgtgct
    G  L  N  E  A  L  P  F  F  L  L  L  I  D  L  S  K  A  C  A
303 ctggccaaatttgcgttgagttcaaactcacaggatgaggtgagagagaatattgccaaa
    L  A  K  F  A  L  S  S  N  S  Q  D  E  V  R  E  N  I  A  K
363 ggaatggctgttttaggacccacctttactcttgatgcccttgtggagtgcctggtgatc
    G  M  A  V  L  G  P  T  F  T  L  D  A  L  V  E  C  L  V  I
423 ggtgtggggacaatgtcaggtgtgcgacagcttgagatcatgtgctgctttggctgcatg
    G  V  G  T  M  S  G  V  R  Q  L  E  I  M  C  C  F  G  C  M
483 tca 485
    S
```

Fig. 1. Nucleotide and deduced amino acid sequences of the partial putative HMGCoAR open reading frame identified in *Carassius auratus* using the ORF Finder software. The identified starting triplets ATG are underlined and highlighted in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)