Retinoid X Receptor α Regulates Glutathione Homeostasis and Xenobiotic Detoxification Processes in Mouse Liver


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ABSTRACT

Retinoid X receptor α (RXRα) plays a pivotal role in regulating liver metabolism. RXRα-mediated gene expression involved in amino acid metabolism was examined using the NIA Mouse 15K cDNA microarray containing 15,000 different expressed sequence tags. Seven amino acid metabolic genes, three of which encode enzymes involved in phase II detoxification process, were identified as RXRα which encode enzymes involved in phase II detoxification process. Seven amino acid metabolic genes, three of which encode enzymes involved in phase II detoxification process, were identified as RXRα. Among these genes, N-acetyl-p-benzoquinone imine (PXR), pregnane X receptor.

RXR regulates many fundamental biological processes, such as reproduction, cell differentiation, bone development, hematopoiesis, and pattern formation during embryogenesis (Mangelsdorf and Evans, 1995). Three distinct RXR genes (RXRα, -β, and -γ) have been characterized in vertebrates. Gene knockout studies have been conducted on all three RXR genes in mouse germ lines with various results. Although mice missing both RXRβ and RXRγ are viable (Krezel et al., 1996), RXRα-null mutants are lethally developed midway in embryonic development because of defects in cardiac development (Kastner et al., 1994). To address the role of RXRα in liver physiology of adult mice, a mutant line with RXRα deficiency, specifically in hepatocytes, was generated by using cre/lox-mediated recombination (Wan et al., 2000a). Fatty acid, cholesterol, carbohydrate, and xenobiotic metabolic pathways mediated by RXRα are compromised in the absence of hepatic RXRα (Cai et al., 2000; Wan et al., 2000a,b, 2003). However, the role of RXRα in regulating glutathione (GSH) homeostasis remains unclear.

Xenobiotic metabolism consists of two major steps in vivo. The first is bioactivation or biotransformation mediated through phase I enzymes such as cytochrome P450 monooxygenases. The second involves conjugation, primarily detoxification, mediated by phase II enzymes, including glutathione S-transferase (GST), UDP-glucuronosyltransferases, and sulfotransferases. Nonetheless, the first step of the detoxification process can paradoxically generate electrophiles or nucleophiles that are often carcinogenic or toxic. Examples are metabolism of nitrosamines, acetaminophen (APAP), CCl4, and aflatoxin by cytochrome P450 enzymes (Smith et al., 2003).

AABBREVIATIONS: RXR, retinoid X receptor; GSH, glutathione; GST, glutathione S-transferase; GPX, glutathione peroxidase; APAP, acetaminophen; CAR, constitutive androstane receptor; GCLC, glutamate-cysteine ligase catalytic subunit; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; t-BOOH, t-butylhydroperoxide; NAPQI, N-acetyl-p-benzoquinone imine; PXR, pregnane X receptor.
metabolism, the overall results illustrate a mechanistic role of GSH synthesis and utilization is unclear. It has yet to be defined whether these effects are the direct results of physiological functions of vitamin A or are caused by one or more secondary ramifications of excessive retinoids.

We hypothesized that RXRα regulates the expression of certain key genes involved in GSH synthesis and utilization, which in turn affect the cellular level of GSH. We further postulated that phase I and II detoxification processes could respond differently to xenobiotics in wild-type and hepatocyte RXRα-deficient mice. Herein, we show that in hepatocyte RXRα-deficient mice, the down-regulation of glutamate-cysteine ligase catalytic subunit (GCLC) significantly reduces the level of GSH in the liver, whereas the decrease in the phase I process of drug metabolism renders resistance to APAP-induced liver injury. In addition to the identification of novel liver RXRα target genes in amino acid synthesis and metabolism, the overall results illustrate a mechanistic role for RXRα in mediating phase I and II detoxification processes.

Materials and Methods

Mice. The cre-mediated tissue-specific knockout mice used in the current study result in deletion of exon 4 of the RXRα gene in the hepatocytes. Exon 4 encodes the major portion of the DNA binding domain, which is essential for RXRα function. Hepatocyte-specific mutation of the RXRα gene was achieved by crossing the albumin-cre transgene against the floxed RXRα allele. The mutated RXRα protein no longer has activity (Wan et al., 2000a). The genotypes of the control and mutant mice are cre−/−RXRαlox/lox and cre+/−RXRαlox/lox, respectively. In the absence of cre-mediated recombination, the conditional flox allele is functionally identical to the wild-type allele (Chen et al., 1998). The presence of cre alone does not cause phenotypic change (Postic et al., 1999). The breeding method used in the current study (i.e., crossing the cre+/−RXRαlox/lox with cre−/−RXRαlox/lox) produced an equal number of wild type and mutant mice for the study. Age-matched male mice were kept (two to three mice/cage) in plastic microisolator cages at 22°C with a 12-h/12-h light/dark cycle. Free access to food and water was provided throughout the entire feeding period. By the end of 10 to 12 weeks, mice were sacrificed and their livers were immediately excised, weighed, frozen in liquid nitrogen, and kept at −80°C until use. For APAP studies, animals were treated with APAP (500 mg/kg, i.p.) for 24 h. Blood samples were collected from the tail veins for ALT analysis. Animals were sacrificed, and liver samples were fixed in 10% formalin and stained with hematoxylin and eosin for histological analysis.

Microarray Analysis. Messenger RNAs were isolated using Oligotex mRNA mini kits (QIAGEN, Valencia, CA). Five micrograms of mRNA from each sample was used for each labeling and hybridization. Cy3 and Cy5 fluorescent-dye labeled mRNAs were hybridized against a National Institute on Aging 15K mouse cDNA chip, which contains ~5,000 different cDNAs from the National Institute on Aging, Intramural Research Program, Howard Hughes Medical Institute/Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT; http://info.med.yale.edu/wmkeck/dna_arrays.htm). Clones showing differential expression in hepatocyte RXRα-deficient mice were compared with wild-type mice, then were further studied by running a blast search against the nucleotide sequence database in GenBank.

Northern-Blotting Analysis. Samples used for the Northern-blotting assay were from the same tissues used for the microarray analysis. Total liver RNA was extracted by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). RNA concentration was determined spectrophotometrically. Twenty micrograms of total RNA was separated by electrophoresis in a denaturing 1.2% (w/v) agarose gel containing 2.2 M formaldehyde. Equal loading per lane was assessed by ethidium bromide staining and hybridization with β-actin cDNA probe. RNA was transferred to a nylon membrane by capillary blotting in 10× standard saline citrate and cross-linked by UV irradiation. Probe labeling and hybridization were performed as described previously (Wan et al., 2003). The fold differential expression was determined by densitometry analysis.

Western-Blotting Analysis. Proteins (50 μg) from the mouse liver homogenates were size-fractionated on a 12% SDS-PAGE gel (GCLC and GSTα) or a 8% native polyacrylamide gel (GPX1). Separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) for 1 h in 250 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% methanol. Rabbit polyclonal antibodies against GPX1 (gift from Dr. Larry Oberley); GSTα (Upstate Biotechnology, Lake Placid, NY), and GCLC (NeoMarkers, Fremont, CA) were used for the immunoblotting. Goat anti-rabbit polyclonal antibody horseradish peroxidase-conjugated (Cell signal, Beverly, MA) was used as the second antibody. The membranes were then subjected to chemiluminescence detection using luminal according to the manufacturer’s instruction (Amersham Biosciences, Piscataway, NJ). The membranes were stripped and re-probed with an anti-β-actin monoclonal antibody (Sigma, St. Louis, MO). The difference in protein levels was determined by densitometry analysis.

ALT Assay. Plasma alanine aminotransferase activity was determined spectrophotometrically with an ALT assay (Diagnostic Chemicals Limited, Charlottetown, PE, Canada) according to the manufacturer’s instructions. Briefly, 200 μl of serum was added to 3 ml of reagent. The blend was incubated at 37°C for 3 min and then assayed for the decrease in absorbance at 340 nm. The activity of ALT was expressed as the number of international units per liter based on the following calculation. Alanine aminotransferase units/liter = [ΔA/min × assay volume (milliliters)] / [sample volume (milliliters)]

Measurement of GSH Levels, Cysteine Levels, and GSH Synthetic Capacity. GSH level was determined by the recycling method (Tietze, 1969). The level of cysteine was measured by a spectrophotometric method (Gaultier, 1967). GSH synthesis rate (in nanomoles of GSH synthesized per minute per milligram of protein) was determined using monochlorobimane as described previously (Huang et al., 1998).

Oxidative Stress Analysis. Hepatocytes were isolated from 10- to 12-week-old wild-type and hepatocyte RXRα-deficient mice by a two-step perfusion with collagenase (Marc et al., 2000). Cells were cultured to form a monolayer on collagen-coated six-well culture plates in 2 ml of culture medium for 2 h at 37°C in an atmosphere of 95% humidity and 5% CO2. The hepatocytes were washed with phosphate-buffered saline and then incubated in the minimal essential medium either with or without 0.01 or 0.1 mM l-butyrohydroper-
oxide (t-BOOH) for 3 h. Cell viability was defined using the index of lactate dehydrogenase (LDH) enzyme leakage from damaged cells, which was expressed as a percentage of total cellular activity. LDH activity was measured by a toxicity assay kit (TOX-7; Sigma, St. Louis, MO) according to manufacturer’s instruction.

**Statistical Analysis.** Data are given as mean ± S.D. Statistical analysis was performed using Student’s t test. Significance was defined by p < 0.05.

**Results**

**RXRα Regulates the Expression of Genes Involved in GSH Synthesis and Utilization.** To examine the effect of RXRα on hepatic gene expression, mRNAs from livers of 3-month-old wild-type and hepatocyte RXRα-deficient male mice were hybridized to NIA 15k mouse cDNA chips. Differences in gene expression profiles were determined by comparing the Cy3 and Cy5 signal strength of each spot between two samples. Consistent results were obtained from three replicates of different mice pairs. Among the 15,000 differential expressed sequence tags presented on the NIA 15k cDNA microarray, 280 cDNA fragments showed either higher or lower expression levels in hepatocyte RXRα-deficient mice than in wild-type ones. The highest up-regulation and lowest down-regulation were 6.4- and 0.17-fold, respectively. Most of these genes were found to have 2- to 3-fold up-regulation or down-regulation, blast search of these 280 expressed sequence tags against the nucleotide database in the GenBank revealed that 146 of them were indistinguishable from identified gene sequences (data not shown). Among the 146 identified genes, seven of them encoded enzymes involved in amino acid metabolism (Table 1); of these, two were up-regulated (2- to 3.3-fold) and five were down-regulated (0.33- to 0.52-fold) in hepatocyte RXRα-deficient mice. None of them had previously been identified as retinoid-modulated genes. Three genes involved in GSH synthesis and utilization were identified in the current study. GCLC, GST, and GPX1 were all down-regulated approximately 3-fold by the deficiency in RXRα. The GCLC enzyme functions in the biosynthetic pathway for GSH, whereas enzymes of GST and GPX1 catalyze the utilization of GSH (Fig. 1A). The differential expression of these genes in livers between the wild-type and hepatocyte RXRα-deficient mice was subsequently confirmed by Northern-blot hybridization (Fig. 1B). GSTμ, an important gene encoding an enzyme of phase II detoxification, was identified as an RXRα target gene in the current study. The down-regulation of the mRNA levels of GCLC and GST μ leads to the proportionally reduced protein concentration in the liver of hepatocyte RXRα-deficient mice. However, the Western blot showed greater -fold change in GPX1 protein levels than the fold change of its mRNA levels determined by Northern-blot analysis between wild-type and hepatocyte-RXRα-deficient mice, suggesting the presence of post-transcriptional regulation (Fig. 1C). We also examined other genes listed in Fig. 1A that were not included in microarray analysis. The expression of cystathionine γ-lyase, glutamate-cysteine ligase modifier subunit, glutathione synthetase, and glutathione reductase seem not to be modulated by RXRα (data not shown).

**RXRα Regulates the Liver GSH Level and Affects Hepatocyte Susceptibility to Oxidative Stress.** The GSH level and GSH synthesis rate in livers of both wild-type and hepatocyte RXRα-deficient mice were analyzed. The down-regulation of GCLC in hepatocyte RXRα-deficient mice leads to 40% and 45% reductions in GSH synthesis rate and GSH level, respectively (Table 2). The abnormal hepatocyte GSH homeostasis was probably not caused by precursor limitation, because no significant change in hepatic cysteine level was observed in RXRα-deficient mice. This suggests that the 3-fold reduction of GCLC gene expression contributed to the marked decrease in GSH synthesis and GSH level observed in the hepatocyte RXRα-deficient mice.

To test whether the level of RXRα-regulated liver GSH influences the susceptibility of hepatocytes to oxidative stress, t-BOOH was used. This tested the oxidative tolerance in primary hepatocytes of wild-type and RXRα-deficient mice. Our result showed that GSH-diminished hepatocytes from RXRα-deficient mice were more susceptible to t-BOOH than those from wild-type mice (Fig. 2). Even at lower t-BOOH concentrations (0.01 mM), the viability of hepatocytes from RXRα-deficient mice decreased dramatically to about 40% within 3 h, whereas that from wild-type mice remained as high as 80% or better. This corresponds to a lower GSH level and reduced expression of GCLC and GSTμ in the liver of hepatocyte RXRα-deficient mice, suggesting that the reduction of GSH could lead to oxidation-induced toxicity.

**RXRα Mediates APAP-Induced Hepatotoxicity via Regulating Cytochrome P450 Enzymes.** Because GSH is required for conjugation and excretion of APAP metabolites, and because GSH depletion is closely associated with APAP induced liver injury, we expected that hepatocyte RXRα-deficient mice might be more sensitive to APAP toxicity than wild-type mice. Animals injected with APAP (i.p., 500 mg/kg) were sacrificed after 24 h. Liver histology and serum levels of ALT were examined. Contrary to our prediction, whereas massive centrilobular necrosis was observed in the liver of wild-type mice, liver morphology was normal in the hepatocyte RXRα-deficient mice (Fig. 3A). Control wild-type and hepatocyte RXRα-deficient animals injected with vehicle displayed no liver injury (data not shown). Levels of the liver enzyme ALT were also significantly higher in the wild-type mice than in the hepatocyte RXRα-deficient mice (Fig. 3B).

APAP is converted to N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome P450 enzymes in vivo. Because NAPQI is highly toxic at the phase I detoxification step, we next tested whether the protection of APAP-induced hepatotoxicity in RXRα-deficient mice was caused by the suppression of the expression of phase I genes that encode enzymes for APAP metabolism. Liver samples were collected from mice given APAP (i.p., 500 mg/kg) for 2 h to avoid the complications related to the massive necrosis noted at later stages. The expression of CYP1A2, CYP2E1, and CYP3A11 were analyzed by Northern blot hybridization. CYP2E1 remained unchanged, but CYP1A2 and CYP3A11 were up-

**TABLE 1**

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<tr>
<td>BG088553</td>
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<td>(S)-Adenosylhomocysteine hydrolase</td>
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<td>BG076460</td>
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<td>Cysteine dioxygenase 1</td>
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<tr>
<td>BG065030</td>
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regulated in wild-type mice, whereas they were down-regulated in hepatocyte RXRα-deficient mice by the APAP treatment (Fig. 4). CYP1A2 has been found to be the preferred form in catalyzing APAP at doses higher than 400 mg/kg (Snawder et al., 1994). This finding suggests that down-regulation of cytochrome P450 genes might be sufficient to protect RXRα-deficient mice from the hepatotoxic effect of APAP even when the GSH level is reduced in the liver.

**Discussion**

The significance of the data presented here is 2-fold. First, RXRα regulates the expression of GCLC, GSTμ, and GPX1,
genes encoding enzymes involved in the phase II detoxification process. Down-regulation of GCLC seems to be the direct mechanism for reducing the rate of GSH synthesis and the reduced GSH levels observed in the livers of hepatocyte RXRα-deficient mice. Second, deficiency in hepatocyte RXRα plays a protective role in defending xenobiotic-stimulated hepatotoxicity. Hepatocyte RXRα-deficient mice are resistant to APAP-induced liver injury because of a decreased expression of the cytochrome P450 genes in the absence of RXRα. Taken together, our results demonstrate that RXRα actively mediates both phase I and phase II detoxification processes. The mechanistic scheme to illustrate the function of RXRα in these processes is shown in Fig. 5.

Deficiency in RXRα results in reduced rates of GSH synthesis and levels of GSH in mouse liver, presumably because of the 3-fold down-regulation of the GCLC gene expression. Glutamate-cysteine ligase catalyzes the rate-limiting step in

<table>
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<th>Hepatic Levels</th>
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<th>RXRα -/-</th>
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<tr>
<td>GSH (nmol/mg of protein)</td>
<td>35.84 ± 2.05</td>
<td>19.65 ± 1.88*</td>
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<tr>
<td>Cysteine (nmol/mg of protein)</td>
<td>1.70 ± 0.13</td>
<td>1.38 ± 0.11</td>
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<tr>
<td>GSH synthesis rate (nmol/mg/min)</td>
<td>2.45 ± 0.36</td>
<td>1.51 ± 0.13*</td>
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* P < 0.05 versus wild-type controls by unpaired t test.
GSH biosynthesis. Lack of its activity, caused by either the catalytic or the modifier subunit, has been found to be tightly associated with reduced GSH levels. This severely impairs cell antioxidation ability. Reduced GSH levels were detected in liver tissues of hepatocyte RXRα-deficient mice because of the decreased GSH synthesis rate. Because expression of the glutamate-cysteine ligase modifier subunit and glutathione synthetase genes was unchanged, the primary factor that limited GSH synthesis in the RXRα-deficient mice could have been the down-regulation of GCLC gene expression. GSH plays an important role in human hepatocyte regeneration (Huang et al., 2001). Using a different model system, the liver regeneration capacity is impaired in RXRα-deficient mice (Imai et al., 2001). This abnormal liver regeneration might be a result of diminished GSH levels such as those observed in the current study. The reduced GSH level could also be a key reason why hepatocytes from RXRα-deficient mice are more vulnerable to t-BOOHH–induced oxidative stress. Oxidative stress and a significant reduction in GSH have been reported in Parkinson’s disease (Merad-Boudia et al., 1998), lung inflammation (Rahman and MacNee, 2000), and familial Alzheimer’s disease (Cecchi et al., 1999). The role of RXRα-mediated GSH synthesis in protecting against oxidative stress may provide new insights into potential mechanisms involved in the development and amelioration of these diseases.

Although the liver GSH level is lower, hepatocyte RXRα-deficient mice are resistant to APAP induced hepatotoxicity because of the reduced expression of cytochrome P450 genes. Our previous data and that in the current study demonstrated that the xenobiotic metabolic cytochrome P450 genes are either down-regulated (CYP2A5, CYP2B10, and CYP3A11) or remained unchanged (CYP1A1, CYP1A2, CYP2D6, and CYP2E1) in the hepatocyte RXRα-deficient mice. However, APAP administration up-regulates genes for its own metabolism, CYP1A2 and CYP3A11, in wild-type mice but down-regulates the same genes in RXRα-deficient mice. This potentially reduces the production of toxic NAPQI in hepatocyte RXRα-deficient mice. CYP2E1 is the major enzyme that metabolizes APAP at a lower dose (150 mg/kg), whereas at a higher dose (400 mg/kg), CYP1A2 becomes the key enzyme (Snawder et al., 1994). Consistent with our findings, Zhang et al. (2002) showed that CYP1A2, CYP3A11, and GSTπ were up-regulated within 2 h after APAP administration, and this was the primary reason for APAP-induced liver injury in wild-type mice. Cytochrome P450 inducers increase the toxicity of APAP. Pretreatment with ethanol, an inducer of CYP2E and CYP3A, promotes much more severe tissue damage after APAP administration (Sinclair et al., 1998). A similar result was also obtained by pretreatment with phenobarbital, a well known inducer of CYP2B and CYP3A (Tuntaterdtum et al., 1993). On the other hand, animals pretreated with cytochrome P450 inhibitors, such as α-bederin and a sublethal dose of bacterial endotoxin, were more tolerant to APAP-induced toxicity (Liu et al., 2000). Accordingly, the inhibition of cytochrome P450 genes by APAP in the hepatocyte RXRα-deficient mice resulted in resistance to APAP-induced liver injury as observed in the present report.

The down-regulation of GSTα in the liver of the hepatocyte RXRα-deficient mice may substantially impair the detoxification functions and defense mechanisms in these animals. GST enzymes detoxify a broad range of endobiotic and xenobiotic chemicals by covalently linking GSH to a hydrophobic substrate, forming less reactive and more polar GSH conjugates. This reaction, termed the phase II detoxification process, is considered one of the major biological defense mechanisms against toxic compounds. APAP is also detoxified by UDP-glucuronosyltransferases. Our unpublished preliminary data indicated that the expression of the UGT1A1 gene.
is slightly down-regulated when RXRα is deficient. This may also contribute to the overall reduced detoxification rate of APAP in the liver of the hepatocyte RXRα-deficient mice. GSTM null polymorphism has been found to be associated with elevated susceptibility to endometriosis and ovarian cancer (Baxter et al., 2001). Total GST activity, particularly GSTM enzyme abundance, is significantly reduced in liver tumors (Zhou et al., 1997). Thus, responses identified in the current study provide a potential mechanism for modulating anti-cancerous capability via RXRα-regulated GSTM gene expression.

Post-transcriptional regulation of GPX1 by selenium has been widely reported (Chang and Reddy, 1991; Bermano et al., 1995; Moriarty et al., 1998). GPX1 contains selenium at its active site as a selenocysteine moiety. Selenium-deficient diet leads to a reduction in GPX1 activity of ~50-fold and a decrease in cytosol GPX1 mRNA abundance by approximately 20-fold but has little affect on nuclear pre-mRNA level in rat hepatocytes (Moriarty et al., 1998). Nuclear run-on assay showed that selenium availability has no impact on GPX1 transcription rate in the livers of mice (Toyoda et al., 1990) and rats (Chang and Reddy, 1991). The mechanism of marked reduction in the GPX1 protein level in the hepatocyte RXRα-deficient mice is unclear. Further study is needed to define whether RXRα-deficiency reduces translational rate of GPX1 or causes the instability of GPX1 protein.

RXRs are an obligate partner for CAR and PXR/steroid X receptor, two major nuclear receptors involved in drug metabolism. CAR and PXR have their unique and redundant role. In the absence of RXRα, the function of both CAR and PXR is affected. Therefore, genes that are regulated by both CAR and PXR can be found in the animal model used in the current study. The RXRα-regulated gene expression discussed herein may happen either directly or indirectly. To further study the mechanism of RXRα-mediated gene expression, we have been analyzing the promoter regions of these RXRα target genes. Putative nuclear receptor binding sites have been found. Whether or not these binding sites are responsible for RXRα-mediated regulation is currently under investigation.

The data presented here significantly contribute to the emerging understanding of nuclear receptor regulated drug metabolism and detoxification. We showed here that RXRα-promoted cytochrome P450 gene expression in response to xenobiotics was detrimental to liver tissues, whereas RXRα-induced GCLC and GSTM expression was crucial for maintaining GSH homeostasis and detoxification capacity. It can be concluded that RXRα-regulated gene expression for enzymes involved in phase I and II detoxification can modulate xenobiotic metabolism, preserve antioxidative capacity, and maintain defense mechanisms.

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