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Lycopene induces Retinoic Acid Receptor transcriptional activation in mice

by

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INTRODUCTION

Lycopene is a fat-soluble carotenoid and gives the red color to tomatoes, tomato products, watermelon, grapefruit and papaya. It has been reported to possess several health benefits. In the last decade, various studies, demonstrated that consumption of lycopene and tomatoes / tomato products is associated with the decreased risk of various diseases such as cancer, cardiovascular diseases and chronic inflammation. However, possible mechanism and metabolites underlying the biological effect of lycopene have not been fully understood. A possible mechanism is that biological metabolite of lycopene may activate nuclear hormone receptors in mammalian cells. Due to their similar chemical structure to β-carotene, pro-vitamin A (pro-vA) activity has been questioned. It is well known that metabolites of β-carotene like all-*trans*-retinoic acid (ATRA) initiate the transcriptional activation of retinoic acid receptor (RAR) pathways. Even though the biological importance of lycopene metabolites has been discussed, it is still unclear how these metabolites attribute to human health and certain pathways. The aim of this study was to investigate the potential of orally administered lycopene, potential lycopene metabolites, tomato extract and ATRA for the induction of the RAR pathways in a transgenic retinoic acid response-element (RARE)-reporter mouse system.

THEORETICAL BACKGROUND

Carotenoids

Carotenoids are known to be responsible for various bright colors in nature and belong to family of compounds over 600 fat-soluble plant pigments. Carotenoids are lipophilic molecules classified by structure as carotenes and xanthophylls. Among the more than 600 different carotenoids, there are well known carotenoids such as β-carotene, lycopene, lutein and zeaxanthin. In the last decade, numerous studies displayed the beneficial role of lycopene in various chronic diseases, cardiovascular diseases (CVD) and cancer.

Characteristics of lycopene

Lycopene is the red pigment abundantly found in tomato, rosehip, pink grapefruit, watermelon and papaya. 85% of daily lycopene intake is from tomato and tomato based products. Lycopene exist in high concentrations in foods like pasta sauce, tomato juice, ketchup, pizza sauce. It is an acyclic carotenoid and due to its 11 conjugated double bonds it has red color. It has no terminal β-ionic ring therefore lycopene has been accepted as non pro-vA precursor so far. Due to its apolar chemical structure, it is a highly lipophilic compound. This lipophilicity makes it easier to be transported in low-density lipoproteins (LDL). Therefore lycopene is suggested to display one of its crucial effects through the protection of LDL from oxidation although lycopene is present in LDL in much lower concentration than other lipophilic carotenoids.

Lycopene absorption, transportation and distribution

Carotenoids are absorbed from the diet along with the same way but distributed, accumulated, metabolized and excreted differently in the body. Process start with the carotenoid release from the food matrix by chewing and initial enzymatic digestion of the food in the mouth and followed by the uptake by intestinal mucosal cells. Then it continues with absorption, transport and cleavage of pro-vA carotenoids within the enterocyte to yield VA. The mechanism proceed with the tissue distribution, metabolism and recycling of carotenoids.

Carotenoids are combined in the form of complex in food matrixes with fibers, lipids and proteins. When the food undergoes three types of processes in the body; absorption, transportation and distribution, firstly, the nutrients are digested and released from the food matrix. When they are released, they come together with the lipids and bile salts to form micelles. Micellar solubilization is a prerequisite to their efficient passage into the lipid-rich membrane of intestinal mucosal cells. Micelles move to intestinal epithelia thus carotenoids can be

taken up, incorporated and dissolved in the lipid core of micelles. Once taken up to the enterocyte, pro-vA can be metabolized, utilized, re-secreted into the intestinal lumen. In the enterocyte they are incorporated into lipoproteins with triacylglycerol, apo-lipoproteins and they are called cyclomicrons. Cyclomicrons can enter the blood via lymph and transport carotenoids to the liver. The action of lipoprotein lipase of hepatic tissue impoverishes the chylomicron of triacylglycerols (triglycerides). Thus cyclomicrons degraded by lipoprotein lipase and carotenoids in cyclomicron remnants are delivered to the liver or may also be taken up by other organs. Carotenoids can be re-secreted as very low density lipoproteins (VLDL).

Lycopene is present in different geometrical isomers and they might contribute to different biological processes. It is known that in serum and tissue *cis*-lycopene is more than all-*trans*-lycopene. In contrast, tomato and tomato based products contain predominantly all-*trans*-lycopene. It was proposed that *cis*-lycopene is more bioavailable than *trans*-lycopene, most likely because of increased solubility in mixed micelles.

Tissue distributions of lycopene

Differences were found in the organ distribution of lycopene. It is present in high concentrations in testis when it is compared with other tissues like liver and adrenal glands.

Potential biological effects of lycopene

Antioxidant effect of lycopene

Free radicals are known to be one of the reasons in the pathogenesis of several chronic diseases. It is well known that oxidative stress contributes to the increased risk of cancer. Lycopene can function as antioxidants terminate the chain reaction before cellular components are damaged due to their ability to quench the singlet oxygen. Lycopene has been demonstrated to be the most potent antioxidant among the carotenoids.

Lycopene and cancer

Results of many studies have proposed that carotenoids may evolve biological processes independently from their antioxidant property. Lycopene is able to contribute modulation of many genes involved in biological processes. It has become an attractive topic of cancer research ever since its antioxidant capacity was proposed. Although lycopene has a detrimental effect on numerous type of cancer incidence, the direct connection between lycopene and cancer is not well established. Details of regulatory pathway remain unclear although observations suggest link between increased consumption of lycopene and decreased risk of cancer. There exist certain genes regulated by lycopene in cancer incidence. Insulin-like growth factors (IGFs) have crucial role in

carcinogenesis, possibly by elevating the risk of cellular transformation by enhancing cell turnover. It has been previously shown that increased risk of IGF levels associated with incidence of cancer.

Another biological structures influenced by lycopene treatments is gap junctions (GJ) which are intercellular channels. GJ are deficient in many human tumors and its restoration or up-regulation is associated with decreased proliferation. Lycopene can up-regulate the gap junctional gene connexin 43 (*Cx43*) thus it contributes gap junctional intercellular communication (GJC). Upregulation of *Cx43* bring about increases in GJC.

Lycopene, inflammation and cardiovascular diseases

There is an association between high lycopene levels and reductions in CVD incidence. It was suggested that, decreased oxidative modification of LDL may be one of the mechanisms by which lycopene may reduce the risk of CVD and atherosclerotic progression.

Retinoid metabolism

Retinoids are the substances compromising vitamin A (retinol) and its natural metabolites, retinaldehyde and retinoic acid (RA) as well as its synthetic derivatives. Those liposoluble vitamins have crucial tasks in many biological processes including fetal development, organogenesis, embryogenesis in addition to the regulation of several aspects of cell metabolism. More than 600 carotenoids have been found in nature and among them, less than 10% of them have pro-vA activity. The most well-known carotenoid is β-carotene since it serves as the major vitamin A (vA) precursor. It is known that enzymatic cleavage of β-carotene can occur either via excentric or central cleavage pathways due to absence or presence of antioxidants. It was demonstrated that enzymatic cleavage of β-carotene by β-carotene oxygenase 1 enzyme result in the formation of two retinal (RAL) molecules as a result of the central cleavage. Retinal molecules can either be reduced to retinol (ROL) subsequently by retinal reductase or oxidized to RA. Eccentric cleavage of β-carotene yields various apo-carotenals and they may be converted subsequently to RAL or oxidized to apo-carotenoic acids. After ROL formation it may be metabolized to retinoic acid.

Lycopene metabolism

In contrast to β-carotene metabolism, lycopene metabolism has not been fully described in biological systems therefore very little information is known about oxidative break down products of lycopene. Firstly, 5, 6-dihydroxy-5', 6'-dihydrolycopene was found in human plasma as a lycopene metabolite. Furthermore, 2, 6

cyclolycopene-1,5-diol A and B were determined as oxidative metabolites of lycopene present in human plasma.

Two carotenoid metabolizing enzymes, β-carotene oxygenase 1 and β-carotene oxygenase 2 (BCO1 and BCO2) may contribute the lycopene metabolism. Lycopene is partly cleaved by BCO1 with a lower affinity. BCO2 catalyzes the asymmetric cleavage of lycopene. BCO2 was cloned from mice and expressed in β-carotene or lycopene synthesizing *E.coli*. It was reported that BCO2 can significantly cleave lycopene. *In vitro* study demonstrated that lycopene is an inefficient substrate for BCO1. It is known that BCO1 and BCO2 are tissue specifically expressed to facilitate the retinoid signaling. It has been previously demonstrated that RA down-regulates the BCO1 mRNA level in chickens and rats. A study by Zaripheh et al. showed that lycopene is the inhibitor of BCO1 as well. It has been found that the *cis* isomer of lycopene can be metabolized into apo-10'-lycopenal by ferret BCO2 *in vitro*. All-*trans*-lycopene supplementation in ferrets caused the formation of apo-10'-lycopenol in the lung. They also demonstrated that apo-10'-lycopenal can be metabolized into apo-10'-lycopenoic acid (apo-10-lycac) or apo-10'-lycopenol. Apo-8'-lycopenal and apo-12'-lycopenal were identified in rat liver.

Nuclear hormone receptors

Nuclear hormone receptors (NHRs) are the superfamily of trancriptional regulators including steroid hormones, thyroid hormones, retinoids, vitamin D and orphan receptors. They play crucial roles in several biological process including development, proliferation, differentation, inflammation and homeostasis. They function as ligand dependent transcriptional factors and activate gene expression upon binding of respective ligands. NHRs are composed of multiple functional domains including weekly conserved NH2-terminal region (A/B), a conserved DNA binding domain (DBD) which is called region C, a linker D region and ligand binding domain called conserved E region. Hormone response elements are DNA sequences in target genes that can bind to hormone receptor resulting in transcriptional regulation. They are present in the regulatory region and located either close to the core promoter or in the enhancer regions several kilobases upstream of the transcriptional initiation site. There are different optimal HREs interactions as palindromes (Pal), inverted palindromes (IPs) or direct repeats. Most potent HRs for non-steroid receptors are configured ad DRs. DR5 is separated by 5 nucleotides and frequently present in RXR-RAR heterodimer.

Nuclear hormone receptors for retinoids

On the basis of structural homologies, NHRs have been divided into two subfamilies (One subfamily includes receptors for steroid hormones (glucocorticoids, progestins, androgens, estrogens, and mineralocorticoids) and the other consists of the 3,3',5-triiodo-L-thyronine (T3) receptor (TR), retinoic acid receptor (RAR), retinoid X receptor (RXR), and vitamin D3 receptor (VDR). Peroxisome prolifetor-activated receptors (PPARs) have features similar to the second subfamily of nuclear hormone receptors in that they form heterodimers with RXR. Nuclear hormone receptors act as transcription factors with appropriate ligand binding and participate in the regulation of target gene expression. Impacts of retinoids are mediated by nuclear hormone receptors including receptors for thyroid and steroid hormones, retinoids as well as vitamin D. It is known that retinoic acid is involved in the regulation of gene expression through the nuclear hormone receptor pathways, RAR and RXR with their respective α, β, δ subtypes coded by separate genes. RXR receptors have an affinity for 9-*cis* retinoic acid, whereas RAR receptors can be activated by all-*trans*-retinoic acid. RAR and RXR are able to make heterodimer and modulate the gene transcription after RA. They can also make RAR-RAR or RXR-RXR homodimers and recognize the DNA response elements in retinoid target genes.

In contrast to retinoic acids, little is known about the impact of lycopene and its metabolites on retinoic acid receptor pathways. In last decade lycopene attracted attention because of its similar chemical structure like β-carotene and its biological and physicochemical properties. However, no pro-vA activity has been found for lycopene.

Scope of the study

Vitamin A is essential for many physiological processes, for instance cell division, differentiation, eye vision, embryogenesis and organogenesis. No pro-VA activity has been found for lycopene. The objective of this study was to determine whether lycopene and lycopene metabolites might induce nuclear hormone receptor pathways activation via RAR-mediated signaling pathways in transgenic RARE—LUC mice like the known endogenous β-carotene metabolite and RAR-activator ATRA. We aimed to determine whether lycopene, lycopene metabolites or tomato extract might induce nuclear hormone receptor pathways. We also wanted to demonstrate the time and gender dependent effect of luciferase expression in RARE-Luc mice upon lycopene administration. We investigated the gene expression profiles of known genes involved in retinoid metabolism, namely metabolizing enzymes, retinoid transport proteins and retinoid target genes in lycopene supplemented mouse in comparison to control vehicle supplemented mouse.

MATERIALS AND METHODS

Chemicals

Beads enriched with lycopene contained 10% (w/v) synthetic lycopene (DSM, Kaiseraugst, Switzerland), control beadlets without lycopene contained modified food starch (30-60%), corn starch (10-30%), sucrose (10-30%), sodium ascorbate (1-5%) and dl-α-tocopherol (0.5-1.5%). The tomato extract (LycoRed, LYC-O-MATO 10 %, Israel) contained 10% lycopene, 1.5% natural tocopherols, 1% phytoene, 1% phytofluene, 0.2-0.45% β-carotene, 0.5% water and 69-74 % fatty acids and acylglycerols and 9-14 % phospholipids.

Experimental diets

Tomato extract and ATRA (BASF, Ludwigshafen, Germany) were given to the mice in separate experiments. Lycopene in beads and control beads (CTRL 2) were dissolved in water, whereas ATRA and tomato extract powder were dissolved in aqueous emulsifier solution (CTRL 1, 25% cremophor EL and 75% H_2O). Lycopene metabolites (apo-14-lycac and apo-10-lycac) were dissolved in DMSO. Therefore control group for these experiments were mouse group treated with DMSO and called CTRL 3.

Mice and Treatments

Retinoic acid response element luciferase construct (RARE-LUC) mice with a CD1 background genetically modified to express firefly luciferase under the control of RARE (retinoic acid response element) were kindly provided by Cgene AS (Cgene, Oslo, Norway). Validation of the RARE-LUC system was based on a previous study. The mice were housed in standard plastic cages at room temperature (20±2°C) and they had free access to both food and water. Standard pelleted laboratory mouse diet (Altromin, type VRF 1, Charles River, Budapest, H) was used with the following diet composition: crude nutrients 19 %, crude ash 7%, crude fat 4.5 %. Both female and male mice of 8-12 wk of age were studied. Single dose oral *gavage* of lycopene, tomato extract, ATRA and control vehicles were applied by sterilized stainless steel feeding needles 18 h before the luciferin injections and the subsequent bioluminescence imaging analyses. Since ATRA and lycopene were dissolved in different vehicle solution two different control groups were used. All mouse experiments were approved and conducted under the guidelines and with ethical approval for the use and care of laboratory animals at the University of Debrecen, Hungary.

Experimental design

Bioimaging experiments

An Andor IQ imaging system (Andor, Belfast, Great Britain), consisting of an Andor-ixon cooled charged coupled device (CCD) camera, housed in Unit-one (Birkerod, Denmark) black box and connected to a computer system, were utilized for data acquisition and analyses. For experiments 1, 2, 4, 5, 6 and 7 the mice were euthanized by cervical dislocation. Subsequently, testis, brain, lung, white adipose tissue (WAT), liver, kidney, spleen, small intestine (s. intestine), large intestine (l. intestine), psv (prostate-seminal vesicule) complex were rapidly excised and placed in tight light chamber for screening before freezing the organs at -80°C. The organs collected from the mice in experiment 2 were used for experiments 2, 8 and 9. All measurements were done in the morning between 9.00 - 11.00 h at different months of the year. The photon signals were quantified by Andor IQ 1.6. Programme. Luciferase expression was presented as integrated intensity / area. Data are presented as means ± SEM values of 4–6 different animals per treatment group. In all bioluminescence imaging experiments, 8-12 wk old mice were used and treated once by oral *gavage* 18 h before luciferin injections.

Organ specific expression based on bioluminescence imaging upon RAR, RXR activators and CTRL treatments (Experiment 1):

The rexinoids are the synthetic RXR ligands that bind and activate specifically to the RXRs. The rexinoid LG268 (also called LG 100268 or LG268) is a potent and specific ligand that binds solely to the RXRs, and not to the RARs. We tested the ability of LG268 to induce luciferase in the DR-5 luc mouse. As a control, we used CD271 (also called adapalene), a synthetic retinoid analog used in dermatological therapy (Differin®) with a higher selectivity for RARβ and γ. Experiments were performed upon CD271 (5 mg/kg bw), LG268 (5 mg/kg bw), CD271 (5 mg/kg bw) and LG268 (5 mg/kg bw) or control treatments. Control contains 25% aqueous cremophor solution. For this experiment, *ex vivo* organ analysis of bioluminescence imaging was conducted. Male mice (*n=6*, per treatment group) were treated 15 min before killing and further organ screening with 120 mg/kg D-luciferin (Bioscience, Budapest, H) via intra-peritoneal injections. Mice were euthanized by cervical dislocation. After sacrificing the mice, organs were collected for bioluminescence imaging and analyzed under CCD camera for bioluminescence signal.

Organ specific expression based on bioluminescence imaging upon lycopene, ATRA CTRL 1 and 2 (Experiment 2):

In the second experiment, we tested organ specific expression based on bioluminescence imaging upon lycopene (100 mg/kg bw), ATRA (50 mg/kg bw), CTRL 1 and 2. Lycopene-treatments (100 mg/kg bw.) were compared with control-bead treatments (CTRL 2), ATRA (50 mg/kg bw in aqueous cremophor solution) with the corresponding vehicle treatments (25% aqueous cremophor solution; CTRL 1). Lycopene was administered at 100 mg/kg of lycopene in 10%-lycopene containing beads resulting in 1000 mg/kg bw given, in comparison to CTRL 2 (1000 mg/kg bw) given by oral *gavage*. For *ex vivo* organ analysis, male mice (*n=6*, per treatment group) were treated 15 min before killing and further organ screening with 120 mg/kg D-luciferin via intra-peritoneal. Then the following organs were collected; testis, brain, spleen, lung, WAT, s. intestine, l. intestine and liver. Bioluminescence imaging was conducted as it is described previously.

Time dependent response of RARE-LUC mice to lycopene and ATRA treatments (experiment 3):

For a time dependent response (experiment 3) upon lycopene and ATRA treatment in RARE-LUC mice *in vivo* full body bioluminescence analysis was used. Firstly, the abdominal part of transgenic mice was shaved to acquire better bioluminescence signals prior to imaging. Male mice (*n=3*, per treatment group) were treated with 120 mg/kg D-luciferin via intra-peritoneal injections and 5 min later anesthetized by intra-peritoneal (10 mg/kg) nembutal (Sigma, Budapest, H) injection and 10 min later screened for whole body bioluminescence. *In vivo* imaging of RARE-LUC mice was measured at several time points following oral *gavage* of mice with lycopene (100 mg/kg bw), ATRA (50 mg/kg bw). Mice were imaged at baseline 0 h (this indicates mouse killing and further examination two minutes after lycopene-treatment, set as 0 h), just after lycopene supplementation and after 6, 12, 18, 24 and 48 h for total body luminescence.

Gender specific response of luciferase gene upon lycopene and bead treatment (experiment 4):

In the fourth experiment, female mice were used (*n=6*, per treatment group) and the same experimental design was followed as in experiment 1 and 2. Organ specific luciferase gene expression was compared in female animal groups upon lycopene (100 mg / kg), ATRA (50 mg / kg) and CTRL1 (cremophor) treatments. Just cremophor treated animal group (CTRL 1) was utilized as control group since no differences were observed between luminescence signal of CTRL 1 and CTRL 2 groups in previous experiments. 8-12 wk old mice groups were treated just one time 18 h before the bioluminescence imaging by oral gavage administration. For *ex vivo* organ analysis, female mice (*n=6*, per treatment group) were treated 15 min before killing and further organ

screening with 120 mg/kg D-luciferin via intra-peritoneal injections. We collected organs for bioluminescence imaging.

Organ specific expression of luciferase signal upon tomato extract treatments (experiment 5):

We treated the two different groups of male mice (*n=6,* per treatment group) with tomato extract in aqueous cremophor suspension and vehicle control (CTRL 1). The amount of tomato extract was adjusted to equal 100 mg lycopene/kg bw used in the previous mouse experiments. The experimental design was comparable as described in experiment 1, 2, and 4. For *ex vivo* organ analysis, male mice (*n=6,* per treatment group) were treated 15 min before killing and further organ screening with 120 mg/kg D-luciferin via intra-peritoneal injections. After dissection, we collected organs for bioluminescence imaging. 8-12 wk old male mice groups were used.

Organ specific expression of luciferase signal upon apo-10- lycac treatments (experiment 6):

In the sixth experiment, we tested organ specific expression, based on bioluminescence imaging upon apo-10-lycac (4 mg/kg bw), ATRA (50 mg/kg bw), CTRL 4 (DMSO) treatments in 8-10 wk old animals. Treatments were given by oral *gavage* administration. Male mice (*n=4*, per treatment group) were treated 15 min before killing and further organ screening with 120 mg/kg D-luciferin via intra-peritoneal injections. After dissection, we collected the organs for bioluminescence imaging.

Organ specific expression of luciferase signal upon apo-14-lycac treatments (experiment 7):

In the sixth experiment, we tested organ specific expression based on bioluminescence imaging upon apo-14-lycac (4.9 mg/kg bw), ATRA (50 mg/kg bw), CTRL 4 (DMSO) and CTRL 1 (cremophor) treatments. Treatments were given by oral *gavage*. 8-12 wk old animals were utilized. Male mice (*n=4*, per treatment group) were treated 15 min before killing and further organ screening with 120 mg/kg D-luciferin via intra-peritoneal injections. After sacrificing the mice, we collected organs for bioluminescence imaging.

Luciferase enzymatic assay

Luciferase Reporter Assay System (Bioscience, Budapest, Hungary) was utilized for the analysis of luciferase activity. Organs were harvested from RARE-LUC mice and stored at -80 ^{o}C for luciferase enzyme activity assay. Tissue homogenization and luciferase assay were conducted according to the manufacturer's instructions. Luciferase activity was measured and quantified using Victor 1420-Multilabel counter (Wallac, Per-form Hungaria KFT, H). Bradford assay was conducted for total protein analysis (experiment 5).

RNA isolation

Beside the bioimaging experiments, qRT-PCR (quantitative Real Time-Polymerase Chain Reaction) was conducted for the analysis of mRNA expression of carotenoid metabolizing enzymes, carotenoid transporter and retinoid target genes. The mice organs from experiment 1 were used for qRT-PCR analysis. RNA isolation was performed from mouse tissues by means of Tri® Reagent (Trizol) isolation manual technique. A tissue sample of ca. 100 mg is homogenized with QIAGEN Tissue Lyser in 1 ml of Trizol, using previously autoclaved QIAGEN metal beads. After 1 minute centrifugation (at 4 °C, 13000 rpm) of the samples, the supernatants are transferred into 1.5 ml Eppendorf tubes. 200 ml of chloroform is added to each sample, the samples are stirred thorought and incubated for 3 minutes at room temperature. After incubation the samples are centrifuged for 15 minutes (at 4 °C, 13000 rpm) and the upper aqueous phase is transferred into a new Eppendorf tube. 500 ml of isopropanol is added to the samples, stirred and incubated for 20 minutes at room temperature. After incubation the samples are centrifuged for 10 minutes (at 4 °C, 13000 rpm). The supernatant is discarded. 800 ml of 70 % ethanol is added, stirred shortly and centrifuged for 5 minutes (at 4 °C, 13000 rpm). The supernatant is discarded. RNA pellets are dried for 20-25 minutes in an Eppendorf concentrator 5301. 40 ml of nuclease free water (NFW) is added to each sample, stirred and incubated for 10 minutes at 65°C in a BIOSAN Dry Block Heating Thermostat. Samples are stirred briefly and cooled down on ice. The concentrations and purities of the RNA samples are measured by NanoDrop ND- 1000 Spectrophotometer. Samples were kept at -20 °C until further usage.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Two-step Taqman QRT-PCR was performed in order to quantify the mRNA expression level of the RARE-Luc mouse genes involved in the retinoid metabolism. Prior to PCR total RNA samples were reverse transcribed into cDNA by enzyme according to supplier's protocol under the following conditions: 10 min at 25°C, 120 min at 42°C, 5 min at 72°C and 10 min at 4°C (Applied Biosystems, 2720 Thermal Cycler). Following reaction mixture has been used. The previously obtained cDNA is amplified by the TaqDNA polymerase enzyme and it is measured quantitatively in order to determine the mRNA expression level of mouse tissue. qRT-PCR was performed by ABI PRISM 7900 sequence detection system as follows: 1 min at 94°C, followed by 40 cycles of 12 sec at 94°C and 30 sec at 60°C. Primers were ordered from Applied Biosystems (Applied Biosystem, Budapest, Hungary) for mouse and probe was from ABI (Life Technologies, Budapest, Hungary). mRNA levels were normalized to the level of cyclophilin expression, which served as an internal control for the amount of RNA used in each reaction. Relative mRNA levels have been calculated using the comparative threshold cycle

(CT) method. For data analysis, Sequence Detector Software was used. Gene targets were as following: *Bco-1*, *Bco-2*, Cytochrome P450 26B1 (*Cyp26b1)*, Cellular retinol binding protein-1 (*Crbp1), Cd36* and Retinoic acid receptor responder protein-2 (Rarres2). mRNA levels were normalized to the level of cyclophilin expression. Cycle threshold values above 40 were scored as under the limit of detection (UDL).

Statistical analysis

Statistical tests for comparison of means were performed using GraphPad Prism version 5. Values are represented as mean ± SEM. For time course experiment, repeated measure of 2-way ANOVA was used to evaluate time dependent changes. Differences between time points were compared with 2-sided Bonferroni's post-test. We also checked the time course experiment with AUC/time (Area Under Curve/time) analysis to find the highest peaks. We analyzed differences between ATRA and lycopene treatments with ROC (Receiver Operating Characteristic) analysis in the time course experiment. For the analysis of qRT-PCR (experiment 6), luciferase protein assay (experiment 5) and tomato extract treatment (experiment 4) student t-test was conducted. We used 1-way ANOVA for organ specific expression of male (experiment 1). 1-way ANOVA was followed by Bonferroni's post-test. We have checked the normality and equality distribution before t-tests and ANOVAs. Differences were considered significant at $*p < 0.05$ and $**p < 0.01$.

RESULTS

RARE response in transgenic RARE-Luc mouse

Organ specific expression of luciferase signal upon synthetic RAR and RXR ligands (experiment 1):

LG268 is a synthetic RXR ligand whereas CD271 is a synthetic RAR ligand. Mice were administered orally with CD271 (5mg/kg bw), LG268 (5mg/kg bw) or together, and sacrificed 18h after the treatment. CD271 induced luciferase activity in brain, testis, spleen, psv, complex s. intestine, thymus, l. intestine, lung, WAT, heart, liver, kidney and skin. LG268 did not induce luciferase activity in any of the tissues examined. It showed similar expression pattern like control treatment except skin. The organ specific expression results of synthetic RAR and RXR ligand treatments demonstrated the compatibility of our transgenic mouse model to our experimental design.

Organ specific expression of luciferase signal upon lycopene and control-bead treatments (experiment 2):

Bioluminescence signal was detected in the basal level for most of the organs. After CTRL 1 and CTRL 2 treatments, highest basal activity was obtained in the testis and brain. It is followed by the spleen, lung, WAT, s / l intestine and liver. We analyzed testis, brain, spleen, lung, spleen, WAT, s / l intestine and liver. Lycopene treatment resulted in significantly induced imaging signal over liver, lung, WAT and l. intestine compared to the control. Mice treated with ATRA induced bioluminescence signal in s. intestine, l. intestine, spleen, lung, WAT and liver (* $P<0.05$). The organ specific expression was analyzed using bioimaging analysis. The organ specific expression resulted in differential patterns upon lycopene and ATRA applications in bioluminescence imaging experiments. Since the basal level of brain and testis was very high, induction was not observed upon supplementations.

Time dependent response of RARE-LUC mice to lycopene and ATRA treatments (experiment 3):

We observed that the bioluminescence signal can be readily detected in the basal state. The bioluminescence signal increased in a time-dependent manner in mice treated with both lycopene and ATRA. Lycopene treatment increased the RARE mediated luciferase expression non-significantly at 12 h and reached the highest induction level at 18 h in comparison to 0 h. It was followed by bioluminescence decay in total body luminescence until 48 h. Lycopene and ATRA treatments did not reveal a significant difference with each other in statistical analysis. During the time course study, the intensity of light emission gradually increased in the abdominal region; mainly in testis, small and l. intestine, liver and adipose tissue regions. However, we could

not identify the organ-specific signals using *in vivo* imaging studies. Time course studies of ATRA treated mice rose significantly after 6 h and 12 h in comparison to 0 h. During the time course experiment, mice treated with ATRA had a higher total body bioluminescence signal, than lycopene. Bioluminescence imaging results can be seen as integrated intensity/area upon lycopene and ATRA treatments in certain time points.

Gender specific response of luciferase gene upon lycopene and control treatment (experiment 4):

Upon organ specific luciferase gene expression analysis in male mice, the same experimental design was applied to female mice after lycopene (100 mg / kg bw) and the corresponding control treatment (CTRL 2). In the reproductive organs, same expression profiles were apparent in different genders, uterus in female mice showed the highest basal level while testis in male mice displayed the highest basal signal. In female mouse, we observed comparable expression pattern with male mouse. Spleen showed the highest luminescence signal.

Organ specific expression of luciferase signal upon tomato extract treatments (experiment 5):

Two different groups of male mice were treated with tomato extract in aqueous cremophor suspension and vehicle control (CTRL 1) for the examination of tissue specific bioluminescence signal. The amount of tomato extract was adjusted to equal 100 mg lycopene / kg bw used in the previous mouse experiments. Upon treatment with tomato extract the bioluminescence in the treated mice was similar to those after lycopene treatment. We observed induction of RARE-activity in lung, liver, spleen, s / l intestine, WAT after tomato extract treatment. Spleen displayed higher bioluminescence signal in comparison to lycopene-treatments.

Organ specific expression of luciferase signal upon apo-10-lycac treatment (experiment 4):

Upon lycopene treatment, we have examined potential lycopene metabolites in our transgenic mouse model. Apo-10-lycac was synthesized. According to our bioluminescence imaging results, we reported that apo-10-lycac treatments can initiate transactivation of RAR in a transgenic reporter animal model in psv complex, lung and WAT.

Organ specific expression of luciferase signal upon apo-14-lycac treatment (experiment 4):

The organ specific expression upon apo-14-lycac and CTRL 4 (DMSO) treatment was analyzed using quantitative bioimaging analysis. The results were compared with ATRA and CTRL1 (cremophor) treatments. The organ specific expression resulted in differential patterns upon apo-14-lycac and ATRA applications in bioluminescence imaging experiments. High basal level was observed in brain and testis like in previous experiments. Therefore, induction was not observed upon supplementations of apo-14-lycac as in the previous

experiments. During the bioimaging experiment ATRA demonstrated higher RARE activity than apo-14-lycac. In s. intestine, l. intestine, psv complex, lung, WAT, liver and kidney induction of RARE activity were found.

Luciferase assay (experiment 8):

In addition to bioimaging results, we focused on the two important organs liver and testis. Further on, we conducted protein luciferase assay for these organs. In this luciferase assay we determined luciferase activity expressed as RLU/µg protein in homogenized organs extracts. We could not find any alteration in testis luciferase activity, while we observed an up-regulation in the liver after lycopene treatments.

Analysis of lycopene influence on gene expression of carotenoid metabolizing enzymes, carotenoid-transporters as well as retinoid target genes in testis and liver (experiment 9):

Firstly, we examined the expression patterns of the two carotenoid metabolizing enzymes (*Bco-1 / Bco-2*) and the non-specific carotenoid transporter (*Cd36)*. We observed down-regulation of *Bco-2* expression in liver and testis upon lycopene administration. Additionally, we found decrease in the *Bco-1* mRNA expression in testis while we did not find alteration for the liver upon lycopene treatment. *Cd36* expression remained stable in testis, but we observed up-regulation of *Cd36* expression after lycopene-administration in liver. The expression of the retinoid target genes: cellular retinol binding protein 1 (*Crbp1*), *Rarres2* and the retinoid metabolizing enzyme *Cyp26b1* was additionally examined in testis and liver. In the liver and testis we found an increased expression of *Crbp1* as well as *Cyp26b1* after lycopene treatment compared to the control. We could detect the retinoid target gene *Rarres2* expression just in liver samples because it was under detection limit in the testis and its expression displayed an increase after lycopene treatments in liver as well.

DISCUSSION

This study demonstrates that lycopene, an acyclic carotenoid with no previously proven pro-vA activity, is able to activate RAR-mediated transcriptional activation pathways in RARE-LUC reporter mice upon oral *gavage* in various organs like l. intestine, lung, liver, WAT and spleen. These RARE-response activities appear to vary tissue specifically after lycopene treatments possibly depending on the expression of *Cd36* as well as carotenoid metabolizing enzymes *(Bco-1, -2).*

RXR, a partner of RAR to form a transcriptional unit, forms various heterodimers with other nuclear receptors. Therefore cross-talk between vA signalling and other signalling pathway can also be assessed *in vivo*. The RARs and RXRs form heterodimers that bind to retinoic acid receptor response elements (RARE) in regulatory regions of target genes. Upon ligand binding, a conformational change is brought about in the heterodimer, mediating release of its association with co-repressors and recruitment of co-activators, which will promote induction of gene expression. With the aim of developing an *in vivo* model that monitor retinoic acid receptor (RAR) transactivation in real-time in intact animals, our mouse model was generated carrying a luciferase (LUC) reporter gene under the control of retinoic acid response elements (RARE) consisting of three copies of a direct repeat with 5 spacing nucleotides (DR5). In this transgenic mouse carrying three RAREs coupled to luciferase reporter gene; luciferase activity was induced by RA. RAR transactivation for luciferase activity by synthetic RAR ligand and RA treatment was previously observed in this mouse model. The RXR agonist LG268 was not able to induce the luciferase signal in any of the tissues.

So far, highest un-induced RARE-mediated luciferase activity has been found in the reproductive organs of male (testis) and female mice as well as in brain. At physiological plasma concentrations, RA cannot be taken up by testis, possibly owing to its inability to cross the blood-testis barrier formed by Sertoli and peritubular cells; therefore testicular RA might be synthesized locally. Sertoli cells are indicated as the main site of endogenous RA production in testis for normal spermatogenesis through the uptake of retinol from plasma RBP following oxidation of retinol to RA. These observations suggest that the lack of RA mediated luciferase induction in testis may stem from inability to take up RA from the circulation. We speculate that the blood–testis barriers and the blood-brain barriers are protective barrier in order to keep this environment stable and that may be the reason, why we could not see any increase of luciferase activity after lycopene treatments to our mice. Our results demonstrate compatibility with previous studies. In addition, reproductive organs like the uterus / ovary and the testis as well as the brain indicate the importance of highly controlled RAR-signaling and homeostasis and that the nutritional influence by carotenoids should be kept to a minimum. Other organs like

liver, WAT, spleen and s / l intestine are easily accessible to nutrients and regulate / balance the organisms due to the nutritional challenges.

The analysis of bioimaging experiments with time dependent ordinal response demonstrated that in the ATRA-treated mice, RARE-activation showed a significant rise after 6 h and maintained a high luciferase activity at least 12 h. Lycopene treatment resulted in a much slower non-significant response after 6 h, there was no difference in total body luminescence speculating that lycopene needs to be metabolized for eliciting bio-activity and further activation of RARE response. In our *in vivo* experiments, we shave the mouse hair in order to get better signals but skin still covers the organs. One drawback of *in vivo* full body screening is that the skin might disturb detection of internal signals. Lower signals induced by lycopene-treatments like observed from *ex* vivo experiments might be influenced more than the strong signals induced by ATRA-treatments. This might also explain the non-significant values from full body *in vivo* screening by lycopene-treatments.

Lycopene treatments induced up-regulation of RARE-response in l. intestine, lung, WAT, spleen and liver using the bioimaging and this RARE-LUC activity could be confirmed with luciferase protein assays in the liver. Luciferase assay was conducted for liver and testis to further focus on these organs due to their different RARE-signaling response to lycopene and their importance in carotenoid metabolism.

Tomato is very important dietary compound and lycopene is the main carotenoid in tomatoes and tomato preparations. We performed supplementation studies also with tomato extract containing high lycopene concentration. The amount of the tomato extract given was adjusted to a comparable lycopene amount for lycopene bead and tomato preparation treatments. Although lycopene was the predominant carotenoid in tomato extract, tomato extract also contained, β-carotene, phytoene and phytofluene as well as other bioactive compounds such as antioxidant polyphenolic compounds or PUFA. In addition to carotenoids, vitamin C (160–240 mg/kg), vitamin E (5–20 mg/kg) and flavonoids contribute the tomato composition.

The results for RARE-activation were quite comparable for lycopene and tomato extract except a much stronger RARE-activation in the spleen with tomato extract. In previous studies in humans, it was already reported that the addition of tomato juice increased various factors of the immune response like the T-lymphocyte functions. In comparison, our study with mice used high concentrations of lycopene which have no relevance for the human nutrition and mainly focused on the question if lycopene or potential novel and non-identified lycopene metabolites originating from lycopene or tomato-extract might also obtain RAR-activation potential. We suggest that other carotenoids or phytochemicals alone or in combination in this tomato extract may positively influence RARE-mediated signaling in mouse spleen.

Further insight into lycopene metabolism has initiated new research field on transactivation of nuclear hormone receptors. We also performed bioluminescence imaging with certain potential lycopene metabolites. Apo-10-lycac and apo-14-lycac were synthesized. The apo-10-lycac has been shown to be an active metabolite, especially in cancer cells. Indeed, Lian et al. demonstrated that treatment of human bronchial epithelial cells with apo-10-lycac resulted in the nuclear accumulation of Nrf2, which is associated with an induction of phase II detoxifying/antioxidant enzymes. This group previously reported that apo-10-lycac activated the retinoic acid receptor β (RARβ) promoter in relatively high concentrations, and induced the expression of RARβ in bronchial and lung cancer cells *in vitro*. Our data strongly suggest that apo-10-lycac is highly active in terms of the regulation of gene expression in lung, prostate and WAT that have an optimal environment for further potential bioactivation to active metabolites. We have demonstrated that apo-10-lycac transactivates RAR in adipose tissue in vivo in adipocytes. Such a transactivational effect was suspected from *in vitro* data in lung. Therefore, apo-10-lycac seems to be an activator of RAR in specific organs, suggesting that an organ specific environment is needed for apo-10-lycac transactivation of RAR-signaling. Consistently we reported that apo-10-lycac treatments can initiate transactivation of RAR in a transgenic reporter animal model, and induce the expression of several RAR target genes.

Apo-14-lycac is another potential lycopene metabolite that we studied. Apo-14-lycac induced RARE-signaling in male mice. In s. intestine, l. intestine, psv, lung, WAT, liver and kidney induction of RARE activity were found. Indeed, apo-14-lycac showed very similar induction pattern in RARE-LUC mice. Unknown lycopene metabolite which might activate the RAR pathway might be apo-14-lycac. HPLC-MS studies have to be done for confirmation. In summary, we suggest that lycopene metabolism into apo-lycopenoids is an important preliminary step toward the expression of lycopene bioactivity.

For further analyses we used testis and liver for qRT-PCR determination of carotenoid metabolizing enzymes and carotenoid transporter. Using these two tissues we determined the regulatory pathways by expression analyses of these enzymes / transporters and we tried to associate enzyme / transporter gene expression with the observed RARE-luciferase data. The two carotenoid metabolizing enzymes *Bco-1* and *Bco-2* may contribute to the lycopene metabolism and the non-specific carotenoid transporter CD36 may contribute to lycopene uptake. However, little is known about the impact of lycopene metabolism and lycopene metabolite induced effects on retinoic acid receptor activation pathways. For confirmation of RARE-activation pathways we also performed additional qRT-PCR experiments for three retinoid response target genes: cellular retinol binding protein 1 (*Crbp1*), retinoic acid receptor responder 2 (*Rarres2*) and cytochrome P450 26B1 (*Cyp26b1*). In the testis and

the liver, a strong up-regulation was found after lycopene-treatments for all three target genes (*Rarres2* was under detection limit in the testis). We hypothesize that the different responses to lycopene treatments between testis and liver may be explained by the fact that there seems to be a relation between *Bco-1* expression (non-significant up-regulation) and induced RARE-activity while alternative explanations involving other retinoid-metabolizing enzymes and retinoid-transporters may also be relevant but have not been investigated in the present study. A down-regulation of *Bco-1* expression in the testis may indicate that prevention of increased lycopene (or other carotenoid) cleavage may inhibit an increased formation of lycopene / or other carotenoid-originating RAR-activators. A down-regulation of *Bco-2* expression was also found in the two organs after lycopene-treatment and was significant for liver and testis but seems not to be related to RARE-signaling.

Feedback mechanisms have previously been demonstrated for RA-treatment induced down-regulation of *Bco-1* expression in chickens and rats. In the mouse testis, a strong down-regulation of *Bco-1* and *Bco-2* expression in response to lycopene treatments suggests feedback inhibition in order to maintain retinoid-homeostasis. These results are consistent with those obtained by Zaripheh et al. 2006 in rat tissue. The expression of *Bco-1* and *-2* seems to be strongly down-regulated in the testis which fits with our observation that lycopene-administration did not induce RARE-activity in this organ. We speculate that the organ specific down-regulation of carotenoid-metabolism is a regulated mechanism to prevent excessive formation of bio-active metabolites.

One remaining question is why we did not see increased RARE-bioluminescence but an increased retinoid target gene expression in the testis. So far, highest un-induced RARE-mediated luciferase activity has been found in the reproductive organs of male (testis) and female mice as well as in brain. At physiological plasma concentrations, RA can't be taken up by testis, possibly owing to its inability to cross the blood-testis barrier formed by sertoli and peritubular cells; therefore testicular RA might be synthesized locally. Sertoli cells are indicated as the main site of endogenous RA production in testis for normal spermatogenesis through the uptake of retinol from plasma RBP following oxidation of retinol to RA. These observations suggest that the lack of RA mediated luciferase induction in testis may stem from inability to take up RA from the circulation. We speculate that the blood–testis barriers and the blood-brain barriers are protective barrier in order to keep this environment stable and that may be the reason, why we could not see any increase of luciferase activity after lycopene treatments to our mice. Our results demonstrate compatibility with previous studies. In addition, reproductive organs like the uterus / ovary and the testis as well as the brain indicate the importance of highly controlled RAR-signaling and homeostasis and that the nutritional influence by carotenoids should be kept to a

minimum. Other organs like liver, WAT, spleen and s / l intestine are easily accessible to nutrients and regulate / balance the organisms due to the nutritional changes.

We suggest that the high basal activity of endogenous RARE-signaling in the testis might just lead to relatively small increase of RARE-bioluminescence but seems to be sufficient for induction of specific retinoid target gene expression. In addition, retinoid signaling and metabolism is under control of follicle-stimulating hormone (FSH) and androgen pathways in the mouse testis while RARE-LUC signaling is exclusively based on the RARE-LUC construct mediated activation and signaling. Additionally, post-translational and transcriptional modifications may cause slightly different results in protein and gene expression based studies.

In summary, we observed that lycopene, potential lycopene metabolites and tomato extract have the ability to strongly up-regulate RAR-mediated transcriptional activation pathways in the RARE-LUC reporter mice. This phenomen was described via RARE-mediated bioimaging, luciferase protein assay and retinoid target gene expression analysis. The RARE-activation potential of lycopene was organ selective and this activation may depend on the expression of non-specific carotenoid transporters and carotenoid metabolizing enzymes. We postulate that several organs are under tight control to maintain an optimized RAR-mediated signaling (brain and reproductive organs in male and female), while other organs like spleen liver, WAT and l. intestine must respond and adapt to the nutritional stimuli. These data suggest that lycopene and metabolites may play an important role in the modulation of retinoid metabolism. The exact regulation of lycopene metabolism, up-take of lycopene and potential novel lycopene metabolites in the mammalian organism is in the focus of further examination in our laboratory.

SUMMARY

Lycopene is an acyclic carotenoid containing eleven conjugated double bonds and lacks the β-ionine ring structure present typically in pro-vA carotenoid, therefore it is suggested to be non pro-vA carotenoid. Lycopene is a lipophilic carotenoid which is responsible for the red color of various fruits and vegetables and is commonly found in tomatoes, watermelon, pink-grapefruit and papaya. Emerging health benefits of lycopene have attracted accumulating attention to this carotenoid. Evidence is increasing that tomatoes / tomato preparations are able to ameliorate diseases with a chronic inflammatory background like cancer incidence for certain cancer types of the prostate, breast, colon, esophagus, stomach, rectum, oral cavity and pharynx. The mechanism of action of these beneficial effects induced by lycopene / tomato preparations remains still unknown, but it is suggested that nuclear hormone receptor mediated pathway activation via lycopene-breakdown products might be responsible.

The aim of this study was to investigate the potential of lycopene, lycopene-metabolite or tomato extract versus control treatments for the induction of the retinoic acid receptor (RAR) in male mice using a transgenic retinoic acid response-element (RARE)-reporter mouse system. The investigation included whole body scanning of the mice as well as organ specific studies with bio-imaging, selected luciferase activity and qRT-PCR of retinoid target genes and proteins involved in carotenoid-metabolism.

Lycopene-treatments induced RARE-mediated cell signaling indicated by quantified bio-imaging, increased luciferase activity. Lycopene supplementations caused the up-regulation of RARE-response in l. intestine, lung, WAT, liver and spleen using the bioimaging and this RARE-LUC activity could be confirmed with luciferase protein assays. The up-regulation of retinoid target gene activation within selected various organs of the mice was observed. Additional experiments focused on RARE-activation in female mice, tomato extract, apo-10-lycac, apo-14-lycac induced RARE-signaling in male mice, treatments displayed comparable RARE-activation like lycopene. In summary, we observed that lycopene, lycopene metabolites and tomato extract have the ability to strongly up-regulate RAR-mediated transcriptional activation pathways in the RARE-LUC reporter mice. However, the responsible biologically active potential lycopene metabolites in the organs are still non-identified.

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1. **Aydemir, G.**, Carlsen, H., Blomhoff, R., Rühl, R.: Lycopene induces retinoic acid receptor transcriptional activation in mice.
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FIRST AUTHORED POSTERS ON INTERNATIONAL MEETINGS

Poster presentations in International Conferences:

Aydın G, Reynaud E, Caris-Veyrat C, Rühl R, Lycopene can induce nuclear hormone receptor pathway in RARE-LUC mouse model" presented at "Nuclear Receptor Signaling: From Molecular Mechanisms to Integrative Physiology" Summer School organized by FEBS, Island of Spetses, Greece, August 23 - 28, 2009

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Aydin G, Rühl, R (2007), Transactivation of nuclear hormone receptor pathways by lycopene, lycopene metabolites on reporter cell culture system, presented at, "Annual Lycocard Meeting" Murcia, Spain, 2007

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