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A review about lycopene-induced nuclear hormone receptor signalling in inflammation and lipid metabolism via still unknown endogenous apo-10´-lycopenoids

Dedicated in memoriam to Paola Palozza (†21.05.2013)

Short title: Lycopene and nuclear hormone receptor mediated signalling

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Abstract:

Lycopene is the red pigment in tomatoes and tomato products and is an important dietary carotenoid found in the human organism. Lycopene-isomers, oxidative lycopene metabolites and apo-lycopenoids are found in the food matrix. Lycopene intake derived from tomato consumption is associated with alteration of lipid metabolism and a lower incidence of cardiovascular diseases (CVD). Lycopene is mainly described as a potent antioxidant but novel studies are shifting towards its metabolites and their capacity to mediate nuclear receptor signalling. *Di-/tetra*-hydro-derivatives of apo-10´-lycopenoic acid and apo-15´-lycopenoic acids are potential novel endogenous mammalian lycopene metabolites which may act as ligands for nuclear hormone mediated activation and signalling. In this review, we postulate that complex lycopene metabolism results in various lycopene metabolites which have the ability to mediate transactivation of various nuclear hormone receptors like RARs, RXRs and PPARs. A new mechanistic explanation of how tomato consumption could positively modulate inflammation and lipid metabolism is discussed.

1. Health beneficial effects of lycopene are linked to various nuclear hormone receptor signalling pathways

High consumption of fruit and vegetables is associated with a lower risk of CVD (Leenders et al., 2013). Dietary carotenoids, in particular lycopene, are among the constituents in various fruits and vegetables and their dietary intake and serum levels are linked to these protective effects. Mechanistic explanations to lycopene effects are mainly focused on its well established antioxidant activity via inhibition and scavenging of reactive oxygen species (ROS) production. However, current research is exploring novel mechanisms such as modulation of inflammatory responses (Palozza et al., 2010) and activation of nuclear hormone receptors (Aydemir et al., 2012; Aydemir et al., 2013; Ben-Dor et al., 2001; Eroglu and Harrison, 2013; Harrison et al., 2012; Lindshield et al., 2007; Stahl and Sies, 1996).

Epidemiological studies suggest a negative association between low serum lycopene concentrations and high risk of coronary events and stroke (Karppi et al., 2012; Rissanen et al., 2001), although this has been refuted by others (Karppi et al., 2013; Sesso et al., 2005). In a cross-sectional study, biomarkers for the risk of coronary biomarkers (total cholesterol and total cholesterol:HDL ratio) were clinically improved in women consuming 24.3 mg of lycopene/day of tomato rich products compared to women consuming the lowest intake of 3.6 mg lycopene/day (Sesso et al., 2012). To further explain this, evidence from experimental studies suggests that lycopene may act through modulation of inflammation in atherosclerotic processes and obesity (Palozza et al., 2010).

Key regulators of metabolic pathways linked to adipogenesis as well as to inflammation mechanisms in the cardiovascular system are the Peroxisome Proliferator Activated Receptors (PPARa, γ, δ/β)(Menendez-Gutierrez et al., 2012). Synthetic ligands of PPAR's have shown to exert beneficial effects identified by lower CVD risk markers (Millar, 2013). PPARa agonists are limiting

postprandial lipoprotein responses and increase HDL-cholesterol via lowering of chylomicron production (Colin et al., 2012). PPARγ agonists down-regulate inflammation via immunomodulation of adipose tissue which results in improved insulin sensitivity (Cipolletta et al., 2012). PPARδ/β agonists inhibit macrophage foam cell formation leading to lower inflammatory responses induced by very low density lipoprotein (Bojic et al., 2012). PPAR's also respond to diet-related ligands (reviewed in (Schupp and Lazar, 2010)) and can therefore modulate target gene expression. This regulation has the potential to modify lipid metabolism and inflammation pathways.

Cell studies using human macrophages have already shown that lycopene is able to lower the formation of atherosclerotic plaques by lowering pro-inflammatory responses via NF-kB activation and ROS production (Lorenz et al., 2012; Palozza et al., 2011b). Lycopene also attenuates foam cell formation during cholesterol homeostasis via prevention of PPARγ activation (Palozza et al., 2011a). These cell studies are further supported by evidence from animal models. Lycopene supplementation in atherosclerotic rabbits induced by high cholesterol diet showed a reduction in total and LDL cholesterol in serum but no modifications in aortic lesions (Lorenz et al., 2012). These results support further evidence from human intervention trials on endothelial function (Stangl et al., 2011).

Lycopene activity may be related to PPAR-mediated signalling, thus we suggest that lycopene-metabolites could act as ligands of PPAR´s which can activate transcriptional signalling. Consequently, the activated PPAR forms together with an RXR are, in our case, the major active transcription factors in diet induced gene expression. Here we describe the current evidence on these novel mechanisms with the aim to propose an explanation focused on inflammation and lipid metabolism to evaluate how lycopene intake is related to beneficial health effects.

2. Lycopene and lycopene-metabolites in the food matrix

The main source of lycopene in Western diets is tomato (*Solanum lycopersicum* L.), the second most consumed vegetable in the world. In the USA, it has been estimated that 80% of lycopene is consumed through tomatoes and tomato products (Clinton, 1998). Lycopene content in tomato can vary depending on the variety of the fruit, its ripeness degree, but also on the environmental conditions (temperature, soil, etc.) (Dumas et al., 2003).

In red tomatoes, lycopene is usually present in its most thermodynamic stable form, the (all-E)-form. Tomato and tomato based products also contain various geometric lycopene-isomers, hydroxy- / oxo- / epoxy-metabolites in addition to apo-lycopenoids (Figure 1), though in lower concentrations than (all-E)-lycopene. (All-E)-lycopene can undergo isomerisation during processing and various lycopene isomers are detectable in processed tomato products (Chanforan et al., 2006). Lycopene 1,2-epoxide and 5,6-epoxide have been found in fresh tomato (Ben-Aziz et al., 1973), whereas only lycopene 1,2-epoxide was found in tomato paste and juice (Khachik et al., 1998). Another lycopene epoxide, namely the 2,6-cyclolycopene-1,5 epoxide has been found in its 2 diastereoisomeric forms in these tomato food products (Khachik et al., 1998). This molecule could be the precursor of 2,6-cyclolycopene-1,5 diol, previously detected in tomato paste (Tonucci et al., 1995). Other oxidative lycopene-metabolites containing alcohol groups (Yokota et al., 1997) were isolated in low quantity from tomato puree and identified as 1,5-*di*-hydroxy-iridanyl-lycopene and 2,6-cyclolycopene-1-methoxy-5-ol and 1,16-*di*-dehydro-2,6-cyclolycopene-5-ol (Yokota et al., 2003).

Lycopene or oxidative lycopene-metabolites can further be converted to apo-lycopenoids via targeted enzymatic cleavage or via unspecific chemical cleavage of lycopene´s hydrocarbon structure. Long-chain apo-lycopenoids, i.e. apo-6'-lycopenal and apo-8'-lycopenal have been found in extracts of tomato paste (Winterstein et al., 1960) with an estimated content

of 5 µg/g. Later, these were also found in lower levels in raw tomatoes, and in addition further three apo-lycopenals (apo-6‘-, apo-8‘-, apo-10‘-, apo-12‘- and apo-14‘-lycopenals) were partly identified and partly predicted to be present in tomato paste and in lower levels in raw tomatoes (Kopec et al., 2010).

3. Lycopene metabolism and molecular mechanisms of action

Lycopene mechanism of action in biological systems has been thoroughly studied and mainly described as being related to its antioxidant activity (Erdman et al., 2009; Stahl and Sies, 1996). In addition, lycopene activity could be mediated through its known and still unknown metabolites (Aydemir et al., 2012; Aydemir et al., 2013; Ben-Dor et al., 2001; Dela Sena et al., 2013; Eroglu and Harrison, 2013; Ferreira et al., 2003; Stahl et al., 2000). These unknown metabolites could be the relevant bioactive molecules because they are comparable to the structure of the retinoic acids which are the major biologically active metabolites of β-carotene. The possibility that lycopene and/or its metabolites act as ligands to initiate nuclear hormone receptor mediated signalling has not been a major research focus but recent observations by us and others point towards this direction (Aydemir et al., 2012; Aydemir et al., 2013; Gouranton et al., 2011). Using reporter animals for the retinoic acid reporter element (RARE), we found that lycopene can activate RARE-mediated signalling in various organs in a similar fashion to that of retinoic acids (Aydemir et al., 2012). Based on our own observations (Aydemir et al., 2013), we hypothesized that retinoid-like lycopene metabolites could act as bioactive molecules which can interact with RAR/RXR (Aydemir et al., 2013; Gouranton et al., 2011). Recently, we identified the endogenous RXR-ligand, 9-cis-13,14-dihydroretinoic acid which may also indirectly originate from tomatoes and tomato products (Rühl et al., 2015). RXR-ligands can also activate various heterodimers like RXR-LXR´s and RXR-PPAR´s. These are involved in glucose and lipid homeostasis (Dawson and Xia,

2011; Perez et al., 2011; Shulman and Mangelsdorf, 2005; Shulman et al., 2004). Thus, RAR and RXR-ligands derived from tomato provide an alternative mechanistic explanation to the beneficial effects of lycopene intake on CVD prevention (Dawson and Xia, 2011; Liu et al., 2000; Miyazaki et al., 2010; Perez et al., 2011). We propose that based on lycopene-mediated RAR- and RXR-pathways the key lycopene derived substances responsible for these RAR- and RXR-mediated effects are the apo-15´-carotenoid acids, in particular *di-/tetra*-hydro-apo-15´-lycopenoic acids (figure 2). These are linear retinoic acid analogues comparable to β-carotene metabolites which are potent activators of several cell and receptor mediated mechanisms.

Other lycopene derivatives such as apo-10´-lycopenoic acid (Aydemir et al., 2013; Ford et al., 2010; Gouranton et al., 2011) and in particular *di-/tetra*-hydro-apo-10´-lycopenoids can also act as ligands of nuclear hormone receptors. Relevant receptors to focus upon as a potential target for these ligands are the PPAR´s. PPAR´s bind a large variety of PUFAs and their metabolites (Forman et al., 1997; Forman et al., 1995; Kliewer et al., 1995; Shiraki et al., 2005). Many of these PUFAs and PUFA-metabolites are of longer chain length than the apo-15´-lycopenoids. PUFA-metabolite chain length is more in the range of apo-10´-lycopenoids or apo-12´-lycopenoids, which suggest that these structures are more likely potential ligands. Apo-10´-carotenoids are described as BCO2-metabolites originating from excentric cleavage of carotenoids and lycopene seems to be a good substrate for this metabolic cleavage pathway (Hu et al., 2006; Lobo et al., 2012). Apo-10´-lycopenoic acid may origin from dietary lycopene either as products of oxidation (via BCO2-cleavage) or isomerisation and oxidation (figure 2) (Amengual et al., 2013; Hu et al., 2006). To date, apo-10´-lycopenoic acid has not been identified to be present endogenously (Gouranton et al., 2011; Hu et al., 2006), but recently 7,8-*di*-hydro-apo-10´-lycopenoic acid has been proposed as a potential endogenous lycopene metabolite in mammals (Gouranton et al., 2011). In addition, apo-10'-lycopenal has been predicted to be present together with other apo-lycopenals in human blood serum

after tomato juice consumption for 8 weeks (Kopec et al., 2010). Apo-10´-lycopenal and apo-10´-lycopenoic acid have been synthesized (Reynaud et al., 2011) and further tested in various *in vitro* and *in vivo* models for nuclear receptor activation potential (Catalano et al., 2013; Gouranton et al., 2011; Reynaud et al., 2011). To support the potential role of these lycopene metabolites as PPAR ligands we present novel results of interaction studies of apo-10'-lycopenoic and apo-10'-lycopenal with RXR and PPAR's in two different systems (Figure 3 and 4).

4. Novel activities of lycopene metabolites and potential lycopene metabolites

In this review, we show novel data originating from COS1-based reporter cell lines. We observed no RXR and PPARa, δ/β and γ activation potential for apo-10'-lycopenal and apo-10'-lycopenoic acids, neither in a potential physiological or nutritional relevant range at lower nM concentrations (data not displayed, because treatments ranging from 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}M were all comparable to control-treatments) nor at higher concentrations of 10^{-5}M (figure 3). This indicates no biological relevant direct interaction with nuclear receptors RXR and PPARa, δ/β, γ.

However, when other indirect *in vitro* reporter techniques like target gene expression analysis in MM6 cells (figure 4) were used, we observed that one common PPAR-target gene, namely ADRP / PLIN2, was significantly induced by apo-10´-lycopenal or -lycopenoic acid at 10^{-5}M (figure 4a) to an extent comparable to the PPARa synthetic ligand GW7647 used at relevant active concentrations of 10^{-6}M (figure 4b). Contrary, the expression of other PPAR / RXR-target genes like the enzymes BCO1 (Boulanger et al., 2003) and BCO2 (Gericke et al., 2013) were either non-affected or non-conclusively regulated by these metabolites, this was also confirmed in studies by our groups (Reynaud et al., 2011). This shows that PPAR-mediated signalling of apo-10´-

lycopenoids needs further attention, in particular for apo-10´-lycopenal and apo-10´-lycopenoic acid metabolites.

Apo-10'-lycopenoic acid has not been found yet as an endogenous metabolite *in vivo* and apo-10'-lycopenal has been predicted to be present in human plasma at a concentration of 0.28 ± 0.10 10^{-9}M following a supplementation diet with tomato juice for 8 weeks (Kopec et al., 2010). The concentrations we used in MM6 cell model were higher than the nutritional relevant levels but comparable to concentrations used in the COS1-based reporter cell models. We hypothesize based on our results (Gouranton et al., 2011) and based on results from other studies (Ip et al., 2013; Ip et al., 2015; Lian and Wang, 2008; Lian et al., 2007; Tan et al., 2014), that further metabolic activation may lead to novel biological active metabolites of apo-10´-lycopenal, apo-10´-lycopenoic acid and/or lycopene. We additionally postulate that these novel metabolites can further directly interact and initiate PPARa, δ/β or γ-RXR mediated signalling in lower relevant endogenous or nutritional concentrations. These novel potential metabolites of apo-10'-lycopenoids have not yet been identified, but we are in the process of further identification and investigation in regard to their physiological and nutritional relevance.

In summary, the lycopene metabolite apo-10'-lycopenal and the potential lycopene-metabolite apo-10'-lycopenoic acid were not able to directly activate the nuclear hormone receptors RXR and PPARs in reporter cell lines. We speculate that further metabolites of apo-10'-lycopenal or apo-10'-lycopenoic acid can interact with PPARs and RXR, as indicated by increased ADPR / PLIN2 (representing a PPAR-RXR target gene) expression in MM6-cell lines.

5. Carotenoid metabolites as nuclear hormone receptor mediated signalling agonists: RXR / PPARa, δ/β, γ

Based on simulation experiments using ligand-docking strategies, our groups are currently working to identify lycopene-metabolites which may have the potential to directly interact with nuclear hormone receptors. Using in silico docking studies, we suggest that *di-* or *tetra*-hydro-apo-10´-lycopenoic acids may be present endogenously or after nutritional interventions with tomato and its products (Gouranton et al., 2011). We propose that *di-* or *tetra*-hydro-apo-10´-lycopenoic acids derivatives may also obtain PPAR-activating potential. Our hypothesis is based on the fact that these derivatives have similar shape and structure elements to those of PPAR-activators as shown in figure 5. Comparison of the docking poses (AutoDoc: (Morrison et al., 1999)) of the endogenous PPARγ-ligand 15-deoxy-d12,14-prostaglandin J2 (15-deoxy-d12,14-PgJ2) and a potential lycopene-metabolite *tetra*-hydro-apo-10´-lycopenoic acid after removal of the fibrate ligand (Nolte et al., 1998) shows similar organization in the PPARγ-binding pocket (figure 6). These observations let us postulate the potential of comparable shaped lycopene-metabolites to be physiologically relevant for PPAR-mediated signalling. Several solutions for the docking of the more flexible lycopene metabolite were found. These multiple binding options are compatible with the large Y-shaped ligand binding pocket of the PPAR subtypes (Markt et al., 2008).

Conclusion

We previously described the potential metabolite 7,8-*di*-hydro-apo-10´-lycopenoic acid (Gouranton et al., 2011) originating from apo-10´-lycopenoic acid as a relevant lycopene metabolite derived from a food matrix and related to tomato / lycopene ingestion. PPAR-mediated systemic effects on lipid and glucose metabolism (in particular insulin sensitivity) are relevant to CVD risk factors. Based on our observations we postulate a connection between tomato intake, lycopene intake, lycopene metabolism and nuclear hormone receptor activation with a focus on the activation of RXR and

PPARa, δ/β, γ mediated pathways by lycopene metabolites. These novel potential lycopene metabolites are suggested to be *di-/tetra*-hydro-derivatives of apo-15´-lycopenoic acid and apo-10´-lycopenoic acid. These metabolites may directly activate RXRs and PPARs in relevant endogenous or nutritional levels and may therefore alter gene expression which could result in physiological events related to inflammation processes, and/or lipid and glucose metabolism. These proposed interactions and pathways are a novel explanation for the protective health effects of lycopene and tomato, in particular those related to lipid metabolism, inflammation and insulin sensitivity. Our hypothesis guarantees to perform further targeted investigations to test and further elucidate these proposed mechanisms.

Our groups will focus on the identification of various bioactive lycopene metabolites namely *di-/tetra*-hydro-derivatives of apo-15´-lycopenoic acid and apo-10´-lycopenoic acid using mainly HPLC-MS techniques. Unfortunately, the commonly used animal models, mice and rats, are not ideal models for carotenoid metabolism to be extrapolated to humans due to their different carotenoid nutri-kinetic. Nevertheless, gerbils can be an alternative because they are a model more suitable for comparisons with humans (Lee et al., 1999). When novel bioactive endogenous lycopene metabolites are identified, targeted organic synthesis can make them available in larger quantities for further biological testing in *in vitro* as well as *in vivo* models. We expect that via our planed experimental strategies soon we will find important new pathways to explain how tomatoes and tomato-products can influence and prevent various chronic diseases relevant to humans. Targeted strategies based on identification of levels of these bioactive compounds derived from food intake can be used as disease biomarkers and will help to develop and plan better nutritional strategies for prevention of various chronic diseases based on altered lipid metabolism and inflammation.

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Figure legends:

Figure 1: Representative examples of lycopene-metabolites present in the human diet and organism: Lycopene isomers, oxidative lycopene metabolites and apo-lycopenoids.

Figure 2: Lycopene metabolism and potential mediation of nuclear hormone receptor activation. Retinoic acid receptor (RAR), retinoid X receptor (RXR), Peroxisomal proliferator-activated receptor (PPAR).

Figure 3: Relative induction of PPARa, PPARβ/δ, PPARγ and RXR activation in reporter cell lines by apo-10´-lycopenoic acid (apo10L-ac), apo-10´-lycopenal (apo10L-ald) or PPAR-selective synthetic or endogenous activators of each specific receptor. WY14643 (PPARa), RSG (Rosiglitazone, PPARγ), GW0742 (PPARβ/δ) or 9CRA (RXR), each n=4..

Used methodologies: COS1 cells were maintained in DMEM medium with 10% FBS, 5% L-glutamine, 1% penicillin streptomycin in 24-well plates and transfections were carried out in triplicates. Cells were transfected with equal amounts of relevant plasmids including Gal-RXRα-LBD for RXR-reporter line or Gal-PPAR$\alpha/\delta/\gamma$-LBD and Gal-RXRα-LBD for PPAR-RXR reporter line, a reporter plasmid (luciferase MH100-TKLuc reporter construct with GAL-binding site (Nagy et al., 1999) and beta-galactosidase (for transfection efficiency calculation). The resulting normalized values are plotted as a bar graph ± the standard error. For details of transfection and measurements see (Rühl et al., 2015).

Figure 4: a) Fold induction of ADRP expression by apo-10´-lycopenoic acid (apo10L-ac), apo-10´-lycopenal (apo10L-ald), each n=3 or b) synthetic and selective activators of PPARa, β/δ and γ (WY14643 (PPARa), GW7845 (PPARγ), GW1516 (PPARβ/δ), each n=3.

Used methodologies; target gene analysis in Mono Mac 6 (MM6) cell in vitro: MM6 cells were maintained in RPMI-1640 medium containing 10% foetal bovine serum, 5% L-glutamine, supplemented with 0.1 % penicillin-streptomycin and kept under controlled atmosphere at 37 °C and 5% CO_2. Cells were subcultured every two days at a density of approximately 10^6 cells/ ml. Prior plating and counted by means of a Bürker chamber, centrifuged at 1000 rpm with a Jouan C312 centrifuge and the obtained cell pellets were resuspended in RPMI-1640 medium containing 10% charcoal stripped serum, 5% L-glutamine, supplemented with 0.1% penicillin-streptomycin. Cells were incubated for 6 hours and apo10L-ac, apo10L-ald and PPARa, δ/β, γ-selective synthetic agonists (at relevant active concentration of 10^{-6}M) were added in an amount of 3 µl / well. Cells were incubated for 48 hours at 37 °C and 5 % CO_2. Total RNA was isolated from cultured cells using Tri reagent solution according to the manufacturer´s instructions. Before real-time quantitative PCR (QRT-PCR), total RNA was reverse transcribed into cDNA using the Super Script II First-Standard Synthesis System (Invitrogen). QRT-PCR was carried out in triplicate using Taqman probes on an ABI Prism 7900. mRNA levels were normalized to the level of cyclophilin, which served as an internal control for the amount of RNA used in each reaction. The resulting normalized values are plotted as a bar graph ± the standard error. Sequence Detector software (version 2.1) was used for data analysis.

Figure 5: PPAR activators: 15-deoxy-d12,14-PgJ2 (endogenous relevant PPARγ activator), Rosiglitazone (synthetic PPARγ activator) and 7,8,11,12-*tetra*-hydro-apo-10´-lycopenoic acid (as a potential lycopene-derived PPAR activator).

Figure 6: Docking poses of 15-deoxy-d12,14-PgJ2 (grey structure) and 7,8,11,12-*tetra*-hydro-apo-10´-lycopenoic acid (yellow structure) bound to PPARγ (PDB code 2i4j). 7,8,11,12-*tetra*-hydro-apo-10´-lycopenoic acid binds in a similar way that others agonists of PPARγ such as the fibrate derivative and the carboxylic acid have contact with Tyr473 and His 323 and His 449 and Ser289 (not shown for clarity).

DOCKING METHODS: The genetic algorithm (Morrison et al., 1999) implemented in AutoDock with the fibrate-bound PPARγ crystal structure ((PDB code 2i4j) (Nolte et al., 1998) upon removal of the ligand was used to generate different PPARγ 15-deoxy-d12,14-PGJ2 and 7,8,11,12-tetra-hydro-apo-10´-lycopenoic acid conformers by randomly changing torsion angles and the overall orientation of the molecules. A volume for exploration was defined in the shape of a three-dimensional cubic grid with a spacing of 0.3 Å that enclosed the residues that are known to make up the inhibitors binding pocket. At each grid point, the receptor's atomic affinity potentials for carbon and hydrogen atoms present in the studied ligands were pre-calculated for rapid intra- and intermolecular energy evaluation of the docking solutions for each inhibitor. To obtain additional validation of the proposed binding mode for the ligands, program GRID (http://www.moldiscovery.com) was also used to search for sites on the enzyme that could be complementary to the functional groups present in this inhibitor. The probes used were C3 (methyl CH3 group), COO- (aliphatic carboxylate). For the GRID calculations, a 18Å × 21Å ×21Å lattice of points spaced at 0.5 Å was established at the binding site. The dielectric constants chosen were 4.0 for the macromolecule and 80.0 for the bulk water. Several solutions for the docking of the more flexible 7,8,11,12-tetra-hydro-apo-10´-lycopenoic acid were found and are compatible with the large Y-shaped LBP of the PPAR subtypes (Markt et al., 2008).

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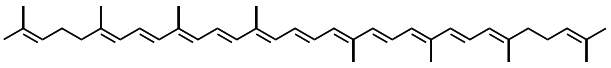
Figure 1:

Fig. 1

(all-*E*)-lycopene



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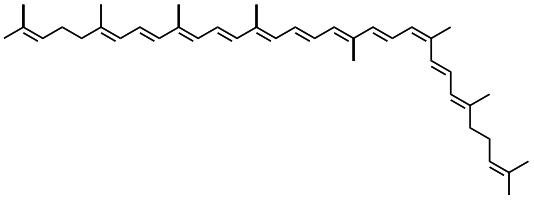

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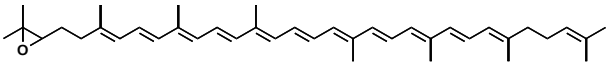

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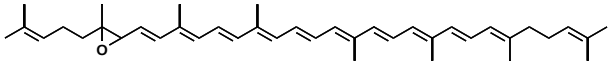

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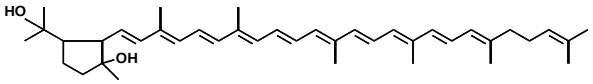
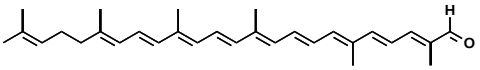


apo-8´-lycopenal



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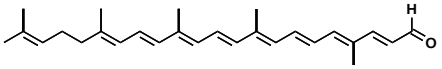
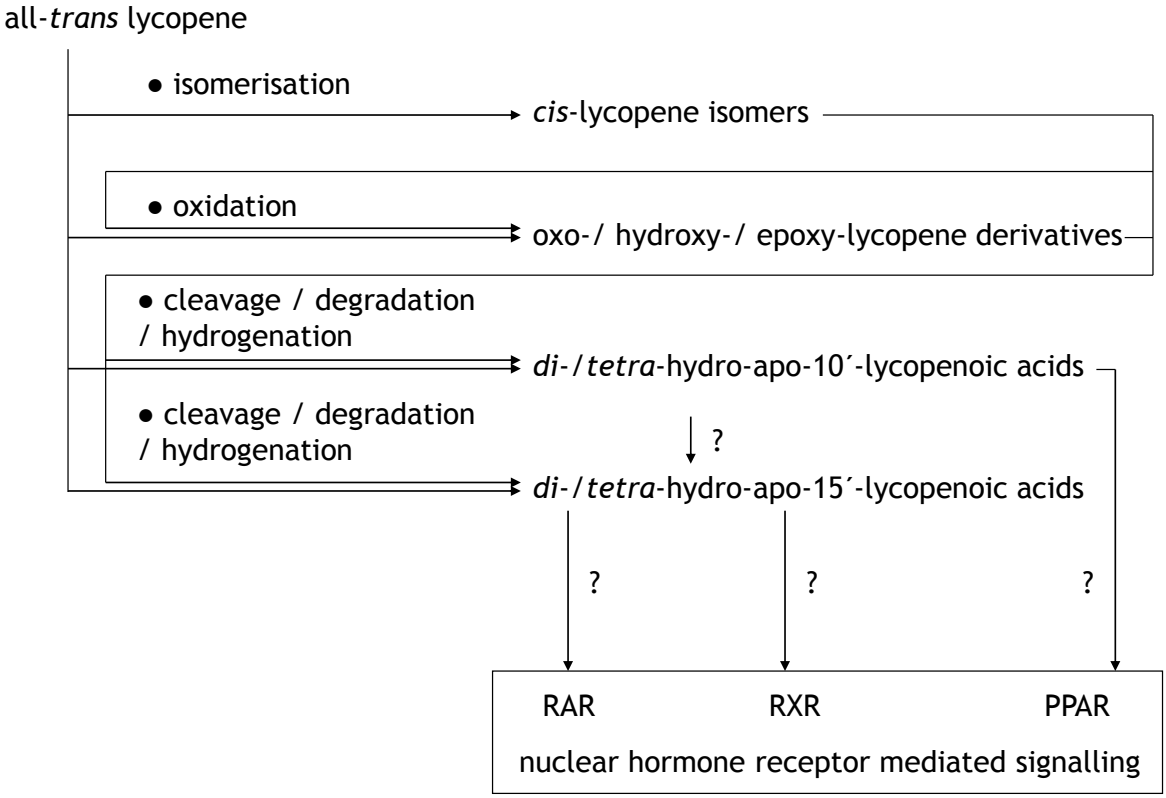


Figure 2:

Fig. 2



673 **Figure 3:**

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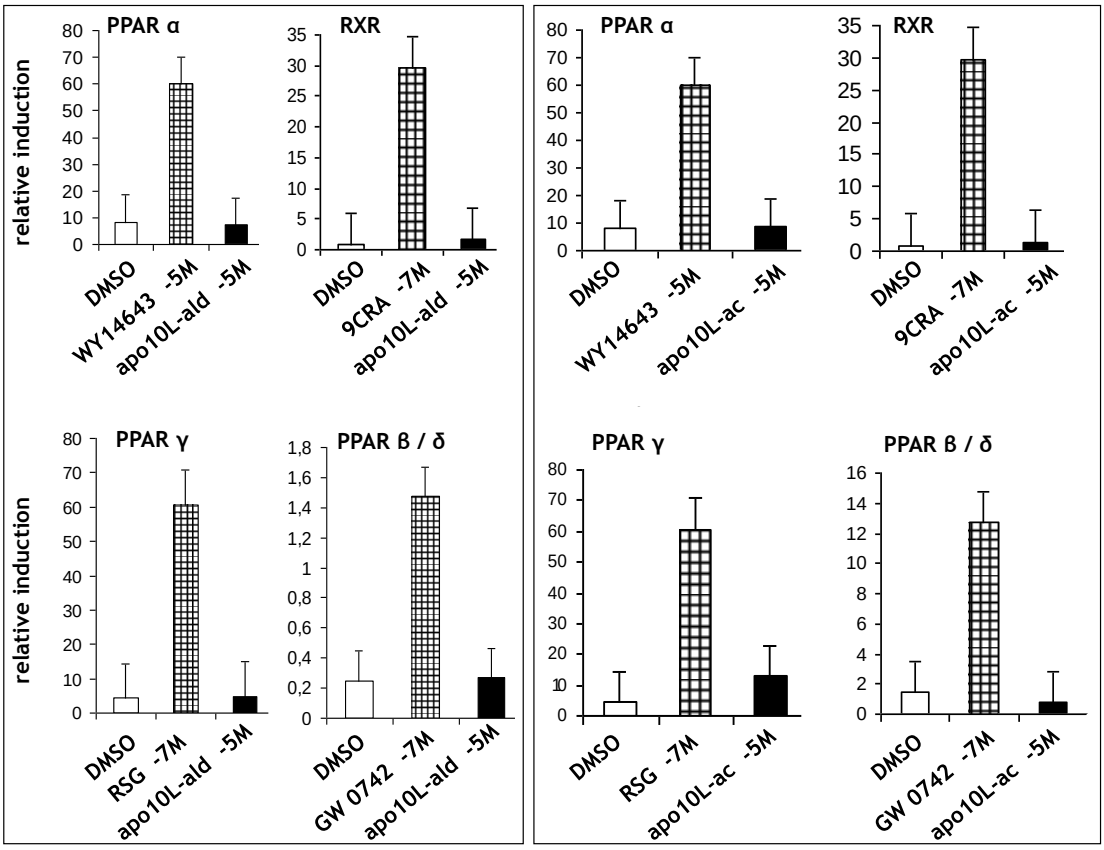

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677 **Figure 4:**

Fig. 4

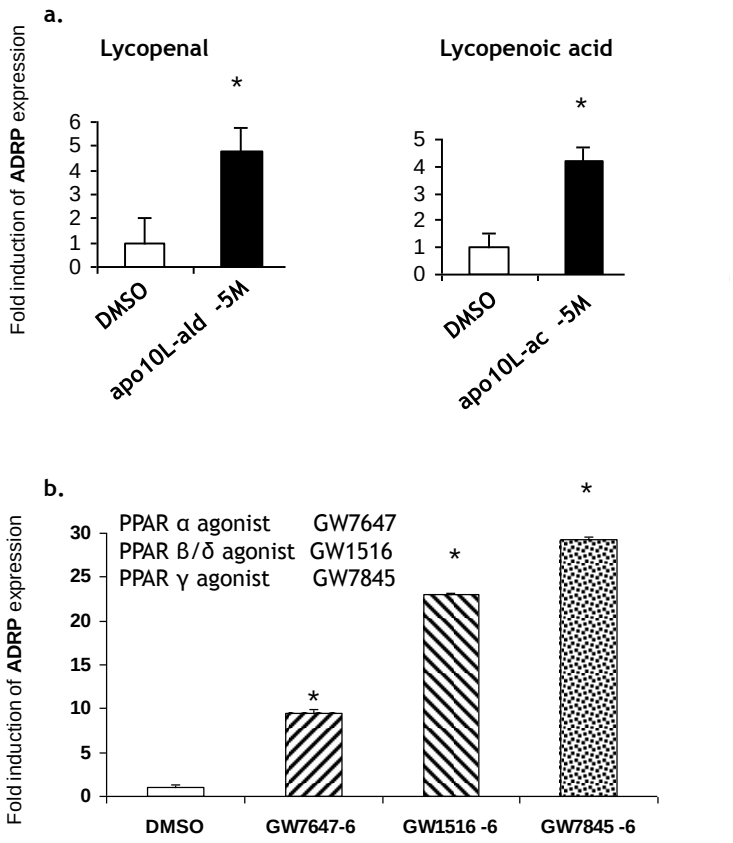

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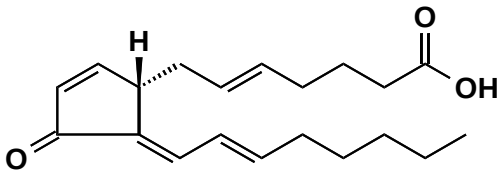
Figure 5:

Fig. 5



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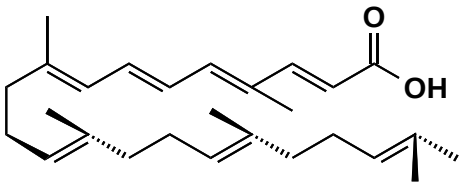
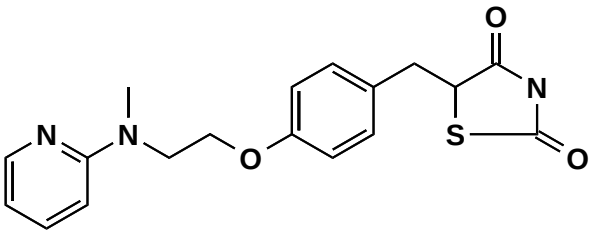


7,8,11,12-*tetra*-hydro-apo-10´-lycopenoic acid



Rosiglitazone

Figure 6:

Fig. 6