THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Lycopene induces Retinoic Acid Receptor transcriptional activation in mice

by

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ABBREVIATIONS

ATRA	- all-*trans*-retinoic acid
AF-1	- activation function 1
AF-2	- activation function 2
AUC	- area under curve
ANOVA	- analysis of variance
Apo-10-lycac	- apo-10'-lycopenoic acid
Apo-14-lycac	- apo-14'-lycopenoic acid
BCO1	- β-carotene oxygenase 1
BCO2	- β-carotene oxygenase 2
B/W	- black and white
Bw	- body weight
CCD	- cooled charged coupled
CD36	- Cluster of Differentiation 36
CHD	- coronary hearth diseases
CRBP1	- cellular retinol binding protein 1
CRABP1	- cellular retinoic acid binding protein 1
CRABP2	- cellular retinoic acid binding protein 2
CT	- comparative threshold
CTRL	- control
cyclo	- cyclophilin A
CYP26A1	- cytochrome P450 26A1
CYP26B1	- cytochrome P450 26B1
Cx43	- connexin 43
CVD	- cardiovascular disease
DBD	- DNA-binding domain
DMSO	- di-methyl-sulfoxide
DR	- direct repeat
FSH	- follicle-stimulating hormone

GJ	- gap junctions
GJC	- gap junctional communication
HRE	- hormone response element
HR	- hormone response
IGF	- insulin-like growth factor
IGFBPs	- insulin-like growth factor binding proteins
IL-6	- inhibit interleukin 6
IP	- inverted palindromes
LBD	- ligand-binding domain
LDL	- low-density lipoproteins
l. intestine	- Large intestine
LRAT	- lecithin retinol acyltransferase
LYC	- lycopene
LUC	- luciferase
NHR	- nuclear hormone receptor
qRT-PCR	- quantitative real time polymerase chain reaction
PAL	- palindromes
PPAR	- peroxisome proliferator-activated receptor
Pro-v	- pro-vitamin
Pro-vA	- pro-vitamin A
Psv	- prostate/seminal vesicules
PUFA	- polyunsaturated fatty acid
RA	- retinoic acid
RAL	- retinal
RALDH	- retinaldehyde dehydrogenase
RARRES	- retinoic acid receptor responder
RARE	- retinoic acid response element
RARE-LUC	- retinoic acid response element / luciferase complex
RBP	- retinol binding protein
RDH	- retinol dehydrogenase
RE	- retinyl ester

ROC	- receiver operating characteristic
ROL	- retinol
ROS	- reactive oxygen species
RAR	- retinoic acid receptor
RARE	- retinoic acid response element
RXR	- retinoid X receptor
SEM	- standard error of mean
s. intestine	- small intestine
TR	- thyroid receptor
UDL	- under detection limit
VA	- vitamin A
VDR	- vitamin D receptor
VLDL	- very low density lipoproteins
WAT	- white adipose tissue
5-LOX	- 5-lipoxygenase

CONTENTS

1. 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 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 in a transgenic retinoic acid response-element (RARE)-reporter mouse system.

2. THEORETICAL BACKGROUND

2.1. 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. The group of β-carotene and lycopene is called as carotene and they possess very lipophilic structure. Therefore, carotenes are prone to be localized in the low-density lipoproteins (LDL) in the circulation (Clevidence and Bieri, 1993, Krinsky and Johnson, 2005). β-carotene possess a pro-vitamin (pro-v) activity and it is widely used as dietary carotenoid in the industry and as food colorants/additives. Of the ~600 naturally occurring carotenoids, ~50 have pro-vitamin A (pro-vA) activity and can be cleaved to ultimately yield retinol (ROL) (Lee, et al., 1999). In the last decade, numerous studies displayed the beneficial role of lycopene in various chronic diseases, cardiovascular diseases (CVD) and cancer.

2.2. Characteristics of lycopene

Lycopene is the red pigment abundantly found in tomato, rosehip, pink grapefruit, watermelon and papaya (Stahl and Sies, 1996). 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 (Tonucci, et al., 1995). It is an acyclic carotenoid and due to its 11 conjugated double bonds it has red color (Kong, et al., 2010). It has no terminal β-ionic ring therefore lycopene has been accepted as non-pro vitamin A precursor so far. The chemical structure of lycopene is shown in *Figure 1*.

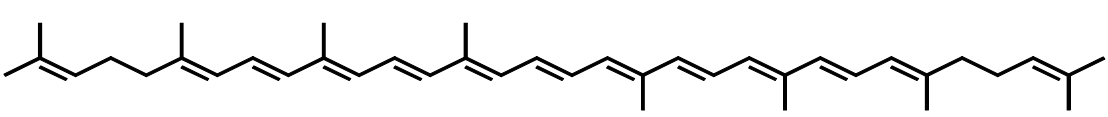

Figure 1. Chemical structure of lycopene

Due to its apolar chemical structure, it is a highly lipophilic compound. Its hydrophobic characteristics make it more soluble in petroleum ether, acetone, methylene chloride, hexane, benzene and chlorofrom (Roldan and Luque de Castro, 2007). This lipophilicity makes it easier to be transported in 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 (Esterbauer and Ramos, 1996). Further physio-chemical characteristics of lycopene is displayed in *Table 1* (Shi, et al., 2002).

	Characteristics of lycopene
Molecular formula	$C_{40}H_{56}$
Molecular weight	536.85 Da
Melting point	172-175 ºC
Crystal form	Long red needle
Powder form	Dark reddish brown
Solubility	Soluble in chloroform, hexane, benzene, acetone, petroleum ether and oil Insoluble in water, ethanol and methanol
Stability	Sensitive to light, oxygen, high temperature, acids and metal ions.

Table 1. Characteristics of lycopene

2.3. 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 (Stahl and Sies, 1992). 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. All those processes have not been fully known (Boileau, et al., 1999, Castenmiller and West, 1998, Stahl, et al., 1992).

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) (Lee, et al., 1999). Lycopene absorbtion, transportation and distribution can be seen in *Figure 2* (Schweigert, 1998).

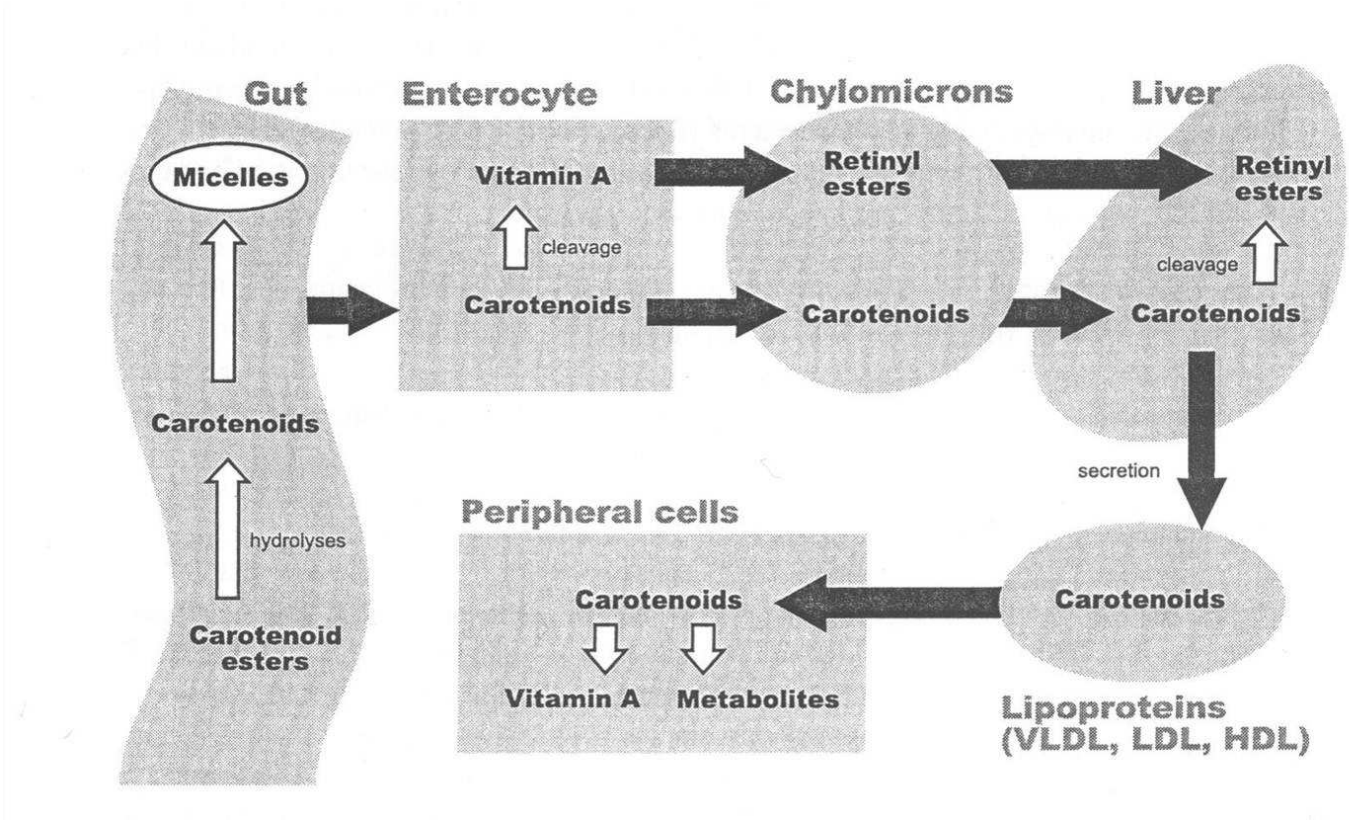

Figure 2. Carotenoid absorbtion, transportation and distribution (Schweigert, 1998).

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. Therefore it was suggested that *cis*-lycopene is more bioavailable than all-*trans*-lycopene (Stahl, et al., 1992). It was proposed that *cis*-lycopene is more bioavailable than *trans*-lycopene, most likely because of increased solubility in mixed micelles. *Cis*-lycopenes are more absorbed than all-*trans*-lycopene therefore cis-lycopene is present in the tissues more due to enhanced solubility in bile acid (Boileau, et al., 1999). Lycopene bioavailability can be influenced by other factors. Fiber, oil or protein amount of food matrix affects the stability of all-*trans*-lycopene bioavailability (Riedl, et al., 1999). Heat may induce the rupture of the cell walls accompanied by release of lycopene from the cells. Additionally, corn oil supplementation increases the lycopene availability. Interindividual differences like LDL assembly may have an impact on plasma lycopene concentration (Bohm and Bitsch, 1999, Stahl, et al., 1992).

2.4. 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 (Kaplan, et al., 1990). *Table 2* shows the tissue distribution of lycopene in various human organs.

Tissue	Lycopene tissue levels (nmol/g wet wt)
Liver	1.28
Kidney	0.15
Adrenal gland	1.90
Testes	4.34
Ovary	0.25
Adipose	0.20
Lung	0.22
Colon	0.31
Breast	0.78
Skin	0.42

Table 2. Tissue distribution of lycopene (Stahl, et al., 1992)

2.5. Potential biological effects of lycopene

Even though lycopene possess non pro-vA activity, it obtains several roles in biological processes like cell differentiation and proliferation, carcinogenesis, organogenesis and mutagenesis. Therefore, potential biological effects of lycopene have been extensively studied. Lycopene has wide range of biological functions such as anticarcinogenic effect and antioxidant effect. Possible mechanisms underlying the disease preventive effect of lycopene can be categorized as below.

2.5.1. Antioxidant effect of lycopene

Antioxidants are involved in prevention of cellular damage induced by free radicals. Cellular protein, lipid and DNA can be oxidatively damaged by reactive oxygen species (ROS), react with very important cellular components, thus carcinogenesis, mutagenesis and cell death occurs in mammalian cells. That's why, free radicals are known to be one of the reason in the pathogenesis of several chronic diseases (Frei, 1994). 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 (Agarwal and Rao, 2000, Matos, et al., 2000). Lycopene has been demonstrated to be the most potent antioxidant among the carotenoids (Di Mascio, et al., 1989). This potential antioxidant property depends on its 11 conjugated double bonds increasing capability to quench singlet oxygen (Miller, et al., 1996).

2.5.2. 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 (Sies and Stahl, 1998).

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 pathways 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 (Seren, et al., 2008). It has been previously shown that increased risk of IGF levels associated with incidence of cancer (Khandwala, et al., 2000). Lycopene modulates the insulin-like growth factor (IGF)-1/IGF binding protein (BP)-3. Lycopene can diminish the cell proliferation via up-regulating the IGF-1. There is a strong association between the IGF-1 level and colorectal (Renehan, et al., 2004), lung (Yu, et al., 1999), prostate (Mantzoros, et al., 1997) breast (Hankinson, et al., 1998) and ovarian (Lukanova, et al., 2002) cancers. It was exhibited that lycopene is able to interfere with the Igf signaling pathways by increasing the amount of IGF-binding proteins (IGFBPs). By binding IGFs, IGFBPs can enhance the activity of IGFs in a tissue specific manner (Drop, et al., 1992).

Another biological structures influenced by lycopene treatments is gap junctions (GJ) which are intercellular channels that links the cytoplasm of two cells, and provide a means of ions, second messengers and small metabolites (Mese, et al., 2007). 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 (Chalabi, et al., 2007) thus it contributes gap junctional intercellular communication. Upregulation of Cx43 bring about increases in gap junctional communication (GJC). It was reported that in human tumors, the expression of connexin43 decreased. Another gene target for cancer formation is 5-lipoxygenase (5-LOX). This enzyme directly stimulates prostate cancer cell proliferation. It is demonstrated that, lycopene can inhibit interleukin (IL-6) and 5-lipoxygenase (Ghosh and Myers, 2002, Hazai, et al., 2006, Siler, et al., 2004). Additionally, activity of 5α-reductase which is responsible for the conversion of androstenedione and testesteron into the most potent natural androgen 5α-dihydrotestesteron has been reduced by lycopene supplementation. It was suggested that lycopene have a beneficial effect on prostate cancer since it downregulates the androgen targets (Herzog, et al., 2005).

2.5.3. Lycopene, inflammation and cardiovascular diseases

In addition, lycopene can decrease inflammatory responses by downregulating inflammatory markers such as interleukin 1β, CXC chemokines MIP-2 and LIX. Therefore it was proposed that lycopene has an anti-inflammatory action in prostate (Herzog, et al., 2005).

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 (Salonen, et al., 1997). The oxidative modification of LDL particles may play a role in the formation of foam cells, atherosclerotic lesions, and CVD (Salonen, et al., 1992) Antioxidants can inhibit the oxidative modification of LDL, may decline atherosclerotic formations and, consequently, may stop clinical complications of atherosclerosis such as myocardial infarction (Salonen, et al., 1997). There are also suggested processes about the contribution of lycopene in the inhibition of atherosclerosis such as intracellular cap junction communication and hormonal and immune system modulation (Lorenz, et al., 2012, Rao and Agarwal, 2000).

2.6. Retinoid metabolism

Retinoids are the substances compromising vitamin A (retinol) and its natural metabolites, retinaldehyde and retinoic acid as well as its synthetic derivatives. Those liposoluble vitamins have crucial tasks in many biological processes including fetal development, organogenesis and embryogenesis in addition to the regulation of several aspects of cell metabolism (Ross, 1993, von Lintig and Vogt, 2000). 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 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 (Yeum, et al., 2000). 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 (Lindqvist, et al., 2005). Retinal molecules can either be reduced to retinol (ROL) subsequently by retinal reductase or oxidized to retinoic acid (RA). Eccentric cleavage of β-carotene yields various apo-carotenals and they may be converted subsequently to RAL or oxidized to apo-carotenoic acids (Silveira and Moreno, 1998). Conversion of β-carotene into ROL occurs in along the enterocytes and subsequently, vitamin A (retinol) esterified with long-chain fatty acids, especially palmitic or stearic acid. Lecithin retinol acyltransferase (LRAT) enzyme catalyzes the esterification of CRBP-II bound all-*trans*-retinol to all-*trans*-retinyl esters (RE), inside the enterocytes (Blomhoff and Blomhoff, 2006, Napoli, 1996, Napoli, 1993). After retinol (ROL) formation, it may be converted to retinyl esters (RE) to be stored in the liver, it may be metabolized to retinoic acid. In addition to those metabolic pathways it may be recycled into the systemic circulation due to the RBP amount. Additionally, retinoic acid is also found in plasma in low concentrations and is taken up by the cells by diffusion (Ross, 1993). Retinoid signaling is shown in *Figure 3*.

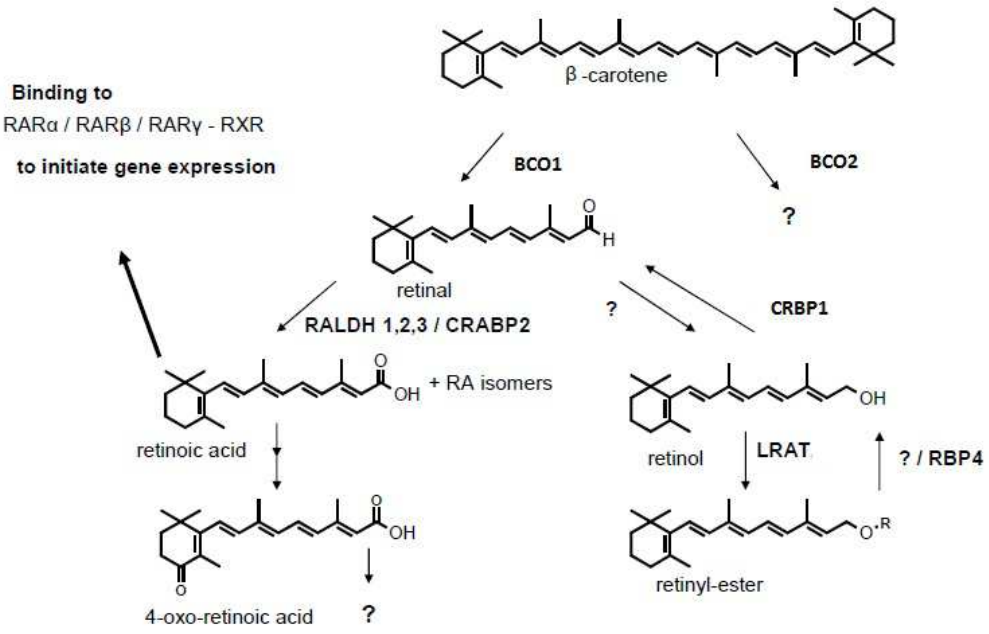

Figure 3. Retinoid signaling pathway. retinoid-metabolizing enzymes: RA - retinoic acid, RDH – retinol dehydrogenase, RALDH – retinaldehyde dehydrogenase, BCO1 – β-carotene oxygenase 1, BCO2 – β-carotene oxygenase 2, CRBP - cellular retinol binding-protein, CRABP – cellular retinoic acid binding-protein, LRAT – lecithin:retinol acyltransferase, RBP4 – retinol binding protein 4, RAR – retinoic acid receptor, RXR – retinoid X receptor,

2.7. 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 (Khachik, et al., 1995, Khachik, et al., 1997). Furthermore, 2,6-cyclolycopene-1,5-diol A and B were determined as oxidative metabolites of lycopene present in human plasma (King, et al., 1997).

Two carotenoid metabolizing enzymes, β-carotene oxygenase 1 and β-carotene oxygenase 2 (Lindqvist, et al., 2005) may contribute the lycopene metabolism. Lycopene is partly cleaved by BCO1 with lower affinity (Redmond, et al., 2001). On the other hand, 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 (Kiefer, et al., 2001). *In vitro* study demonstrated that lycopene is an inefficient

substrate for BCO1 (Ershov Yu, et al., 1994). 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 (Bachmann, et al., 2002). A study by Zaripheh et al. showed that lycopene is the inhibitor of BCO1 as well (Zaripheh, et al., 2006).

It has been found that a *cis* isomer of lycopene can be metabolized into apo-10'-lycopenal by ferret BCO2 *in vitro* (Hu, et al., 2006). 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 (Gajic, et al., 2006). *Figure 4* shows the tomato carotenoid metabolism.

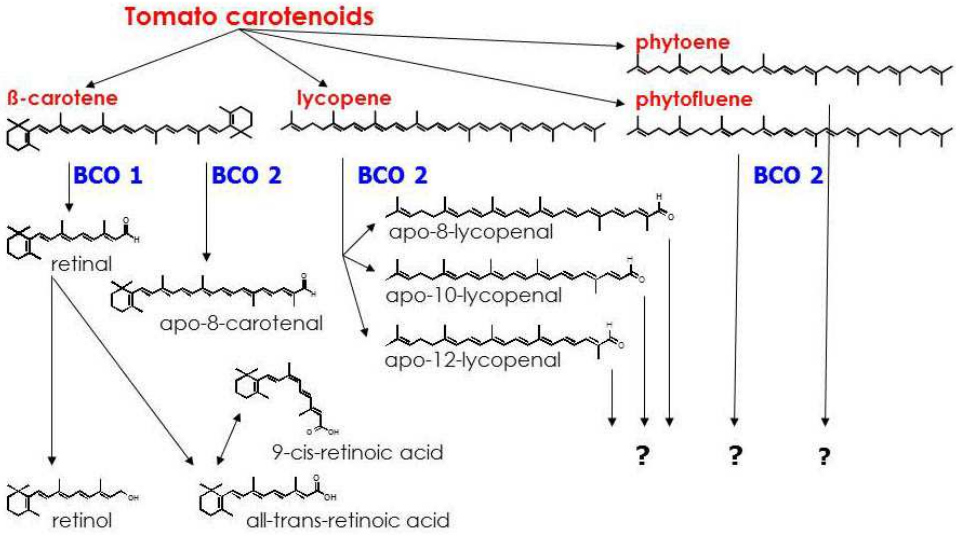

Figure 4. Metabolism of tomato carotenoids. Two carotenoid metabolizing enzymes, β-carotene oxygenase 1 and β-carotene oxygenase 2 contribute the lycopene metabolism. Lycopene is partly cleaved by BCO1 with a lower affinity. BCO2 catalyzes the asymmetric cleavage of lycopene. β-carotene was cleaved by BCO1 and BCO2, at the end of the process retinol and retinoic acid forms. BCO1; β-carotene oxygenase , BCO2; β-carotene oxygenase 2.

2.8. 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. Ligand binding cause conformational change in the receptors hence these receptors can interact to the specific sequences of DNA in the promoter region of target gene resulting in trancriptional regulation of gene expression. NHRs are composed of multiple functional domains including weakly 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. NH2 terminal A/B region has a variable length and possess an autonomous activation function (AF-1). AF-1 is ligand independent transcriptional domain. The conserved C domain is the DNA-binding domain composed of two highly conserved zinc-finger-like motifs. It is responsible for the recognition of specific DNA sequences. D region links the DBD to conserved E/F region. LBD region is at the C terminal half of the receptor contains ligand binding domain, dimerization surface and second activation function (AF-2). Unlike AF-1, AF-2 is known to be ligand dependent and conserved among the nuclear hormone receptor superfamily (Aranda and Pascual, 2001, Danielian, et al., 1992, Nagy and Schwabe, 2004). *Figure 5* displays the molecular architecture of nuclear hormone receptors (Aranda and Pascual, 2001).

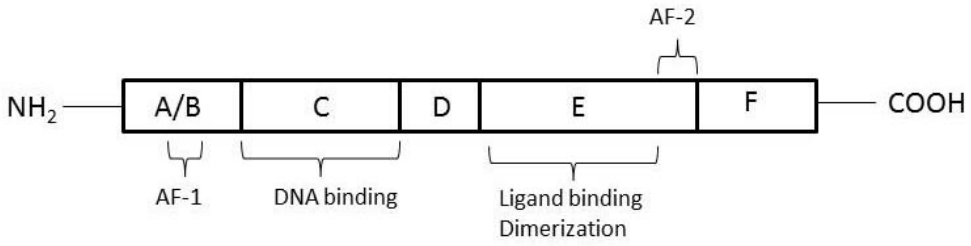

Figure 5. The structure of nuclear hormone receptors. *A/B domain;* ligand independent transcriptional activation Autonomous activation function (AF-1), *C domain;* DNA binding domain, *D domain;* Linker, *E domain;* Ligand binding domain, dimerization surface, Autonomous activation function (AF-2), *F domain;* ligand dependent transactivation domain

Hormone response (HR) 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. Sequences of 6 bp constitutes core recognition motif. There are different optimal HREs interactions as palindromes (Pal), inverted palindromes (IPs) or direct repeats (Werner, et al., 2002). Most potent HRs for non-steroid receptors are configured as DRs. *1-to-5 rule* describes binding of different receptors to direct repeats with a spacing of one to five nucleotides (DR-*1* to. DR-5). DR5 is separated by 5 nucleotides and frequently present in RXR-RAR heterodimer (Aranda and Pascual, 2001, Mangelsdorf, 1994).

2.9. 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 seperate genes (Evans, 1998, Leid, et al., 1992). RXR receptors have an affinity for 9-*cis* retinoic acid, whereas RAR receptors can be activated by all-*trans*-retinoic acid (Chambon, 1996, Giguere, et al., 1987, Petkovich, et al., 1987). RAR and RXR receptors are distributed in distinct tissues thus it is demonstrated that they have different tasks and functions in the regulation of transcriptional gene expression (Mangelsdorf, 1994).

RAR and RXR are able to make heterodimer and modulate the gene trancription after RA. They can also make RAR-RAR or RXR-RXR homodimers and recognize the DNA response elements in retinoid target genes (Altucci and Gronemeyer, 2001). These elements involve three or more repetitions of the AGGTCA sequence. The sequences of the retinoic acid response element (RARE), i.e., for the RXR-RAR heterodimer, are separated by two or five nucleotides (DR2 and DR5), with the DR5 spacing being actually the most frequent. On the other hand, the RXR-RXR homodimer response element (RXRE) recognizes these same sequences when they are separated by only one nucleotide (Aranda and Pascual, 2001, Chambon, 1996, Molnar, et al., 2004).

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. pro-vA activity has been demonstrated by α- and β-carotene in addition to β-cryptoxanthin. However, no pro-vA activity has been found for lycopene. The possibility that a metabolite of lycopene can mediate transcriptional regulation of nuclear hormone receptors caused new theories.

2.10. Scope of the study

Vitamin A is essential for many physiological processes, for instance cell division, differentiation, eye vision, embryogenesis and organogenesis. Vitamin A derivative retinoic acid (RA) plays crucial roles in controlling vitamin A signalling via nuclear hormone receptors. Retinoic acid receptors (RAR) and Retinoid X receptors (RXR) are activated by RA (Chambon, 1996, Giguere, et al., 1987, Petkovich, et al., 1987). RAR and RXR are able to form heterodimers and modulate the gene trancription after RA binding.

No pro-vA activity has been found for lycopene. So far, the evidences have not been found to demonstrate the transcriptional regulation of nuclear hormone receptors by lycopene metabolites. 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 or lycopene metabolites might induce nuclear hormone receptor pathways activation via RAR-mediated signaling pathways in transgenic RARE—LUC mice like the known β-carotene metabolite and endogenous RAR-activator ATRA.
- ✓ to demonstrate the time and gender dependent effect of luciferase expression in RARE-Luc mice upon lycopene administration.
- ✓ to investigate organ specific luciferase expression upon tomato extract treatment.
- ✓ to investigate 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.

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3. MATERIALS AND METHODS

Chemicals

Beads enriched with lycopene contained 10% (w/v) synthetic lycopene (DSM, Kaiseraugst, Switzerland), control beads 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 values are based on information from DSM, Switzerland. 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 (Jaensson-Gyllenbäck, et al., 2011, Svensson, et al., 2008, van de Pavert, et al., 2009) genetically modified to express firefly luciferase under the control of RARE (retinoic acid response element) (Carlsen, et al., 2002) were kindly provided by Cgene AS (Cgene AS, Oslo, Norway). Validation of the RARE-LUC system was based on a previous study (Carlsen, et al., 2002). Transgenic reporter mice containing three copies of RARE, canonical DR5 sites derived from RAR-β2 promoter, coupled to luciferase gene to monitor real-time RAR activation in living mice (DR5-LUC mice).In a variety of different DR5- luciferase constructs in P19 embryonic carcinoma cells (DR5 derived from either RAR-β2 promoter or the CYP26 promoter, one or multiple copies of the response elements, different directions, etc) transcription induction was checked with 1 μM. The construct for generating transgenic mice consists of three RARE repeats derived from RAR-β2 promoter, coupled to a minimal TATA-box from the herpes simplex virus thymidine kinase (TK) promoter and the luciferase reporter gene, flanked with insulator sequence. It is well known that the expression of transgenes driven by weak

promoters may be heavily influenced by enhancers/silencers nearby sequences of the integration site of the transgene. In addition, methylation may extinguish their promoter activity gradually in used long term growth. To overcome these concerns, two copies of a 250 bp core fragment from “insulator” β-globin hypersensitive site 4 (HS4), flanked the transgene after ligation into both ends of the construct. P19 embryonic carcinoma cells transfected with the construct showed a 20–fold luciferase induction in 1 μM RA for 24h treatment, either with or without insulator (data not shown). Using the insulated and non-insulated DR5-luc constructs, a number of PCR based genotyping-positive mice were obtained. Several RA inducible strains were obtained when we used the constructs flanked with the insulator sequence.

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, Hungary) 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 (Table 3). 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.

Number	Experiments	Mice, n / treatment group	Gender	Age	Treatment way, frequency	Treatment types
1	Analysis of organ specific localization of RARE signal by bioimaging for positive controls	6	Male	8-12 wk	Oral gavage, one time	CD271, LG 268, CD271+LG268, CTRL [1]
2	Analysis of organ specific localization of RARE signal by bioimaging	6	Male	8-12 wk	Oral gavage, one time	Lycopene, CTRL 2[2], ATRA, CTRL 1[3]
3	Analysis of time dependent response by bioimaging	3	Male	8-12 wk	Oral gavage, one time	Lycopene, ATRA[4]
4	Analysis of gender dependent response by bioimaging	6	Female	8-12 wk	Oral gavage, one time	Lycopene, CTRL 2, ATRA, CTRL 1
5	Analysis of tomato extract treatment by bioimaging	6	Male	8-12 wk	Oral gavage, one time	Tomato extract[5], CTRL 1
6	Analysis of apo-10-lycac treatment by bioimaging	4	Male	8-10 wk	Oral gavage, one time	apo-10-lycac, CTRL 4[6]
7	Analysis of apo-14-lycac treatment by bioimaging	4	Male	8-12 wk	Oral gavage, one time	apo-14-lycac, CTRL 4
8	Analysis of luciferase enzymatic activity	6	Male	8-12 wk	Oral gavage, one time	Lycopene, CTRL 2, ATRA, CTRL 1
9	Analysis of mRNA expression	6	Male	8-12 wk	Oral gavage, one time	Lycopene, CTRL2

Table 3. Summary of different experiments including bioluminescence imaging analysis of time dependent course, organ specific activity, gender dependent response, tomato extract treatment as well as luciferase enzymatic activity and qRT-PCR analysis for RARE-LUC mice[7,8]

[1] CTRL indicates control treatment for CD271 and LDG268 and contains 25% aqueous cremophor solution

[2] CTRL 2 (1000 mg / kg bw) indicates control treatment for lycopene with lycopene-free beadlets

[3] CTRL 1 indicates control treatment for ATRA and contains 25% aqueous cremophor solution

[4] ATRA was given 50 mg / kg bw in aqueous cremophor solution.; lycopene was given 100 mg / kg bw

[5] Tomato extract equalized to 100 mg / kg bw lycopene, in 25% aqueous cremophor suspension.

[6] CTRL 4 indicates control treatment for apo-10-lycac and apo-14-lycac and contains DMSO.

[7] Treatment schedule for *ex vivo* experiment (experiment 1, 2, 4, 5, 6, 7): 0 h - oral treatment; 15 h 45 min - luciferin injection; 16 h - killing of the mice and organ withdraw; immediate bioimaging; and following storage of the organs in -80°C for luciferase assay and qRT-PCR analysis.

[8] In the case of *in vivo* experiment (experiment 3), mice were anesthetized by nembutal 5 min after the luciferin injection and mice were not killed during the experiment.

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 (*Table 3*) the mice were euthanized by cervical dislocation. Subsequently, testis, brain, lung, WAT, liver, kidney, spleen, small intestine (s. intestine), large intestine (l. intestine) 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. Bioluminescence images were taken with 5 min integration time. Normalization was done by dividing photon signals into region of interest and the result was defined as integrated intensity/area. All measurements were done in the morning between 9.00 - 11.00 h at different months of the year. Sufficient mice in sufficient age and gender could not be provided at the same time for the experiment 5, therefore the direct inter-comparison of results for the experiments 2 and 4 was not performed. photon signals has been compared as heat map.

For bioluminescence experiments gray-scale and pseudo-images of organs were acquired by cooled CCD camera (-81^{o}C) and 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. Details were shown in *Table 3*.

Ex vivo bioluminescence imaging experimental set up has been summarized for experiment 1, 2, 4, 5, 6 and 7 in *Figure 6.A* whereas *in vivo* bioluminescence imaging experimental design for experiment 3 has been summarized in *Figure 6.B.*

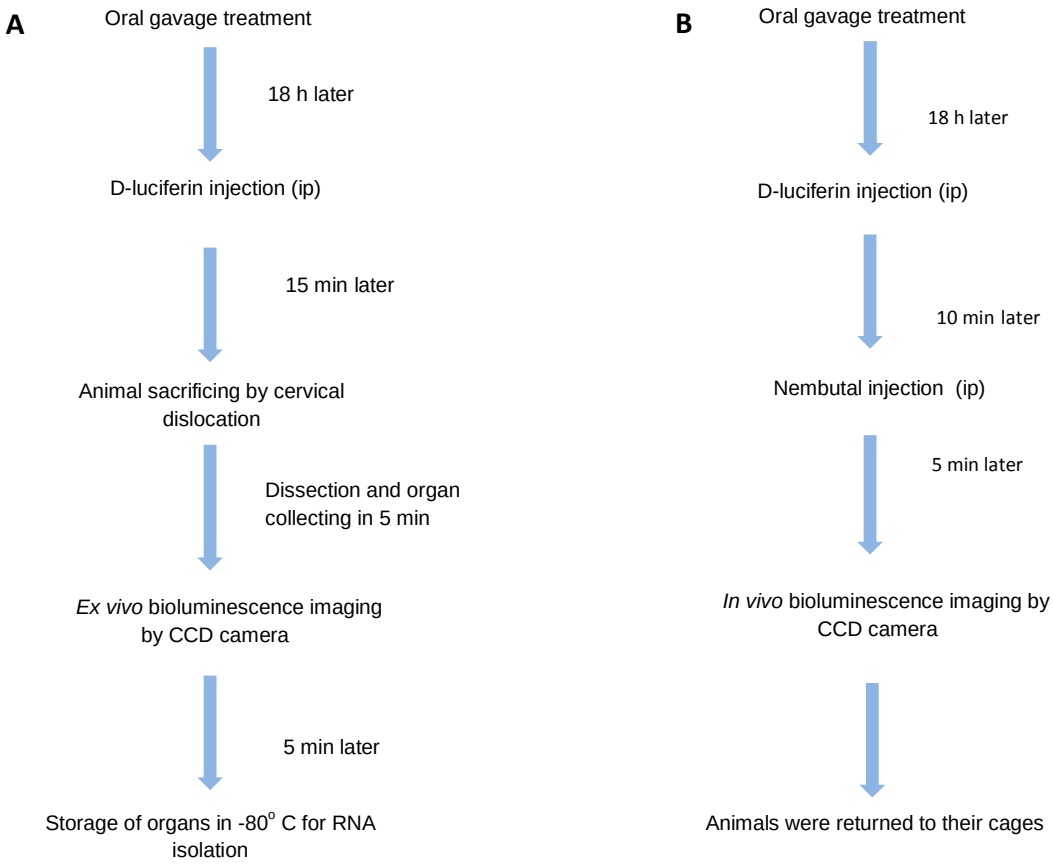


Figure 6.*A)* Summary of experimental design of *ex vivo* imaging in bioluminescence imaging experiments for experiment 1, 2, 4, 5, 6 and 7 *B) In vivo* bioluminescence imaging setup for experiment 3.

In all bioluminescence imaging experiments, 8-12 wk old mice were used and treated once by oral *gavage* 18 h before luciferin injections and bioimaging analysis (Detailed information was displayed in *Table 3*).

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 LDG268 (also called LG 100268 or LG268) is a potent and specific ligand that binds solely to the RXRs, and not to the RARs (Suh, et al., 2002). 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 (Rühl, et al., 2001).

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 (*Table 3*). 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, Hungary) via intra-peritoneal injections. Mice were euthanized by cervical dislocation. Experimental set up is shown in *Figure 6.A*. After sacrificing the mice, we collected testis, brain, spleen, psv complex, lung, WAT, s. intestine, l. intestine, thymus, heart, kidney, liver and skin for bioluminescence imaging. Organs were 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-beadlet 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* (*Table 3*).

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. We used cervical dislocation for the killing animals. Experimental set up is shown in *Figure 6.A*. 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, Hungary) injection and 10 min later screened for whole body bioluminescence. Experimental set up is shown in *Figure 6.A*. *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 beadlet 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 (*Table 3*). 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. (data not shown). 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. Mice were euthanized by cervical dislocation Experimental set up is shown in *Figure 6.A*. We collected uterus-ovary complex, brain, spleen, lung, WAT, s. intestine, l. intestine and liver 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. Experimental set up is shown in *Figure 6.A.* After dissection, we collected testis, brain, spleen, lung, WAT, s. intestine, l. intestine and liver for bioluminescence imaging. 8-12 wk old male mice groups were used (*Table 3*).

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 (*Table 3*). 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. Mice were killed by cervical dislocation. Experimental set up is shown in *Figure 6.A.* After dissecting the mice, we collected testis, brain, spleen, lung, WAT, s. intestine, l. intestine and liver 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 (*Table 3*). 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. Mice were euthanized by cervical dislocation. Experimental set up is shown in *Figure 6.A.* After sacrificing the mice, we collected testis, brain, spleen, prostate/seminal vesicule complex, lung, WAT, s. intestine, l. intestine, liver and kidney 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, Hungary). 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.

compound	concentration Amount	volume in µl 1 reaction
5xSSII buffer		8,00
DTT	0.1 M	4,00
dNTP	2.5 mM	8,00
random hexamer primer	3 µg/µl	0,20
SSIIMnlv	200U/µl	0,20
volume of Mastermix +		20,40
RNA	3 µg	19,60
total volume		**40,00**

Table 4. Protocol for reverse transcriptase PCR for mouse tissue

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 (Gundersen, et al., 2007) 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). The following reaction mixture is utilized for FAM-TAMRA assays (*Table 5*).

Compound	Concentration	volume in µl (1 reaction)
Water		2,10
MgCl2	0.1 M	1,20
Taq-buffer	10 x	1,00
dNTP	2.5 mM	0,50
Taqpol	5 U/µl	0,06
primer -	100 µM	0,04
primer +	100 µM	0,04
probe	20 µM	0,06
volume of Mastermix +		5,00
cDNA (1:10 dilution)		5,00
total volume		10,00

Table 5. Protocol for FAM-TAMRA assays in mouse tissue

In case of TaqMan ROX-MGB assays, the reaction mixture is modified (5 ml cDNA is added to 5 ml mastermix (*Table 6*).

Compound	Concentration	volume in µl (1 reaction)
Water		1,90
MgCl2	25mM	1,20
Taq-buffer	10 x	1,00
dNTP	2.5 mM	0,50
Taqpol	5U	0,06
50 x ROX		0,20
20 x oligo mix (ABI)		0,15
volume of Mastermix +		5,01
cDNA (1:10 dilution)		5,00
total volume		10,01

Table 6. Protocol for TaqMan ROX-MGB assays, in mouse tissue

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. Cycle threshold values above 40 were scored as under the limit of

detection (UDL). Relative mRNA levels have been calculated using the comparative threshold cycle (CT) method. For data analysis, Sequence Detector Software (Lindqvist, et al., 2005) 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, which served as an internal control for the amount of RNA used in each reaction. Cycle threshold values above 40 were scored as under the limit of detection (UDL).

TaqMan ROX-MGB assay was utilized for qRT-PCR. Assay ID's of genes are as below.

Gene symbol	Gene names	Assay ID
Bco1	β-carotene oxygenase 1	Mm01251350_m1
Bco2	β-carotene oxygenase 2	Mm00460051_m1
Crbp1	*Cellular retinol binding protein-1*	Mm00441119_m1
Cyp26b1	cytochrome P450 subfamily 26A polypeptide 1	Mm00514486_m1
Cd36	Cluster of Differentiation 36	Mm01208559_m1
Rarres2	*Retinoic acid receptor responder protein-2*	Mm00503579_m1

Table 7. Assay ID's of the genes of TaqMan ROX-MGB assay.

Gene name	Sequences
Probe for cyclophilin	5'-/56-FAM/CGC GTC TCC TTT GAG CTG TTT GCA/ 36-TAMSp/-3'
Cyclophilin primer + (forward primer)	5'-CGA TGA CGA GCC CTT GG-3'
Cyclophilin primer - (reverse primer)	5'-TCT GCT GTC TTT GGA ACT TTG TC-3'

Table 8. Sequences of probes and primers in FAM-TAMRA assay.

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 $**p < 0.01$.

4. RESULTS

Induction of luciferase gene expression following systemic administrations was demonstrated; time and gender dependent effect of luciferase expression were analysed in RARE-Luc mice. Additionally, organ specific luciferase expression upon lycopene, tomato extract, non-lycopene containing beads and ATRA treatment were investigated. Besides the bioimaging experiments, qRT-PCR was conducted for the analysis of mRNA expression of RAR target genes and the genes which are possibly involved in metabolism of lycopene. The latter was done in order to get an insight about possible pathways of lycopene bioactivation.

4.1. RARE response in transgenic RARE-Luc mouse

4.1.1. 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. *Figure 7* presents bioimaging results for luciferase activity in various tissues. CD271 induced luciferase activity in brain, testis, spleen, psv complex, s. intestine, thymus, l. intestine, lung, WAT, heart, liver, kidney.

LG268 did not induce luciferase activity in any of the tissues examined. It showed similar expression pattern like control treatment except skin. Combined treatments resulted in activities similar to the treatment with only CD271. The organ specific expression results of synthetic RAR and RXR ligand treatments demonstrated the compatibility of our transgenic mouse model to our experimental design. We confirmed the dependency of RAR transactivation for luciferase activity CD271 (synthetic RAR ligand) treatment.

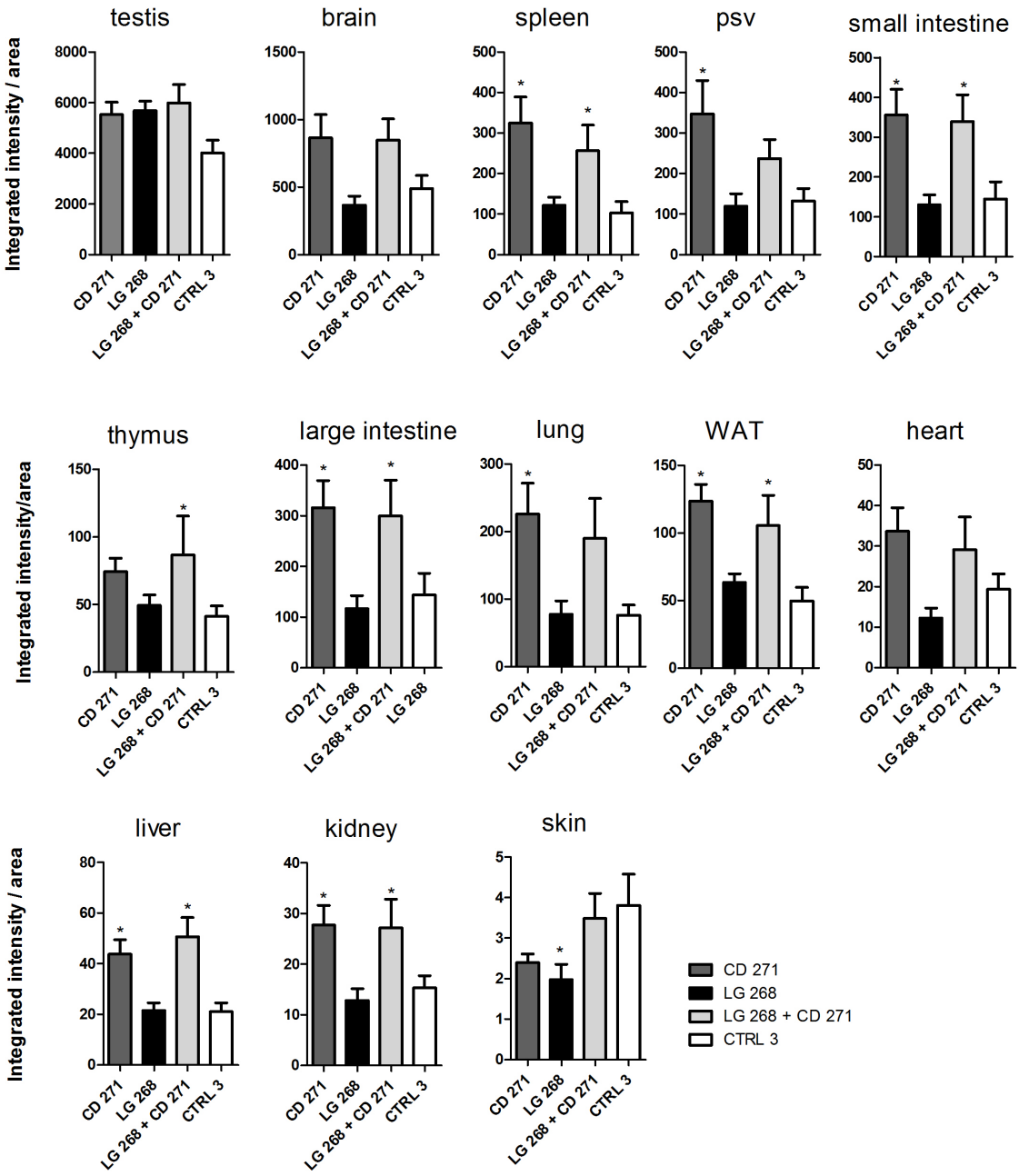


Figure 7. Quantification was based on intensity of light emission calculated by the Andor IQ imaging systems software displayed as integrated intensity / area. The order of the graphs is starting from the highest to the lowest integrated intensity / area in CD271. Control contains 25% aqueous cremophor solution. Experiments were conducted upon CD271 (5 mg / kg bw), LG268 (5 mg/kg bw), CD271 (5 mg/kg bw) + LG268 (5 mg/kg bw) or control treatments. psv; prostate / seminal vesicule complex, wat; white adipose tissue. Values are expressed as mean ± SEM, $n = 6$. (*$p<0.05$).

4.1.2. Organ specific expression of luciferase signal upon lycopene and control-beadlet 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 (See *Figure 8* for further definition). It is followed by the spleen, lung, WAT, s. intestine, l. intestine and liver (*Figure 8*). We analyzed testis, brain, spleen, lung, spleen, WAT, s. intestine, 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 (*Figure 8*) (* $P<0.05$).

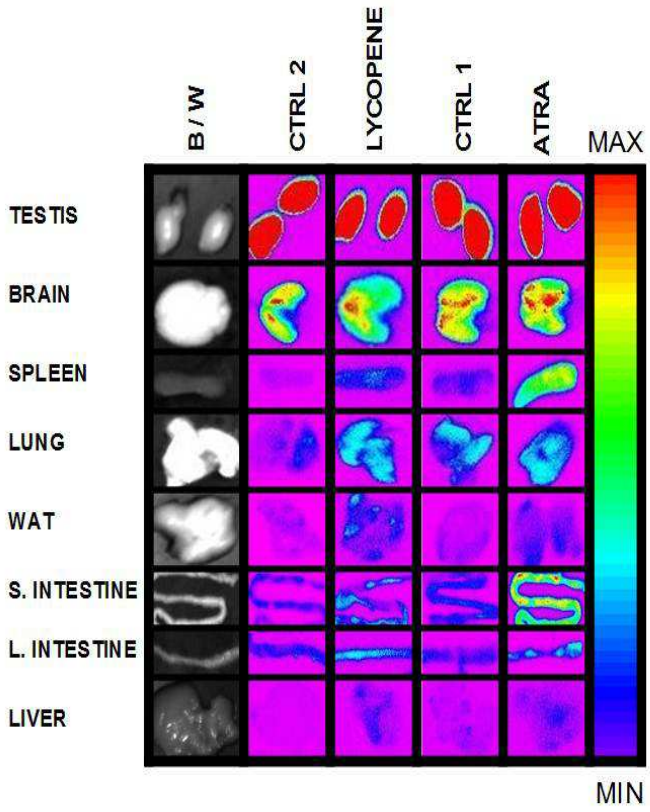


Figure 8. Representative bioimaging pictures of i*n vivo* bioluminescence imaging of male RARE-LUC mice displaying black and white (B/W) photograph for orientation in addition to corresponding bioluminescence images of organ specific expression based on bioluminescence imaging in RARE-LUC male mice upon a single oral gavage treatment with lycopene (100 mg / kg bw), ATRA (50 mg / kg bw) and corresponding CTRL 1 and 2 treatments (experiment 1). The color pattern at the right side of the figure shows the maximum (max) and the minimum (min) bioluminescence signal based on integrated intensity / area. WAT; white adipose tissue, s. intestine; small intestine, l. intestine; large intestine

The organ specific expression was analyzed using quantitative bioimaging analysis (*Figure 9*). 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. During the bioimaging experiment, ATRA demonstrated higher RARE activity than lycopene. The results after plotting graphs can be seen in *Figure 9.*

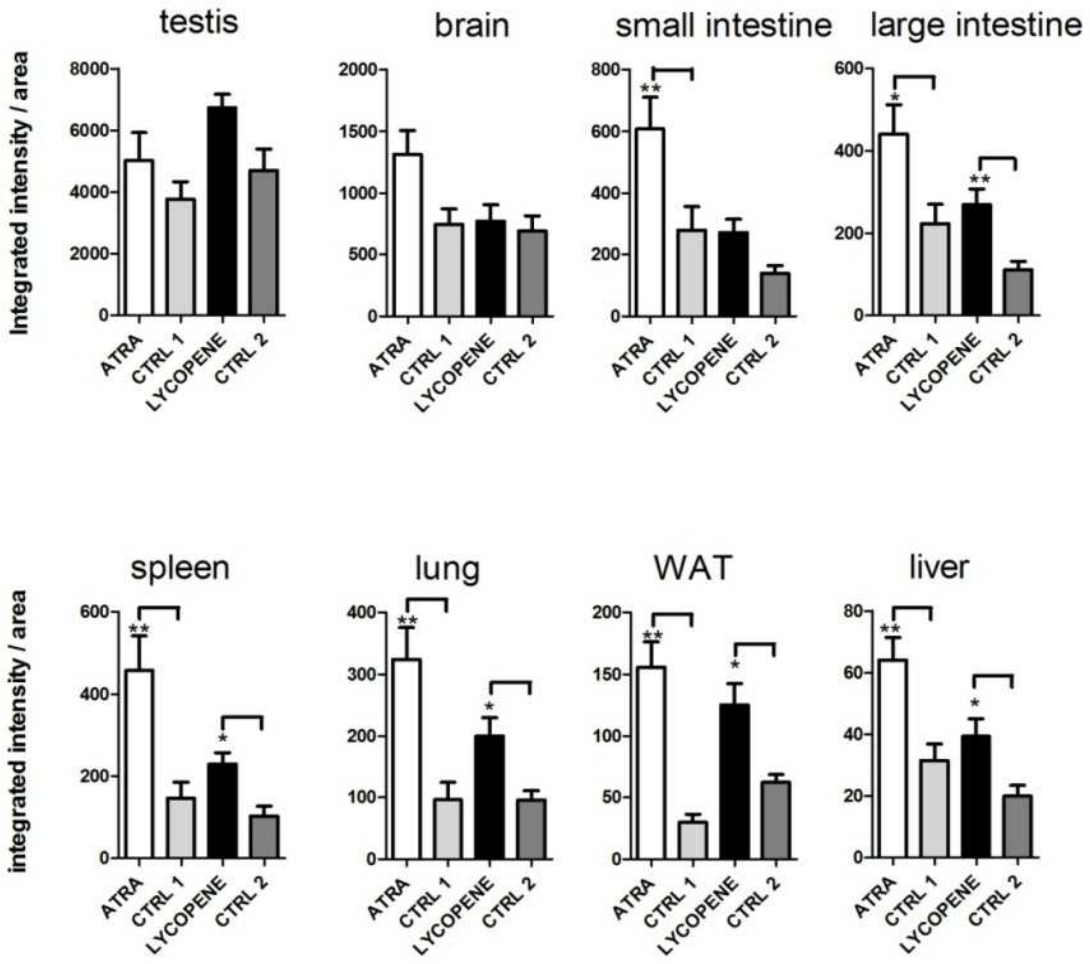
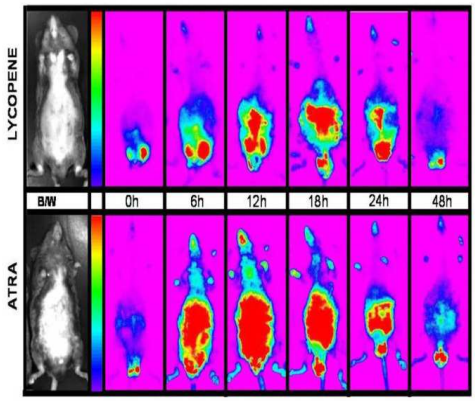


Figure 9. Quantification was based on intensity of light emission calculated by the Andor IQ imaging systems software displayed as integrated intensity / area. The order of the graphs is starting from the highest to the lowest integrated intensity / area data after ATRA-treatment. Values are expressed as mean ± SEM, n = 6. (* p<0.05; ** p<0.01). CTRL 1 indicates control treatment for ATRA and contains 25% aqueous cremophor solution whereas CTRL 2 (1000 mg / kg bw) indicates control treatment for lycopene with lycopene-free beads.

4.1.3. 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 (*Figure 10.A* and *B*). Lycopene treatment increased the RARE mediated luciferase expression non-significantly at 12 h and reached the highest induction level at 18 h (Yokoyama, et al., 2000) 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, s. intestine, l. intestine, liver and adipose tissue regions. However, we could not identify the organ-specific signals using *in vivo* imaging studies except for testis. 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, which peaked after 6 h, than after treatment with lycopene. Bioluminescence imaging results can be seen as integrated intensity/area upon lycopene and ATRA treatments in certain time points (*Figure 10.B*)

A.



B.

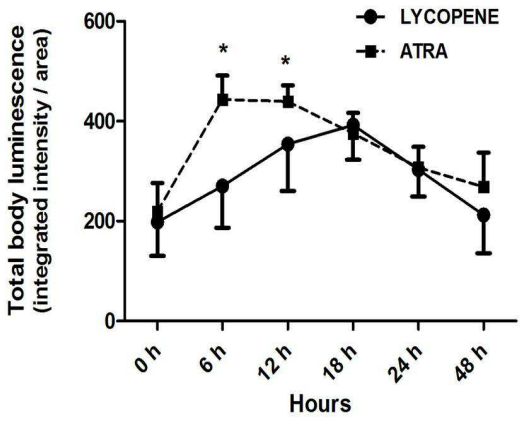


Figure 10.A*)** *In vivo* bioluminescence imaging of male RARE-LUC mice displaying black and white (B/W) photograph for orientation in addition to corresponding bioluminescence images of selected organs of male RARE-LUC mice upon lycopene (100 mg / kg bw) and ATRA (50 mg/kg) (experiment 3) treatments. The color pattern at the right side of the figure shows the maximum and minimum bioluminescence signal based on integrated intensity / area. ***B) Time dependent RARE-LUC signaling in mice upon lycopene and ATRA treatments (experiment 2). Mice were imaged at baseline (0 h) and after 6, 12, 18, 24 and 48 h for total body luminescence. Statistical evaluation was made using repeated measures of 2-way ANOVA test in comparison to the baseline level (0 h). Values are expressed as mean ± SEM, *n=3*. * shows statistically significant upregulation upon treatment. CTRL 1 indicates control treatment for ATRA and contains 25% aqueous cremophor solution whereas CTRL 2 (1000 mg / kg bw) indicates control treatment for lycopene with lycopene-free beads.

4.1.4. 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). *Figure 11.A* and *B* shows the bioluminescence imaging of RARE-LUC female mice upon lycopene and CTRL 2 treatments. In the reproductive organs, same expression profiles were apparent in different genders, uterus in female mice showed the highest basal level (*Figure 11.B*) while testis in male mice displayed the highest basal signal. In female mouse, we observed comparable expression pattern with male mouse. Among the organs spleen showed the highest luminescence signal.

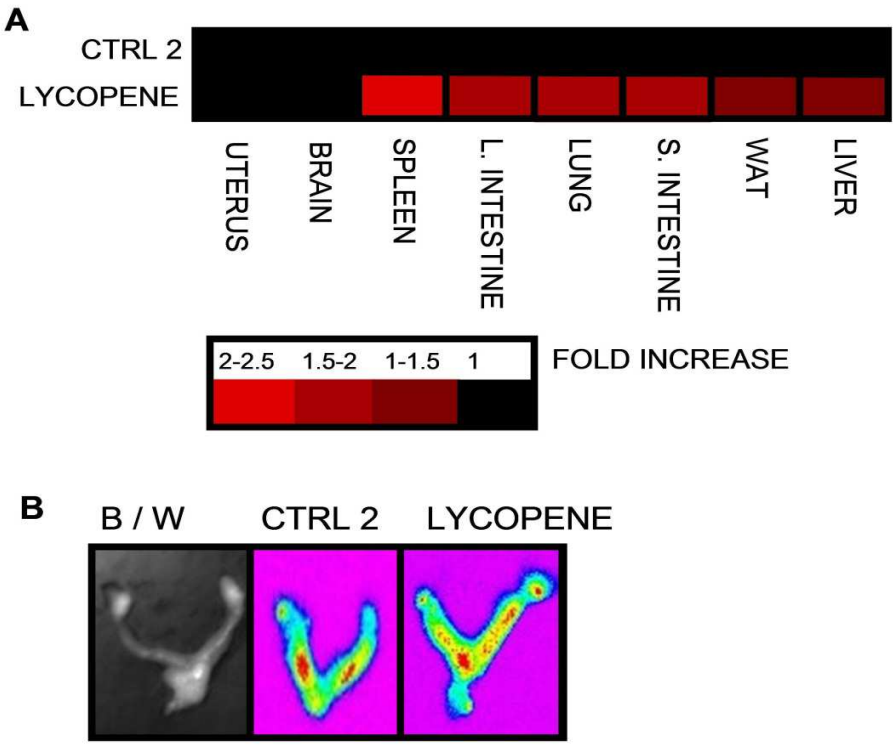


Figure 11.*A*) Heat map generated from bioluminescence data reflecting integrated intensity / area upon lycopene (100 mg / kg bw) and CTRL 2 treatments in female RARE-LUC mice (*n=6*) (experiment 3). ***B***) Representative pictures of uterus as black and white (B/W) for orientation and corresponding bioluminescence images. WAT; white adipose tissue, s. intestine; small intestine, l. intestine; large intestine.

4.1.5. 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 (*Table 3*).

Upon treatment with tomato extract the bioluminescence in the treated mice was similar to those after lycopene treatment (*Figure 12*). We observed induction of RARE-activity in lung, liver, spleen, s. intestine, l. intestine, WAT after tomato extract treatment (*Figure 12*). After tomato extract treatment, spleen displayed higher bioluminescence signal (6 x up-regulation, based *Figure 12*) in comparison to lycopene-treatments.

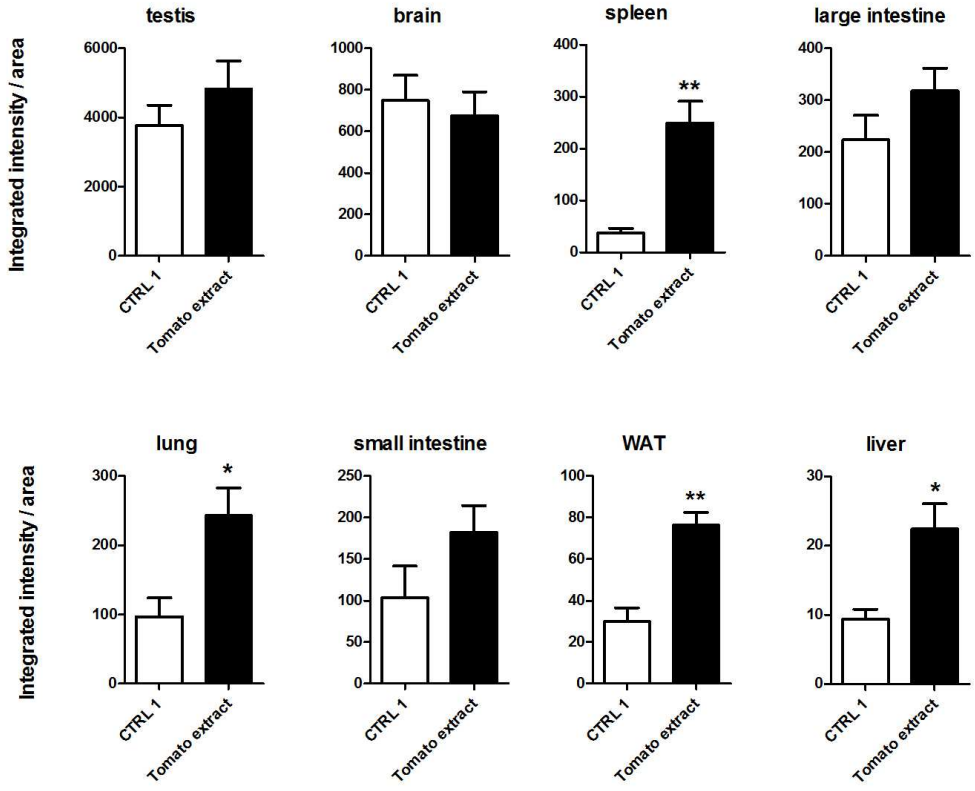


Figure 12. Bioluminescence data reflecting integrated intensity / area upon CTRL 1 and tomato extract treatments in male RARE-LUC mice (experiment 5). * shows statistically significant upregulation upon treatment. (* $p<0.05$; ** $p<0.01$).

4.1.6. 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 (Reynaud, et al., 2011). 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 (Gouranton, et al., 2011).

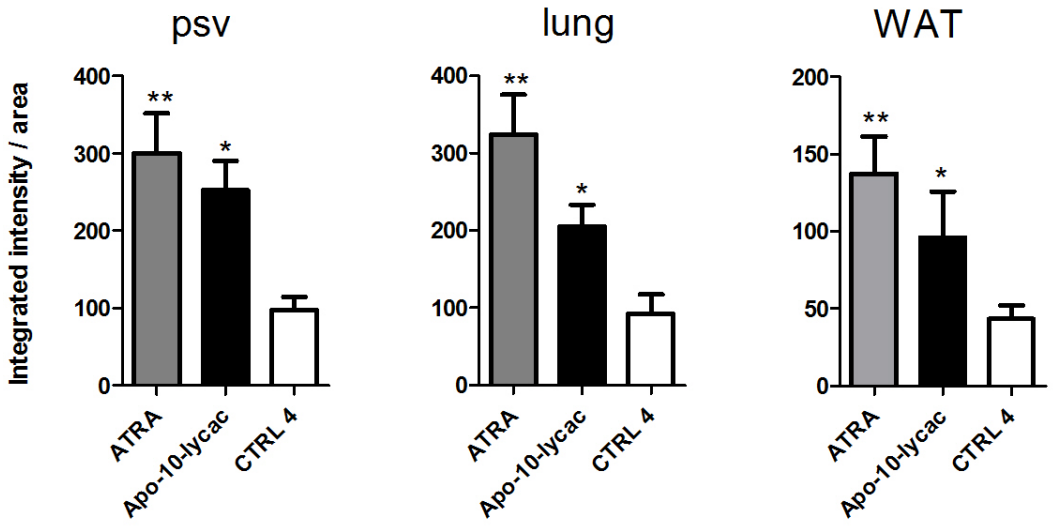


Figure 13. Quantification was based on intensity of light emission calculated by the Andor IQ imaging systems software displayed as integrated intensity / area. The order of the graphs is starting from the highest to the lowest integrated intensity / area data after ATRA-treatment. CTRL 4 represents DMSO. psv; prostate and seminal vesicules complex, WAT; white adipose tissue. Values are expressed as mean ± SEM, n = 6. (* p<0.05;** p<0.01) Bioluminescence imaging results of DMSO and cremophor showed similarity (data not shown). Therefore cremophore results were used as control for both treatments.

4.1.7. 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. The results after plotting graphs can be seen in *Figure 14.*

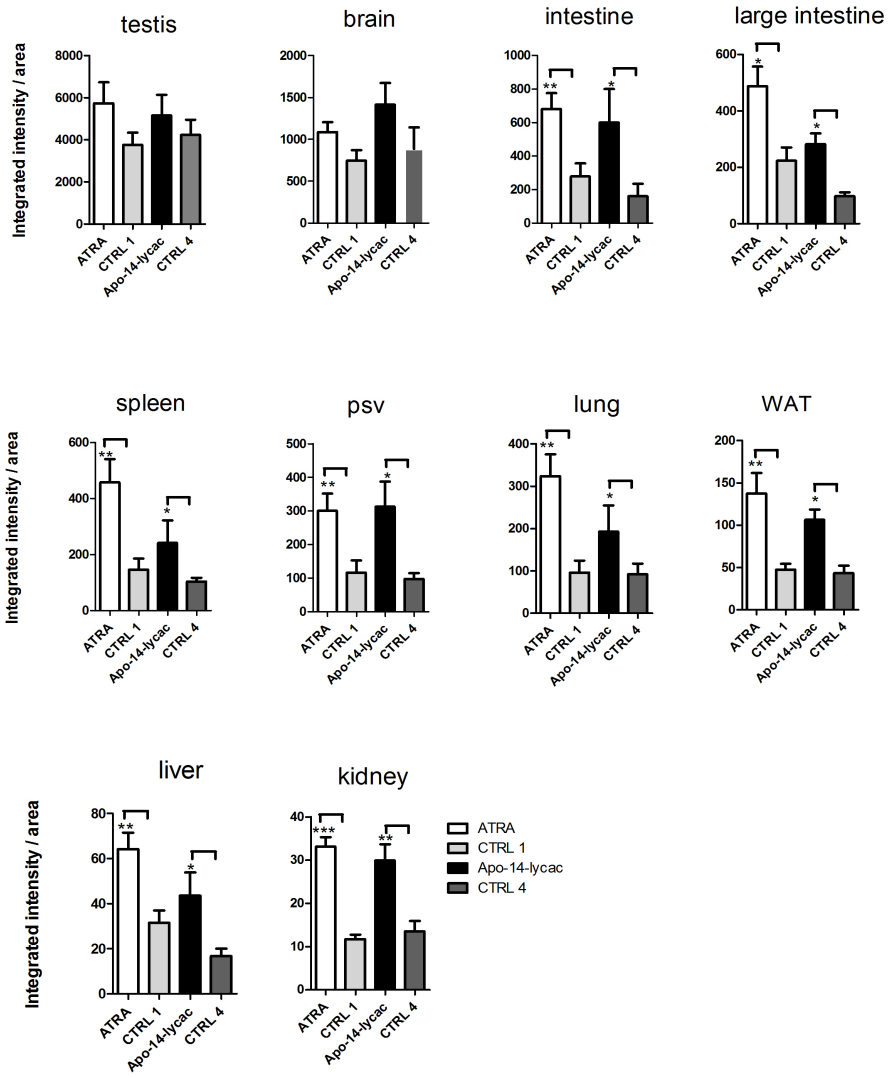


Figure 14. Quantification was based on intensity of light emission calculated by the Andor IQ imaging systems software displayed as integrated intensity / area. The order of the graphs is starting from the highest to the lowest integrated intensity / area data after ATRA-treatment. DMSO was used as CTRL 4. psv; prostate and seminal vesicules complex, WAT; white adipose tissue. Values are expressed as mean ± SEM, $n = 6$. (* $p<0.05$;** $p<0.01$)

4.1.8. 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 (*Figure 15*).

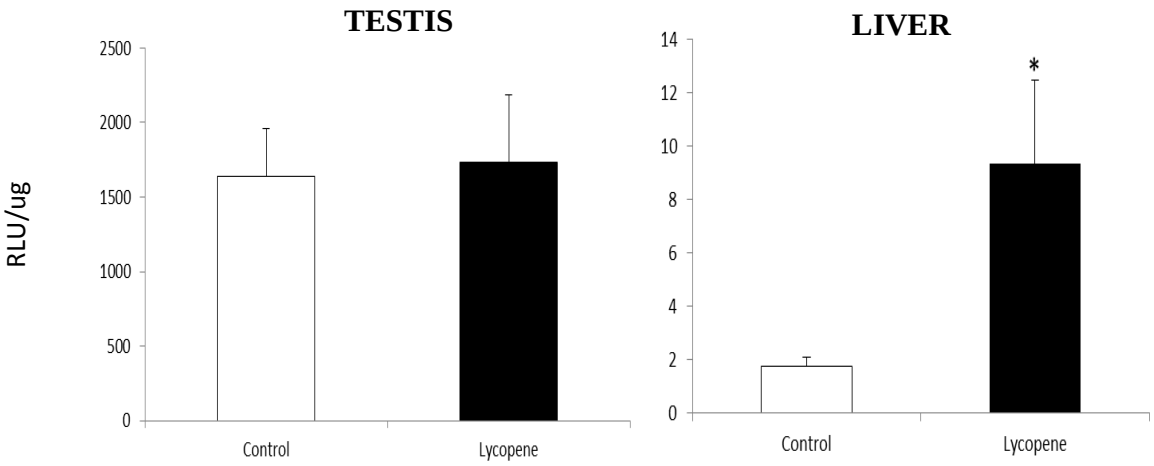


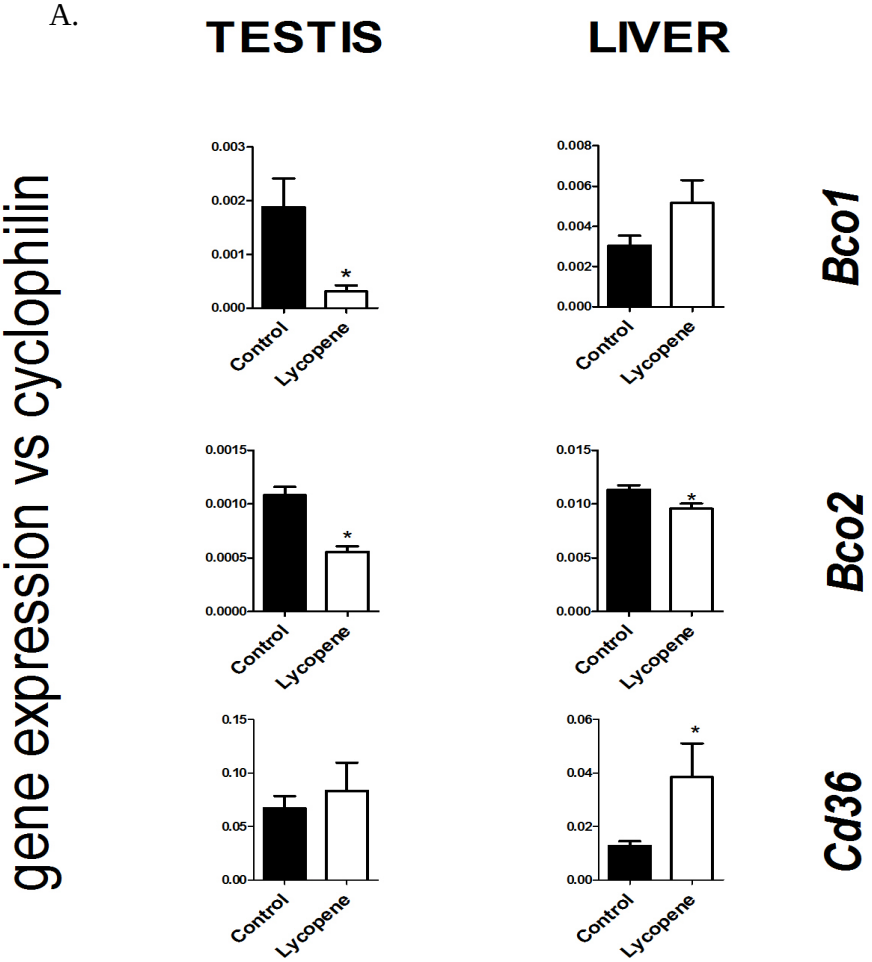
Figure 15. Luciferase protein expression / total protein (*RLU/µg*) analysis for liver and testis upon lycopene and CTRL 2 treatments in male RARE-LUC mice (experiment 5). Values are expressed as mean ± SEM, $n = 6$. (* $p<0.05$;** $p<0.01$)

4.2. 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 (close to significance, P=0.085) upon lycopene treatment (*Figure 16*). *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 (*Figure 16*).

A.



B.

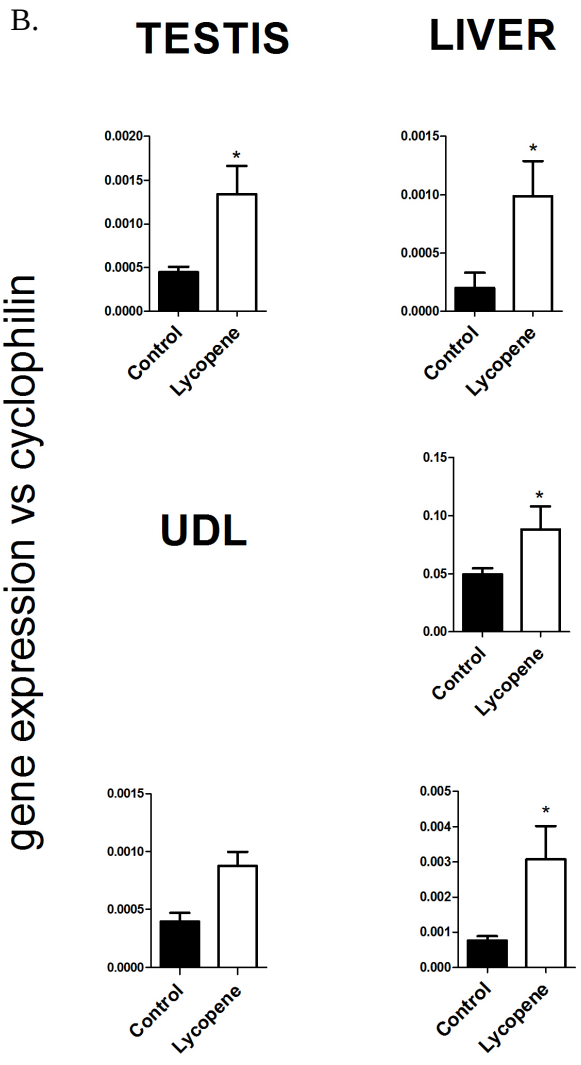


Figure 16.A-B. qRT-P CR analysis of testis and liver tissues for *Bco-1, Bco-2, Cd36, Cyp26b1, Rarres2, Crbp1* upon lycopene and CTRL 2 treatments in male RARE-LUC mice (experiment 9)[9]. Values are expressed as mean ± SEM, $n = 6$. (* $p<0.05$;** $p<0.01$).

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[9] Cyclophilin was used as endogenous housekeeping gene.

5. 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 non-specific carotenoid-transporter (*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 vitamin A 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 (Chambon, 1996, Kastner, et al., 1995). 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) (unpublished data). 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 (unpublished data).

So far, highest un-induced RARE-mediated luciferase activity has been found in the reproductive organs of male (testis) and female mice (Zhuang, et al., 1994) as well as in brain (unpublished data). 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 (Kurlandsky, et al., 1995). Sertoli cells are indicated as the main site of endogenous RA production in testis for normal spermatogenesis through the uptake of retinol from plasma retinol binding protein (RBP) following oxidation of retinol to RA (Cavazzini, et al., 1996). 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

(unpublished data) 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 (Lufkin, et al., 1993, van Pelt and de Rooij, 1991). 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 (*Figure 9*) 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 (*Figure 15*). 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 beadlet and tomato preparation treatments (*Table 3*). 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 (Clinton, 1998, Khachik, et al., 1992). In addition to carotenoids, vitamin C (160–240 mg/kg), vitamin E (5–20 mg/kg) and flavonoids contribute the tomato composition (Davies and Hobson, 1981).

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

(Bessler, et al., 2008, Watzl, et al., 1999). 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 (Reynaud, et al., 2011). 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 (Lian and Wang, 2008). 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* (Lian, et al., 2007). Our data strongly suggest that apo-10-lycac is highly active in terms of the regulation of gene expression in lung, psv complex and white adipose tissue (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 (Lian, et al., 2007). 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 complex, 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 (Gouranton, et al., 2011, Reynaud, et al., 2011).

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 (Moussa, et al., 2011). 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; *P=0.085*, see *Figure 16.A*) 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 (*Figure 10*).

Feedback mechanisms have previously been demonstrated for RA-treatment induced down-regulation of *Bco-1* expression in chickens and rats (Bachmann, et al., 2002). 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 (Reynaud, et al., 2011). These results are consistent with those obtained by Zaripheh et al. 2006 in rat tissue (Zaripheh, et al., 2006). 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.

We can summarize the results of bioluminescence imaging, protein assay and qRT-PCR results as a color diagram in *Figure 17*.

Experiments		TESTIS	LIVER
Experiment 2	RARE-bioimaging		
Experiment 6	Protein assay		
Experiment 9	*Bco-2*		
	Bco-1		

Figure 17. Summary of qRT-PCR of carotene metabolizing enzymes *(Bco-1 and 2),* luciferase protein assay, organ specific bioluminescence imaging based RARE activity after lycopene treatment compared to the CTRL2 in RARE-LUC male mice. Dark red indicates up-regulation (>2), light red indicates up-regulation (>1,2 - <2), white indicates under detection limit, grey indicates no change, dark blue indicates down-regulation, (>1,2 - >2).

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 (Zhuang, et al., 1994) 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 (Kurlandsky, et al., 1995). 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 (Cavazzini, et al., 1996). 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 (unpublished data) 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 (*Figure 11.B*) and the testis (*Figure 8*) as well as the brain indicates the importance of highly controlled RAR-signaling and homeostasis and that the nutritional influence by carotenoids should be kept to a minimum (Lufkin, et al., 1993, van Pelt and de Rooij, 1991). 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.

Though testis has high lycopene content, lycopene supplementation does not influence the luciferase activity. There exist specific pathways and receptors in testis (Lei, et al., 1997, Zechel, 2005). For instance germ cell nuclear factor is a receptor with unknown function and has been just found in testis. It takes place in

differentiation process in testis. Additionally TR4 receptor is also specific to testis. It is known that TR4 competes to RAR to its higher binding affinity and represses the RA induced transactivation (Lee, et al., 1998). Such receptors can prevent the activation of RAR pathways in testis. Thus, we may not observe increased RAR activity by bioimaging in testis.

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 (Braun, et al., 2000, Guo, et al., 2001, O'Shaughnessy, et al., 2007) while RARE-LUC signaling is exclusively based on the RARE-LUC construct mediated activation and signaling (Jaensson-Gyllenbäck, et al., 2011, Svensson, et al., 2008, van de Pavert, et al., 2009). 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 laboratories.

6. SUMMARY

Lycopene is an acyclic carotenoid containing eleven conjugated double bonds and lacks the β-ionine ring structure present typically in pro-vA carotenoids, 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.

7. REFERENCES

7.1. REFERENCES

1. S. Agarwal and A. V. Rao. (2000). Tomato lycopene and its role in human health and chronic diseases. Cmaj. 163, 739-44

2. L. Altucci and H. Gronemeyer. (2001). Nuclear receptors in cell life and death. Trends Endocrinol Metab. 12, 460-8

3. A. Aranda and A. Pascual. (2001). Nuclear hormone receptors and gene expression. Physiological Reviews. 81, 1270-95

4. H. Bachmann, A. Desbarats, P. Pattison, M. Sedgewick, G. Riss, A. Wyss, N. Cardinault, C. Duszka, R. Goralczyk and P. Grolier. (2002). Feedback regulation of beta,beta-carotene 15,15'-monooxygenase by retinoic acid in rats and chickens. J Nutr. 132, 3616-22

5. H. Bessler, H. Salman, M. Bergman, Y. Alcalay and M. Djaldetti. (2008). In vitro effect of lycopene on cytokine production by human peripheral blood mononuclear cells. Immunol Invest. 37, 183-90

6. R. Blomhoff and H. K. Blomhoff. (2006). Overview of retinoid metabolism and function. J Neurobiol. 66, 606-30

7. V. Bohm and R. Bitsch. (1999). Intestinal absorption of lycopene from different matrices and interactions to other carotenoids, the lipid status, and the antioxidant capacity of human plasma. Eur J Nutr. 38, 118-25

8. A. C. Boileau, N. R. Merchen, K. Wasson, C. A. Atkinson and J. W. J. Erdman. (1999). Cis-lycopene is more bioavailable than trans-lycopene in vitro and in vivo in lymph-cannulated ferrets. J Nutr. 129, 1176-81.

9. T. W. Boileau, A. C. Moore and J. W. J. Erdman. (1999). Carotenoids and Vitamin A. Antioxidant Status, Diet, Nutrition, and Health; Papas AM, ed. CRC Press LLC:Boca Raton, 133-158

10. K. W. Braun, W. A. Tribley, M. D. Griswold and K. H. Kim. (2000). Follicle-stimulating hormone inhibits all-trans-retinoic acid-induced retinoic acid receptor alpha nuclear localization and transcriptional activation in mouse Sertoli cell lines. J Biol Chem. 275, 4145-51

11. H. Carlsen, J. Ø. Moskaug, S. H. Fromm and R. Blomhoff. (2002). In vivo imaging of NF-kappa B activity. J Immunol. 168, 1441-6

12. J. J. Castenmiller and C. E. West. (1998). Bioavailability and bioconversion of carotenoids. Annu Rev Nutr. 18, 19-38

13. D. Cavazzini, M. Galdieri and S. Ottonello. (1996). Retinoic acid synthesis in the somatic cells of rat seminiferous tubules. Biochim Biophys Acta. 1313, 139-45

14. N. Chalabi, L. Delort, S. Satih, P. Dechelotte, Y. J. Bignon and D. J. Bernard-Gallon. (2007). Immunohistochemical Expression of RAR{alpha}, RAR{beta}, and Cx43 in Breast Tumor Cell Lines After Treatment With Lycopene and Correlation With RT-QPCR. J Histochem Cytochem. 55, 877-883

15. P. Chambon. (1996). A decade of molecular biology of retinoic acid receptors. Faseb J. 10, 940-54

16. B. A. Clevidence and J. G. Bieri. (1993). Association of carotenoids with human plasma lipoproteins. Methods Enzymol. 214, 33-46

17. S. K. Clinton. (1998). Lycopene: chemistry, biology, and implications for human health and disease. Nutr Rev. 56(2 Pt 1), 35-51

18. P. S. Danielian, R. White, J. A. Lees and M. G. Parker. (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. EMBO J. 11, 1025-33

19. J. N. Davies and G. E. Hobson. (1981). The constituents of tomato fruit--the influence of environment, nutrition, and genotype. Crit Rev Food Sci Nutr. . 15, 205-80

20. P. Di Mascio, S. Kaiser and H. Sies. (1989). Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys. 274, 532-8

21. S. L. Drop, A. G. Schuller, D. J. Lindenbergh-Kortleve, C. Groffen, A. Brinkman and E. C. Zwarthoff. (1992). Structural aspects of the IGFBP family. Growth Regul. 2, 69-79

22. V. Ershov Yu, V. Bykhovsky and A. A. Dmitrovskii. (1994). Stabilization and competitive inhibition of beta-carotene 15,15'-dioxygenase by carotenoids. Biochem Mol Biol Int. 34, 755-63

23. H. Esterbauer and P. Ramos. (1996). Chemistry and pathophysiology of oxidation of low density lipoprotein. Rev. Physiol. Biochem. Pharmacol. 127, 31-64

24. R. M. Evans. (1998). The steroid and thyroid hormone receptor superfamily. Science. 240, 889–895

25. B. Frei. (1994). Reactive oxygen species and antioxidant vitamins: mechanisms of action. Am J Med. . 97, 5S-12S

26. M. Gajic, S. Zaripheh, F. Sun and J. W. Erdman, Jr. (2006). Apo-8'-lycopenal and apo-12'-lycopenal are metabolic products of lycopene in rat liver. J Nutr. 136, 1552-7

27. J. Ghosh and C. E. Myers. (2002). Molecular mechanisms of prostate cancer cell death triggered by inhibition of arachidonate 5-lipoxygenase: involvement of Fas death receptor-mediated signals. Adv Exp Med Biol. 507, 415-20

28. V. Giguere, E. S. Ong, P. Segui and R. M. Evans. (1987). Identification of a receptor for the morphogen retinoic acid. Nature. . 330, 624-9

29. E. Gouranton, G. Aydemir, E. Reynaud, J. Marcotorchino, C. Malezet, C. Caris-Veyrat, R. Blomhoff, J. F. Landrier and R. Rühl. (2011). Apo-10'-lycopenoic acid impacts adipose tissue biology via the retinoic acid receptors. Biochim Biophys Acta. . 1811, 1105-14

30. T. E. Gundersen, N. E. Bastani and R. Blomhoff. (2007). Quantitative high-throughput determination of endogenous retinoids in human plasma using triple-stage liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom. 21, 1176-86

31. X. Guo, P. Morris and L. Gudas. (2001). Follicle-stimulating hormone and leukemia inhibitory factor regulate Sertoli cell retinol metabolism. Endocrinology. 142, 1024-32

32. S. E. Hankinson, W. C. Willett, G. A. Colditz, D. J. Hunter, D. S. Michaud, B. Deroo, B. Rosner, F. E. Speizer and M. Pollak. (1998). Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet. 351, 1393-6

33. E. Hazai, Z. Bikádi, F. Zsila and S. F. Lockwood. (2006). Molecular modeling of the non-covalent binding of the dietary tomato carotenoids lycopene and lycophyll, and selected oxidative metabolites with 5-lipoxygenase. Bioorg Med Chem. 14, 6859-67

34. A. Herzog, U. Siler, V. Spitzer, N. Seifert, A. Denelavas, P. B. Hunziker, W. Hunziker, R. Goralczyk and K. Wertz. (2005). Lycopene reduced gene expression of steroid targets and inflammatory markers in normal rat prostate. Faseb J. 19, 272-4

35. K. Q. Hu, C. Liu, H. Ernst, N. I. Krinsky, R. M. Russell and X. D. Wang. (2006). The biochemical characterization of ferret carotene-9',10'-monooxygenase catalyzing cleavage of carotenoids in vitro and in vivo. J Biol Chem. 281, 19327-38

36. E. Jaensson-Gyllenbäck, K. Kotarsky, F. Zapata, E. K. Persson, T. E. Gundersen, R. Blomhoff and W. W. Agace. (2011). Bile retinoids imprint intestinal CD103(+) dendritic cells with the ability to generate gut-tropic T cells. Mucosal Immunol. 4, 438-47

37. L. A. Kaplan, J. M. Lau and E. A. Stein. (1990). Carotenoid composition, concentrations, and relationships in various human organs. Clin Physiol Biochem. . 8, 1-10

38. P. Kastner, M. Mark and P. Chambon. (1995). Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? Cell. 83, 859-69

39. F. Khachik, G. R. Beecher and J. C. J. Smith. (1995). Lutein, lycopene, and their oxidative metabolites in chemoprevention of cancer. J Cell Biochem Suppl. 22, 236-46

40. F. Khachik, M. B. Goli, G. R. Beecher, J. Holden, W. R. Lusby, M. D. Tenorio and M. R. Barrera. (1992). The effect of food preparation on qualitative and quantitative distribution of major carotenoid constituents of tomatoes and several green vegetables. J Agric Food Chem. 40, 390-398

41. F. Khachik, C. J. Spangler, J. C. Smith, Jr., L. M. Canfield, A. Steck and H. Pfander. (1997). Identification, quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum. Anal Chem. 69, 1873-81

42. H. M. Khandwala, I. E. McCutcheon, A. Flyvbjerg and K. E. Friend. (2000). The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. Endocr Rev. 21, 215-44

43. C. Kiefer, S. Hessel, J. M. Lampert, K. Vogt, M. O. Lederer, D. E. Breithaupt and J. von Lintig. (2001). Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. J Biol Chem. 276, 14110-6

44. T. J. King, F. Khachik, H. Bortkiewicz, L. H. Fukushima, S. Morioka and J. S. Bertram. (1997). Metabolites of dietary carotenoids as potential cancer preventive agents. Pure Appl. Chem. 69, 2135-40

45. K. W. Kong, H. E. Khoo, K. N. Prasad, A. Ismail, C. P. Tan and N. F. Rajab. (2010). Revealing the Power of the Natural Red Pigment Lycopene. Molecules. 15, 959-87.

46. N. I. Krinsky and E. J. Johnson. (2005). Carotenoid actions and their relation to health and disease. Mol Aspects Med. . 26, 459-516

47. S. B. Kurlandsky, M. V. Gamble, R. Ramakrishnan and W. S. Blaner. (1995). Plasma delivery of retinoic acid to tissues in the rat. J Biol Chem. 270, 17850-7

48. C. M. Lee, A. C. Boileau, T. W. Boileau, A. W. Williams, K. S. Swanson, K. A. Heintz and J. W. Erdman, Jr. (1999). Review of animal models in carotenoid research. J Nutr. 129, 2271-7

49. Y. F. Lee, W. J. Young, J. P. Burbach and C. Chang. (1998). Negative feedback control of the retinoid-retinoic acid/retinoid X receptor pathway by the human TR4 orphan receptor, a member of the steroid receptor superfamily. J Biol Chem. 273, 13437-43

50. W. Lei, T. Hirose, L. X. Zhang, H. Adachi, M. J. Spinella, E. Dmitrovsky and A. M. Jetten. (1997). Cloning of the human orphan receptor germ cell nuclear factor/retinoid receptor-related testis-associated receptor and its differential regulation during embryonal carcinoma cell differentiation. J Mol Endocrinol. 18, 167-76

51. M. Leid, P. Kastner and P. Chambon. (1992). Multiplicity generates diversity in the retinoic acid signalling pathways. Trends Biochem. Sci. 17, 427-433

52. F. Lian, D. E. Smith, H. Ernst, R. M. Russell and X. D. Wang. (2007). Apo-10'-lycopenoic acid inhibits lung cancer cell growth in vitro, and suppresses lung tumorigenesis in the A/J mouse model in vivo. Carcinogenesis. 28, 1567-74

53. F. Lian and X. D. Wang. (2008). Enzymatic metabolites of lycopene induce Nrf2-mediated expression of phase II detoxifying/antioxidant enzymes in human bronchial epithelial cells,. Int. J. Cancer. 123, 1262–1268.

54. A. Lindqvist, Y. G. He and S. Andersson. (2005). Cell type-specific expression of beta-carotene 9',10'-monooxygenase in human tissues. J Histochem Cytochem. 53, 1403-12

55. M. Lorenz, M. Fechner, J. Kalkowski, K. Frohlich, A. Trautmann, V. Bohm, G. Liebisch, S. Lehneis, G. Schmitz, A. Ludwig, G. Baumann, K. Stangl and V. Stangl. (2012). Effects of lycopene on the initial state of atherosclerosis in New Zealand White (NZW) rabbits. PLoS One. 7, e30808

56. T. Lufkin, D. Lohnes, M. Mark, A. Dierich, P. Gorry, M. P. Gaub, M. LeMeur and C. P. (1993). High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. Proc Natl Acad Sci U S A. 90, 7225-9

57. A. Lukanova, E. Lundin, P. Toniolo, A. Micheli, A. Akhmedkhanov, S. Rinaldi, P. Muti, P. Lenner, C. Biessy, V. Krogh, A. Zeleniuch-Jacquotte, F. Berrino, G. Hallmans, E. Riboli and R. Kaaks. (2002). Circulating levels of insulin-like growth factor-I and risk of ovarian cancer. Int J Cancer. 101, 549-54

58. D. J. Mangelsdorf. (1994). Vitamin A receptors. Nutr. Rev. 52, S32-S34

59. C. S. Mantzoros, A. Tzonou, L. B. Signorello, M. Stampfer, D. Trichopoulos and H. O. Adami. (1997). Insulin-like growth factor 1 in relation to prostate cancer and benign prostatic hyperplasia. Br J Cancer. 76, 1115-8

60. H. R. Matos, P. Di Mascio and M. H. Medeiros. (2000). Protective effect of lycopene on lipid peroxidation and oxidative DNA damage in cell culture. Arch Biochem Biophys. 383, 56-9

61. G. Mese, G. Richard and T. W. White. (2007). Gap junctions: basic structure and function. J Invest Dermatol. 127, 2516-24

62. N. J. Miller, J. Sampson, L. P. Candeias, P. M. Bramley and C. A. Rice-Evans. (1996). Antioxidant activities of carotenes and xanthophylls. FEBS Lett. 384, 240-42

63. J. Molnar, N. Gyemant, I. Mucsi, A. Molnar, M. Szabo, T. Kortvelyesi, A. Varga, P. Molnar and G. Toth. (2004). Modulation of multidrug resistance and apoptosis of cancer cells by selected carotenoids. In Vivo. 18, 237-44

64. M. Moussa, E. Gouranton, B. Gleize, C. E. Yazidi, I. Niot, P. Besnard, P. Borel and J. F. Landrier. (2011). CD36 is involved in lycopene and lutein uptake by adipocytes and adipose tissue cultures. Mol Nutr Food Res. 55, 578-84

65. L. Nagy and J. W. Schwabe. (2004). Mechanism of the nuclear receptor molecular switch. Trends Biochem Sci. 29, 317-24

66. J. L. Napoli. (1996). Retinoic acid biosynthesis and metabolism. FASEB J. 10, 993-1001

67. J. L. Napoli. (1993). Retinoic acid synthesis from beta-carotene in vitro. Methods Enzymol. 214, 193-202

68. P. J. O'Shaughnessy, M. Abel, H. M. Charlton, B. Hu, H. Johnston and P. J. Baker. (2007). Altered expression of genes involved in regulation of vitamin A metabolism, solute transportation, and cytoskeletal function in the androgen-insensitive tfm mouse testis. Endocrinology. 148, 2914-24

69. M. Petkovich, N. J. Brand, A. Krust and P. Chambon. (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature. 330, 444-50

70. A. V. Rao and S. Agarwal. (2000). Role of antioxidant lycopene in cancer and heart disease. J Am Coll Nutr. 19, 563-9

71. T. M. Redmond, S. Gentleman, T. Duncan, S. Yu, B. Wiggert, E. Gantt and F. X. Cunningham, Jr. (2001). Identification, expression, and substrate specificity of a mammalian beta-carotene 15,15'-dioxygenase. J Biol Chem. 276, 6560-5

72. A. G. Renehan, M. Zwahlen, C. Minder, S. T. O'Dwyer, S. M. Shalet and M. Egger. (2004). Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet. 363, 1346-1353

73. E. Reynaud, G. Aydemir, R. Rühl, O. Dangles and C. Caris-Veyrat. (2011). Organic synthesis of new putative lycopene metabolites and preliminary investigation of their cell-signaling effects. J Agric Food Chem. 59, 1457-63

74. J. Riedl, J. Linseisen, J. Hoffmann and G. Wolfram. (1999). Some dietary fibers reduce the absorption of carotenoids in women. J Nutr. 129, 2170-6

75. J. M. Roldan and M. D. Luque de Castro. (2007). Lycopene: The need for better methods for characterization and determination. Trends Analyt Chem. 26, 163-70

76. A. C. Ross. (1993). Cellular metabolism and activation of retinoids: roles of cellular retinoid-binding proteins. FASEB J. 7, 317-27

77. R. Rühl, R. Thiel, T. S. Lacker, S. Strohschein, K. Albert and H. Nau. (2001). Synthesis, high-performance liquid chromatography-nuclear magnetic resonance characterization and pharmacokinetics in mice of CD271 glucuronide. J Chromatogr B Biomed Sci Appl. 757, 101-9

78. J. T. Salonen, K. Nyyssönen, R. Salonen, E. Porkkala-Sarataho, T. P. Tuomainen, U. Diczfalusy and I. Björkhem. (1997). Lipoprotein oxidation and progression of carotid atherosclerosis. Circulation. 95,

79. J. T. Salonen, S. Yla-Herttuala, R. Yamamoto, S. Butler, H. Korpela, R. Salonen, K. Nyyssönen, W. Palinski and J. L. Witztum. (1992). Autoantibody against oxidised LDL and progression of carotid atherosclerosis. Lancet. 339, 883-87

80. F. J. Schweigert. (1998). Metabolism of carotenoids in mammals. In Britton, G., Liaaen‐Jensen, S., Pfander, H. (eds) Carotenoids. 3, Biosynthesis & Metabolism, Birkhäuser Verlag, Basel, 249–284

81. S. Seren, R. Lieberman, U. D. Bayraktar, E. Heath, K. Sahin, F. Andic and O. Kucuk. (2008). Lycopene in cancer prevention and treatment. Am J Ther. 15, 66-81

82. J. Shi, M. Le Maguer and M. Bryan. (2002). Lycopene from tomatoes In: Shi J, Mazza G, Le Maguer M, eds. . In Functional Foods-Biochemical and Processing Aspects.Eds.; CRC Press: USA. 2, 135-68

83. H. Sies and W. Stahl. (1998). Lycopene: antioxidant and biological effects and its bioavailability in the human. Proc Soc Exp Biol Med. . 218, 121-4

84. U. Siler, L. Barella, V. Spitzer, J. Schnorr, M. Lein, R. Goralczyk and K. Wertz. (2004). Lycopene and vitamin E interfere with autocrine/paracrine loops in the Dunning prostate cancer model. FASEB J. 18, 1019-21

85. E. R. Silveira and F. S. Moreno. (1998). Natural retinoids and β-carotene: from food to their actions on gene expression. J Nutr Biochem. 9, 446-56

86. W. Stahl, W. Schwarz, A. R. Sundquist and H. Sies. (1992). cis-trans isomers of lycopene and beta-carotene in human serum and tissues. Arch Biochem Biophys. 294, 173-7

87. W. Stahl and H. Sies. (1996). Lycopene: A biologically important carotenoid for humans? Arch Biochem Biophys. 336, 1-9

88. W. Stahl and H. Sies. (1992). Uptake of lycopene and its geometrical isomers is greater from heat-processed than from unprocessed tomato juice in humans. J Nutr. 122, 2161-6

89. N. Suh, W. W. Lamph, A. L. Glasebrook, T. A. Grese, A. D. Palkowitz, C. R. Williams, R. Risingsong, M. R. Farris, R. A. Heyman and M. B. Sporn. (2002). Prevention and treatment of experimental breast cancer with the combination of a new selective estrogen receptor modulator, arzoxifene, and a new rexinoid, LG 100268. Clin Cancer Res. 8, 3270-5

90. M. Svensson, B. Johansson-Lindbom, F. Zapata, E. Jaensson, L. M. Austenaa, R. Blomhoff and W. W. Agace. (2008). Retinoic acid receptor signaling levels and antigen dose regulate gut homing receptor expression on CD8+ T cells. Mucosal Immunol. 1, 38-48

91. L. H. Tonucci, J. M. Holden, G. Beecher, F. Khachik, C. S. Davis and G. Mulokozi. (1995). Carotenoid content of thermally processed tomato-based food products. J. Agric. Food Chem. 43, 579-586

92. S. A. van de Pavert, B. J. Olivier, G. Goverse, M. F. Vondenhoff, M. Greuter, P. Beke, K. Kusser, U. E. Höpken, M. Lipp, K. Niederreither, R. Blomhoff, K. Sitnik, W. W. Agace, T. D. Randall, W. J. de Jonge and R. E. Mebius. (2009). Chemokine CXCL13 is essential for lymph node initiation and is induced by retinoic acid and neuronal stimulation. Nat Immunol. 10, 1193-9

93. A. M. van Pelt and D. G. de Rooij. (1991). Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells. Endocrinology. 128, 697-704

94. J. von Lintig and K. Vogt. (2000). Filling the gap in vitamin A research. Molecular identification of an enzyme cleaving beta-carotene to retinal. J Biol Chem. 275, 11915-20

95. B. Watzl, A. Bub, B. R. Brandstetter and G. Rechkemmer. (1999). Modulation of human T-lymphocyte functions by the consumption of carotenoid-rich vegetables. Br J Nutr. 82, 383-9

96. S. Werner, K. Buser, A. Kapp and T. Werfel. (2002). The incidence of atopic dermatitis in school entrants is associated with individual life-style factors but not with local environmental factors in Hannover, Germany. Br J Dermatol. 147, 95-104

97. K. J. Yeum, A. L. dos Anjos Ferreira, D. Smith, N. I. Krinsky and R. M. Russell. (2000). The effect of alpha-tocopherol on the oxidative cleavage of beta-carotene. Free Radic Biol Med. 29, 105-14

98. A. Yokoyama, T. Hamazaki, A. Ohshita, N. Kohno, K. Sakai, G. D. Zhao, H. Katayama and K. Hiwada. (2000). Effect of aerosolized docosahexaenoic acid in a mouse model of atopic asthma. Int Arch Allergy Immunol. 123, 327-32

99. H. Yu, M. R. Spitz, J. Mistry, J. Gu, W. K. Hong and X. Wu. (1999). Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis. J Natl Cancer Inst. 91, 151-156

100. S. Zaripheh, T. Y. Nara, M. T. Nakamura and J. W. Erdman, Jr. (2006). Dietary lycopene downregulates carotenoid 15,15'-monooxygenase and PPAR-gamma in selected rat tissues. J Nutr. 136, 932-8

101. C. Zechel. (2005). The germ cell nuclear factor (GCNF). Mol Reprod Dev. 72, 550-6

102. Y. H. Zhuang, T. Ylikomi, M. Lindfors, S. Piippo and P. Tuohimaa. (1994). Immunolocalization of retinoic acid receptors in rat, mouse and human ovary and uterus. J Steroid Biochem Mol Biol. 48, 61-8

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