Published in final edited form as:

J Agric Food Chem. 2015 February 25; 63(7): 2013–2025. doi:10.1021/jf5056584.

Egg yolks inhibit activation of NF- κ B and expression of its target genes in adipocytes after partial delipidation

Qiwen Shen¹, Ken M. Riedl², Rachel M. Cole¹, Christopher Lehman¹, Lu Xu^{1,3}, Hansjuerg Alder⁴, Martha A. Belury¹, Steven J. Schwartz², and Ouliana Ziouzenkova^{1,#}

¹Department of Human Sciences, The Ohio State University, Columbus, Ohio, 43210, USA

²Department of Food Science and Technology, The Ohio State University, Columbus, Ohio, 43210, USA

³Division of Minimally Invasive Surgery, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China

⁴Nucleic Acid Shared Resource, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio, 43210, USA

Abstract

How composition of egg yolk (EY) influences NF- κ B, a key transcription pathway in inflammation, remains unclear. We performed partial delipidation of EY that removed 20–30% of cholesterol and triglycerides. The resulting polar and non-polar fractions were termed EY-P and EY-NP. NF- κ B activation in response to EY from different suppliers and their fractions was examined in 3T3-L1 adipocytes using a NF- κ B response element reporter assay and by analyzing expression of 248 inflammatory genes. Although EY-P and EY contained similar level of vitamins, carotenoids, and fatty acids, only delipidated EY-P fraction suppressed NF- κ B via down-regulation of toll like receptor-2 and up-regulation of inhibitory toll interacting protein (Tollip) and lymphocyte antigen 96 (Ly96). Our data suggest that anti-inflammatory activity of lutein and retinol were blunted by non-polar lipids in EY likely via crosstalk between SREBP and NF- κ B pathways in adipocytes. Thus, moderate delipidation may improve their beneficial properties of regular eggs.

[#]Corresponding author: O.Z., 1787 Neil Avenue, 331A Campbell Hall, Columbus, OH, 43210, ziouzenkova.1@osu.edu, Telephone: 614 292 5034, Fax: 614 292 8880.

Keywords

Egg yolk; delipidation; inflammation; lipids; NF- κ B; vitamins

Introduction

Chronic inflammation accompanies all degenerative metabolic disorders, including obesity, type 2 diabetes, cardiovascular diseases, cancer, and aging.^{1, 2} Moreover, inflammation is causatively linked to the development and progression of these disorders.^{3–5} In different diseases, inflammation is mediated by specific cytokines that are produced from different cell types. In obesity, chronic inflammation is associated with the production of inflammatory chemokines (CXC) and CC chemokine ligands (CCL) by circulating and resident immune cells, as well as by differentiated adipocytes, steatotic hepatocytes and other peripheral tissues accumulating lipid.^{6–8} Reduction of inflammation delays the progression of metabolic disorders and is considered to be an effective strategy for their prevention.^{5, 9, 10}

The production of the wide spectrum of molecules mediating inflammation is under the control of several transcription factors. These include Rel, signal transducers and activators of transcription family (STAT), interferon (IRF) and other families of transcription factors.^{11–15} Among these transcription factors, NF- κ B has been established as a key regulator of acute and chronic inflammation.¹² NF- κ B is a heterodimeric complex of Rel proteins (e.g. p65/p50). Inhibitory I κ B proteins can bind to the p65/p50 complex and sequester this inactive complex in the cytosol. NF- κ B activation is mediated by a family of pattern recognition receptors that includes nine receptors toll-like receptors 1-9 (TLR1-9).¹² Activation of TLR receptors leads to the binding of adaptor molecules and activation of signaling cascades, which results in the phosphorylation of I κ B and its degradation.¹² The free NF- κ B complex binds to the cognate response elements (*Nf κ B-RE*) in the promoter or enhancer regions of target genes. NF- κ B target genes include families of CXC and CCL cytokines, chemokines, adhesion molecules, integrins, differentiation factors.¹⁶ The activation of TLR by viral/bacterial metabolic products and cytokines induces acute inflammatory response.¹²

In metabolic disorders, NF- κ B activation can be provoked by high-fat, high-cholesterol diets.¹² Studies demonstrate that fatty acids and lipoproteins can bind to TLR receptors,¹² whereas cholesterol can trigger the activation of NF- κ B by mechanisms that remain unclear.¹⁷ In experimental models, the inhibition of NF- κ B improves insulin resistance and hepatic steatosis and decreases obesity and atherosclerosis.¹⁰ Inhibition of NF- κ B can also be achieved through the diet. Polyunsaturated fatty acid (PUFA), antioxidants, and lipophilic vitamins can inhibit NF- κ B activation via a variety of mechanisms. PUFA and bioactive hormone-like metabolites of vitamin A, retinoic acid, and vitamin D3 serve as cognate ligands for anti-inflammatory transcription factors peroxisome proliferator-activated receptors (PPARs), retinoic acid receptor (RAR), and vitamin D receptor (VDR), respectively.^{18–21} Activated PPARs, RAR, and VDR interfere with NF- κ B activation by sequestering p65 in the nucleus, encoding inhibitory I κ B, promoting catabolism of pro-

inflammatory lipids, decreasing reactive oxygen species (ROS) production and other mechanisms.^{21–23} Vitamin E and polyphenols have been shown to inhibit NF- κ B activation by disabling signaling cascades, counteracting ROS production as antioxidants, and through activation of redox-active transcription factors, *e.g.* nuclear factor (erythroid-derived 2)-like 2 (NRF2).²²

Many dietary products have complex bioactive macro- and micronutrient composition. Recent research highlighted the anti-inflammatory²⁴ and pain-reducing²⁵ properties of egg yolk (EY). These effects have been attributed to specific lipid-soluble extracts²⁵ and water-soluble proteins²⁴. EY contains an unique combination of vitamins A, D, E, carotenoids, such as lutein and zeaxanthin, and phospholipids that can potentially diminish inflammation and oxidative stress.²⁶ However, the EY also contains cholesterol and fatty acids that can contribute to pro-inflammatory pathways.²⁷ The role of EY composition on the regulation of NF- κ B has not been investigated. In this paper, we show that partial delipidation of the EY without changes to the EY's carotenoid/vitamin content, is sufficient to markedly reduce activation of NF- κ B and its target cytokines.

Materials and methods

Reagents and supplies

We purchased reagents for cell culture from Invitrogen (Carlsbad, CA). Other reagents were acquired from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Vectors were purchased: Cignal Lenti NF κ B Reporter (GFP) Kit from SABiosciences/Qiagen (Valencia, CA); mCherry constitutive expression vector from Genecopoeia (Rockville, MD). We bought four types of regular eggs from Sauder's egg (EY1), Kroger medium egg (EY2), Kroger large egg (EY3), and EggLand's Best egg (EY4). Cholesterol was purchased from MP Biomedicals (Santa Ana, CA).

EY extraction

15mL whole EY was diluted in 35mL phosphate buffered saline (PBS) (1:3.33, v/v). This solution was termed 'EY'. Hexane (4mL) was added to 1mL of EY and inverted vigorously by hand for 1min. The hexane phase was removed and dried under nitrogen, and then resuspended in 1mL ethanol. The hexane extract of the EY was termed 'Non-polar, (EY-NP)' and the remaining phase was 'Polar, (EY-P)' (Fig 1A). These EY, EY-NP, and EY-P extracts were used for cholesterol/cholesteryl ester quantification, triglyceride quantification, gas chromatography, high performance liquid chromatography (HPLC), and cell stimulation.

Cell culture studies

Nfkb-RE/GFP cell line was derived from murine 3T3-L1 preadipocyte clone stably transfected with lentiviral *Nfkb*-RE GFP reporter construct and control mCherry overexpression construct as described.²⁸ The *Nfkb*-RE/GFP cell line derivation procedure and its characterization in presence of pro- and anti-inflammatory mediators was described before.²⁸ *Nfkb*-RE/GFP cells were cultured in *standard culture medium* (high glucose DMEM containing 10% CS and 1% penicillin-streptomycin). Similar to the parent 3T3-L1

cell line, *Nfkb*-RE/GFP cells preadipocytes were capable of differentiation.²⁸ Differentiation was induced (day 0) in confluent preadipocytes using *differentiation media I* (DMI) containing 3-isobutyl-1-methylxanthine (0.5mM), dexamethasone (1 μ M), insulin (1.7 μ M), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin in DMEM. *Differentiation media II* (DMII) contained 10% FBS, insulin (1.7 μ M), and 1% penicillin-streptomycin in DMEM. Every 48h post adipogenesis induction, cell media was replaced with DMI using standard protocol.²⁹ Prior to measuring fluorescence, cells were washed twice with PBS and lysed with an appropriate volume (*i.e.* 120 μ L/well for 24-well plate, 150 μ L/P60 dish) of RIPA buffer (Boston BioProducts, Ashland, MA) containing complete protease inhibitor (Roche Diagnostics Corporation, Indianapolis, IN). Cells were incubated on ice for 15min, and then the plate was scratched using a tip to lyse residual cells. Cell lysate (80 μ L/well) was transferred to a black 96-well plate (Fisher Scientific Company, Hanover, IL) to measure fluorescence at wavelengths Ex/Em 485/528 for GFP, and then, at Ex/Em 587/640 for mCherry using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). *Nfkb*-RE activation was calculated as ratio of GFP to mCherry control fluorescence (GFP/mCherry), or GFP/protein if mCherry fluorescence was below detection limit. 3T3-L1 preadipocytes were cultured and differentiated under the same conditions as *Nfkb*-RE/GFP preadipocytes.

mRNA analyses

The quantitative analysis of mRNA was performed using the nCounter mouse inflammation expression V2 Panel kit (NanoString Technologies, Seattle, WA). NanoString's nCounter technology is based on direct detection of target molecules using color-coded molecular barcodes, providing a digital simultaneous quantification of the number of target molecules.²⁹ All data were normalized to 6 housekeeping genes quantified in the same samples. Total mRNA (100ng in 5 μ L) was hybridized overnight with nCounter Reporter (20 μ L) probes in hybridization buffer and in excess of nCounter Capture probes (5 μ L) at 65°C for 16–20h. The hybridization mixture containing target/probe complexes was allowed to bind to magnetic beads containing complementary sequences on the Capture Probe. After each target found a probe pair, excess probes were washed followed by a sequential binding to sequences on the Reporter Probe. Biotinylated capture probe-bound samples were immobilized and recovered on a streptavidin-coated cartridge. The abundance of specific target molecules was then quantified using the nCounter Digital Analyzer. Individual fluorescent barcodes and target molecules present in each sample were recorded with a CCD camera by performing a high-density scan (600 fields of view). Images were processed internally into a digital format and were normalized using the NanoString nSolver software analysis tool. Counts were normalized for all target RNAs in all samples based on the positive control RNA to account for differences in hybridization efficiency and post-hybridization processing, including purification and immobilization of complexes. The average was normalized by background counts for each sample obtained from the average of the eight negative control counts. Subsequently, a normalization of mRNA content was performed based on six internal reference housekeeping genes *Cltc*, *Gapdh*, *Gusb*, *Hprt1*, *Pgk1*, and *Tubb* using nSolver Software (NanoString Technologies, Seattle, WA).

Protein assay of cell lysates

The protein content was measured using a BCA kit (Thermo Fisher Scientific; Rockford, IL). The absorbance (562nm) was measured using the Synergy H1 Hybrid Multi-Mode Microplate Reader.

Lipid content analysis of EY fractions

The EY, EY-P, and EY-NP fractions were prepared by hexane extract procedure as described above. To enable enzymatic reaction, EY-NP fraction was resuspended in 200 μ L chloroform and 300 μ L Cholesterol Assay Buffer (from the Cholesterol Quantitation Kit), instead of 1mL ethanol. The suspension was mixed well by tapping, and then sonicated to evaporate chloroform. 10 μ L EY-NP from this stock (in 300 μ L cholesterol assay buffer) was diluted with DI water (to 33 μ L) to achieve an original concentration in 1ml of EY. All EY fractions were then diluted in Cholesterol Assay Buffer according to manufacturer's instruction and L-Type Triglyceride M Kit (Wako Diagnostics; Richmond, VA) was performed. The absorbance (650 nm) was measured using the Synergy H1 Hybrid Multi-Mode Microplate Reader.

Cholesterol Enzymatic Kit (Wako Diagnostics) was performed according to manufacturer's description. The absorbance (600nm) was measured using the Synergy H1 Hybrid Multi-Mode For cholesterol ester analysis, the EY and EY-NP fractions were prepared by hexane extract procedure as described above; however, EY-NP fraction was resuspended in 200 μ L Cholesterol Assay Buffer (NP). 0.3 μ L EY, 0.5 μ L EY-P, and 10 μ L EY-NP were diluted with Cholesterol Assay Buffer (total volume 50 μ L). Cholesterol Quantitation Kit was performed according to manufacturer's description. The absorbance (570nm) was measured using the Synergy H1 Hybrid Multi-Mode Microplate Reader.

Fatty acid analysis

Total lipids were extracted from all EY fractions with 2:1 (v/v) chloroform:methanol and washed with 0.88% KCl.³⁰ Fatty acid methyl esters were prepared using 5% hydrochloric acid in methanol at 76°C.³¹ Analysis of fatty acid methyl esters was completed by gas chromatography with column and conditions as previously described.³² Retention times were compared to standards (Matreya, LLC, Pleasant Gap, PA, Supelco, Bellefonte, PA, and Nu-Check Prep Inc, Elysian, MN) and fatty acids are reported as percent of total identified fatty acids.

HPLC- tandem mass spectrometry analysis (HPLC/MS/MS)

Prior to analysis, EY-P fractions were further extracted using hexane/ethanol/acetone/toluene (10:6:7:7, v/v/v/v, termed HEAT).³³ The EY-NP extracts and HEAT-extracted EY-P fractions were injected on a YMC C30 column (4.6 $\times$ 150mm, 5 μ m, Waters Corp.) and separated with a gradient solution 1 (80/18/2 MeOH/water/2% ammonium acetate) and solution 2 (78/20/2 MtBE/MeOH/2% ammonium acetate at 1.8mL/min and 40°C. Diode array spectra were collected with an Agilent 1260 diode-array detector with 60mm path length flow cell (Agilent Technologies). Eluent was introduced to a QTrap 5500 (ABSciex, Concord, Canada) mass spectrometer via an atmospheric pressure chemical ionization (APCI) probe operated in positive ion mode. Multiple reaction monitoring (MRM) MS/MS

transitions were used to detect vitamins and carotenoids. Lutein/zeaxanthin and retinol were quantified based on external standards.

Statistical analysis

Data was shown as mean $\pm$ SD based on 3 to 4 independent experiments. Statistical comparison of groups was examined by both Student's *t* test and Mann-Whitney U test. Correlation analysis was performed using Pearson test. $P < 0.05$ was considered statistically significant.

Results

Hexane extraction procedure moderately reduces cholesterol and triglycerides in EY content

EY contains many lipophilic compounds that can potentially promote and suppress inflammation.^{26, 27} To assess how lipophilic compounds influence properties of whole EY, we performed hexane extraction procedure on the EY (Fig. 1A) and characterized the composition of the non-polar (EY-NP), and polar (EY-P) fractions. To account for variability related to egg production, we performed experiments using EY obtained from 4 different suppliers. Due to a high level of amphipathic phospholipids in EY, hexane extraction removed approximately 20% of total cholesterol from EY (100%) (Fig. 1B). EY contained 65% of free cholesterol (43% in EY-P and 22% in EY-NP fractions) and 35% of cholesteryl ester (27% in EY-P and 8% in EY-NP fractions). Hexane extraction also removed 30% of triglycerides that were found in the EY (100%) (Fig. 1B, **insert**). Thus, the majority of cholesterol was retained in the EY-P fraction, while the EY-NP fraction contained only 20–30% of cholesterol and triglycerides content from the EY.

EY-P and EY-NP fractions have similar composition of major fatty acids

Next we compared the composition of fatty acids in the EY, EY-P, and EY-NP fractions after their hydrolysis from complex lipids. The major fatty acids in all fractions were saturated palmitic (16:0), monounsaturated oleic (18:1), and polyunsaturated omega 6 linoleic (18:2) acids that comprised approximately 27%, 37%, and 19% of all fatty acids, respectively, in the EY, EY-P, or EY-NP fractions (Fig. 2 A–C). We found significant, but minor differences between different fractions (less than 2% compared to total fatty acid content) in the less abundant saturated (18:0, 14:0, 20:0), monounsaturated (16:1n7), and polyunsaturated (22:5n3) fatty acids. In the EY-P fraction, 22:5n3 was less compared to EY (Fig. 2D), while the content of saturated (18:0, 14:0) and monounsaturated (16:1n7) fatty acids was higher in EY-P compared to EY-NP fractions. Overall, the relative composition of fatty acids was similar among the EY, EY-P, and EY-NP fractions.

Lipophilic vitamins and carotenoids were retained exclusively in EY-P fraction

Simultaneous determination of vitamins E (α -tocopherol), D (D3), A (retinol and retinyl esters), and carotenoids (free and esterified lutein and zeaxanthin esters) was performed using HPLC/MS/MS. Carotenoids and retinol were quantified based on external standards. The EY-NP fraction contained no detectable levels of lipophilic vitamins and carotenoids (Fig. 3). All lipid soluble vitamins and carotenoids were found in the EY-P fraction,

probably due to their interaction with phospholipids. The contents of lutein and zeaxanthin were the most variable (~25%) among the EY-P fractions from different EY suppliers (Fig. 3A). The variability of retinol content in EY was less than 6% (Fig. 3B). Vitamins E and D3 were not quantified but were detected in the EY-P fractions from all suppliers (Fig. 3C). Thus, the EY-P fraction contained all lipophilic vitamins and carotenoids from the EY.

In summary: The EY-NP fraction contained 20–30% of cholesterol and triglycerides compared to EY (100%). The EY-P fraction contained 70–80% of cholesterol and triglycerides and all lipophilic vitamins and carotenoids seen in EY (100%).

EY-P fraction inhibits NF- κ B pathway in adipocytes

1. EY-P fraction inhibits the activation of NF- κ B and its target genes in adipocytes—To assess whether similar composition of the EY and EY-P fractions exerted comparable activation of NF- κ B, we performed reporter assays in the 3T3-L1-*Nf κ b*-RE preadipocyte cell line. This reporter cell line was previously validated using both pro-inflammatory agents and anti-inflammatory antioxidants.²⁸ We compared *Nf κ b*-RE activation in adipocytes stimulated with the EY, EY-P, and EY-NP fractions. Although both the EY and EY-P fractions contained carotenoids and vitamins, *Nf κ b*-RE activation was suppressed only in adipocytes stimulated with the EY-P fractions. Compared to *Nf κ b*-RE activation in non-treated adipocytes, stimulation with EY increased *Nf κ b*-RE activation to 126%. In contrast, the EY-P fraction from the same EYs reduced *Nf κ b*-RE activation 40% compared to control adipocytes (Fig. 4A). The suppression of *Nf κ b*-RE activation by the EY-P fraction was seen in eggs from different suppliers. The variability of response to the EY, EY-P, and EY-NP fractions from EY obtained from different suppliers reached 33%, 20%, and 30%, respectively (data not shown). The suppression of *Nf κ b*-RE activation by EY-P fraction occurs in a concentration-dependent manner (Fig. 4B).

The inhibitory effect EY-P fraction on NF- κ B target genes was further examined in the classical preadipocyte 3T3-L1 cell line. We stimulated differentiated 3T3-L1 preadipocytes with the EY, EY-P, and EY-NP. Then we simultaneously quantified expression of multiple genes in these cells. First, we analyzed the expression of classical NF- κ B target genes, such as MCP1 (CCL2). MCP1 was the most abundantly expressed cytokine in 3T3-L1 adipocytes followed by MCP3 (CCL7) and MCP2 (CCL8) (Fig. 4C). In spite of differences in basal expression, all *Mcp* genes were markedly suppressed by the EY-P fraction. EY-P inhibited expression of *Mcp1* by 59% (Fig. 4D), *Mcp2* by 61% (Fig. 4E), and *Mcp3* by 49% (Fig. 4F) compared to non-stimulated adipocytes. The EY and EY-NP fractions did not significantly influence expression of these genes.

We also explored the specificity of cytokine production in response to the EY fractions. All INFs (*Inf1*: 7 $\pm$ 3, *Inf2*: 51 $\pm$ 6, *Inf3*: 7 $\pm$ 3 AU) were expressed at low levels in 3T3-L1 adipocytes. The EY fractions did not significantly influence expression of all *Inf*s (data not shown). *Cxcl5* and *Cxcl1* cytokines co-regulated by NF- κ B and STAT1 transcription factors,^{3, 14} whereas mitogene-inducible gene (Mig), interferon gamma-induced protein 10 (Ip10), and Rantes are co-regulated by NF- κ B, STAT1, and IRF transcription factors^{11, 34–37} (Fig. 5A). Stimulation of 3T3-L1 adipocytes with the EY-P fraction markedly decreased expression of *Cxcl5* (Fig. 5B) and *Cxcl1* (Fig. 5C) to 9% and 22%, respectively, compared

to non-treated control adipocytes. In contrast, all EY, EY-P and EY-NP fractions lead to a modest (less than 40%) reduction in expression of *Ip10* (Fig. 5D), *Mig* (data not shown), or *Rantes* (Fig. 5E) compared to the control. The reduction of *Ip10* and *Mig* expression was only significant in adipocytes stimulated with the EY-P fraction, and *Rantes* only with the EY fraction compared to control adipocytes. None of the EY fractions significantly influenced the expression of other *Ccl* cytokines, *Tnfa*, and *Il6* (data not shown). Given that the EY-P fraction led to the most potent (10-fold) reduction in the expression of *Cxcl5*, which was known to be regulated by both STAT1 and NF- κ B,^{3, 14, 38} we also analyzed the expression of *Stat1*. We found 10-fold reduction of *Stat1* expression in presence of EY-P in adipocytes (Fig. 5F). Thus, in spite of similar content of lipophilic vitamins in the EY-P and EY fractions, EY-P inhibited the activation of *Nfkb*-RE and expression of NF- κ B target genes and *Stat1*, whereas EY and EY-NP exerted similar level of NF- κ B activation as non-treated adipocytes. The expression of genes regulating apoptosis, such as *Bcl2l1* and *FasI*, was not different in control and treated cells (Data are not shown).

2. Inhibition of NF- κ B activation by EY-P fraction is associated with selective regulation of Tlr receptors and their inhibitor proteins—Among nine *Tlr* receptors, *Tlr2* was the most abundantly expressed receptor in 3T3-L1 adipocytes followed by *Tlr3* and *Tlr4* (Fig. 6A). *Tlr2* expression was similar in non-treated adipocytes and adipocytes treated with EY-NP fraction (Fig. 6B). EY moderately reduced expression of *Tlr2* (to 75%), whereas the EY-P fraction decrease *Tlr* expression to 31% compared to control adipocytes. The expression of two other major, *Tlr3* and *Tlr4* receptors was not significantly affected by the treatment with any EY fractions (Fig. 6C,D). However, the EY-P fraction significantly increased the expression of *Ly96* (to 224%) compared to control adipocytes (Fig. 6E). The interaction of *Ly96* with TLR4 prevents ligand binding to this receptor and the activation of NF- κ B. Similar up-regulation (to 145%) of expression by the EY-P fraction was also observed in an inhibitory adaptor protein, *Tollip*, compared to control adipocytes (Fig. 6F). Therefore, decreased activation of NF- κ B was associated with inhibited expression of major *Tlr* either via direct inhibition of expression of *Tlr2* or indirectly via up-regulation of the expression of inhibitory *Ly96* and *Tollip* by the EY-P but not the EY-NP and EY fractions.

3. Dissimilar regulation of expression by EY-NP and EY-P fractions influence proteins with potential binding sites for both SREBP and NF- κ B—To obtain a clue for specific regulation of *Tlr* and inhibitory *Ly96* and *Tollip* by the EY-P, but not the EY and EY-NP fractions, we analyzed the putative binding sites of *Srebp*. This transcription factor has been established as a sensor of cholesterol/phospholipid ratio on the membranes or by signaling cascades (*e.g.* insulin).³⁹ We found 3 potential high score SREBP binding sites in *Tlr2* (score 92–93), and 2 SREBP binding sites in *Tollip* (score 86) and one in *Ly96* (score 88). *Tlr2* and *Ly96* could be potentially regulated by NF- κ B, because they also contained 5 and 2 potential NF- κ B binding sites, respectively. *Stat1* contains 2 SREBP and 9 NF- κ B binding sites. To examine the putative contribution of SREBP to dissimilar responses among the EY, EY-P and EY-NP fractions we examined the effect of SREBP inhibitor, fatostatin,⁴⁰ on NF- κ B activation in *Nfkb*-RE/GFP preadipocyte cell line. Fatostatin significantly inhibited *Nfkb*-RE activation by 52% compared to the activation induced by the EY-NP fraction (Fig. 7A). Thus, SREBP partially contributed to the

activation of *Nfκb*-RE associated with the EY-NP fraction. Next we stimulated of *Nfκb*-RE/GFP preadipocytes with water soluble cholesterol during differentiation. Cholesterol induced concentration-dependent NF-κB activation that reached 170% plateau compared to non-stimulated adipocytes (Fig. 7B). Stimulations of *Nfκb*-RE/GFP preadipocytes with α-tocopherol (Fig. 7E), *all trans* retinol (Fig. 7C) and lutein had the opposite effect. However the significant concentration-dependent inhibition of NF-κB occurred only in presence of *all trans* retinol and lutein. Each of these compounds suppressed 30% of NF-κB activation compared to non-stimulated adipocytes.

Discussion

Our data demonstrate that EY contained a mixture of compounds that can effectively suppress the key inflammatory transcription pathway NF-κB *in vitro*; however, these effects were blunted in the presence of non-polar cholesterol and TG. EY-P compounds efficiently inhibited:

1. Initiation of receptor-mediated signaling activating NF-κB pathway;
2. Binding of transcriptional complex to regulatory DNA sequences; and
3. Expression of target cytokines.

The receptors regulating NF-κB in adipocytes exhibit specific pattern. 3T3-L1 adipocytes expressed predominantly three *Tlrs*: *Tlr2>Tlr3=Tlr4*. EY-P fraction suppressed expression of *Tlr2*, whereas the expression of seven other *Tlrs* remained unchanged. The inhibition of other TLRs, was mediated via inhibitory adaptor protein Tollip and TLR4 inhibitor Ly96. Polar lipids in the EY-P fraction induced expression of these inhibitory proteins. These changes in TLRs were associated with inhibition of NF-κB activation and the expression of NF-κB-dependent genes by EY-P compounds. Unexpectedly, EY supported NF-κB activation, even though the EY and EY-P fractions shared similar content of vitamins, carotenoids, and fatty acids. Partial removal of non-polar cholesterol and TG from the EY was required to reveal its inhibitory effects on the NF-κB pathway. Although these studies were performed in adipocytes, EY fractions expect to exert similar effects in other cells that are under NF-κB and SREBP transcriptional control. Example includes mucosal epithelial cells exposed to EY-containing products such as mayonnaise. In other pathophysiological situations *in vivo*, EY can potentially have beneficial effects without delipidation.^{41, 42}

The inhibition of *Nfκb*-RE activation together with the suppressed expression of NF-κB-regulated cytokines, and TLR2 receptor suggest that EY-P may exert anti-inflammatory properties. The association of anti-inflammatory properties with EY-P, but not EY fractions, appears to be independent of the variation in EY composition, since we showed these effects using regular quality eggs from four different suppliers. The highest variability in composition was associated with cholesterol content in the EY-NP fraction and lutein and zeaxanthin content in the EY-P fraction; however, all EY-P fractions inhibited expression of genes in the NF-κB cascade after partial delipidation. Although numerous studies showed that NF-κB pathways are regulated by fatty acids,¹² in our study similar fatty acid composition among the EY fractions suggest their unlikely role in dissimilar NF-κB activation. The mechanism by which cholesterol regulates NF-κB remains unclear. In

macrophages, cholesterol mobilization to lipid rafts contributes to TLR activation.⁴³ Activation of NF- κ B via TLR4 is blocked by mevinolin, suggesting the activation of TLR4 may require binding of cholesterol or its metabolic precursors.⁴⁴ Decades of studies with statins, inhibitors of cholesterol synthesis, showed that anti-inflammatory effects of these pharmaceuticals depend on inhibition of NF- κ B in a variety of tissues.^{45–47} The regulation of cholesterol metabolism and fatty acid synthesis is controlled by a SREBP family of transcription factors. In adipocytes, SREBP is activated by insulin/JNK pathways³⁹ and glucose/JAK/STAT pathways⁴⁸ that regulate differentiation, glycolytic pathways, fatty acid synthesis, and lipid droplet formation in adipocytes.⁴⁹ In our studies, all proteins participating in NF- κ B activation cascade, TLR2, Tollip, and Ly96, had putative binding sites for SREBP. Given that detailed analysis of promoters was beyond the scope of our first study, we accessed the global impact of SREBP in the regulation of NF- κ B by EY-NP fraction using its specific inhibitor fatostatin.⁴⁰ *Nfkb-RE* activation was significantly reduced by fatostatin compared to non-treated adipocytes, whereas cholesterol induced *Nfkb-RE* activation in a concentration-dependent manner. However, effects of fatostatin was less pronounced than that achieved by EY-P compounds. Vitamin A and lutein inhibited *Nfkb-RE* activation at low concentrations and could be potentially accounted for the inhibition mediated by EY-P fraction. Thus, partial delipidation of EY may lead first to the disruption of SREBP-dependent genes involved in NF- κ B activation, and then enable lutein and retinol to exert their potential anti-inflammatory effects (Fig. 8).

A remaining question is: why only partial delipidation was needed to improve the inhibition of NF- κ B by the EY-P fraction? Although specific studies are needed to answer this question, a possible explanation can be found in the relationship between membrane composition and activation of SREBP. Recent studies showed that SREBP activation could be triggered by reduced phospholipid concentrations in the membranes.⁵⁰ After partial delipidation, the EY-P fraction has an increased ratio of phospholipid to cholesterol, which, possibly, prevents SREBP activation. The decrease in ratio of cholesterol to phospholipid could also improve the delivery of lipophilic vitamins and carotenoids to adipocytes.

Our study also revealed that partial delipidation of EY can influence inflammation via STAT1. This transcription factor is a critical mediator of interferon-mediated inflammation⁵¹ and promotes a variety of diseases, including hepatitis³ and type 1 diabetes in experimental animal models.⁴ *Stat1* controls apoptosis in pancreatic β cells⁵² via transcription of *Cxcl1* and *Cxcl2*. These cytokines which promote neutrophil migration into pancreatic islets.¹⁴ Insulin resistance in obese patients⁵ and patients with hepatitis³ were attributed to another STAT1-regulated cytokine, CXCL5. CXCL1 and CXCL5 are also NF- κ B target genes.^{14, 38} The deficient NF- κ B activation and *Stat1* expression in our studies markedly diminished expression of *Cxcl1* and *Cxcl5* expression (by 80% and 90% decrease in the presence of EY-P compared to control). It is unlikely that INF was the major regulator of *Stat1* in adipocytes, because the expression of all *Inf* was very low in adipocytes and was not regulated by EY fractions (data not shown). Accordingly, the expression of INF γ -regulated *Ip10* and *Rantes* was only moderately regulated by EY fractions (Fig. 5). Recent studies showed that CXCL5 could be another mechanism for the activation of STAT1 in adipocytes.⁵³ Suppression of NF- κ B activation and CXCL5 expression by EY-P fraction can

also result in deficient *Stat1* expression. Regardless of these still speculative mechanisms, the results of our study suggest that regular EY contains compounds that can selectively regulate expression of inflammatory cytokines through two major transcriptional pathways, NF- κ B and STAT1. Identification of these compounds and/or their mixtures can help to develop novel therapeutics and functional foods, which can potentially improve the treatment of metabolic diseases. Our data help to recognize the pivotal role of lipophilic compounds in EY that can sensitize inflammatory pathways and disable anti-inflammatory effects of *all trans* retinol and lutein associated with the EY-P fraction. We propose partial delipidation of EY as an efficient solution to improve beneficial effects of EY.

Acknowledgments

We express our gratitude to Dr. Paolo Fadda at the Nucleic Acid Shared Resource at The Ohio State University (OSU) for excellent technical and intellectual support and Dr. Kirsteen Maclean at NanoString Technologies for her help with data analysis. HPLC-MS/MS analyses were conducted by the Nutrient & Phytochemical Analytic Shared Resource (OSU-Comprehensive Cancer Center (OSUCCC)). This research was supported by Award Number 20020728 from American Egg Board and Award Number 10040042 from Novo Nordisk Pharmaceuticals as well as by the Food Innovation Center, Office for International Affairs and Center for Advanced Functional Foods Research and Entrepreneurship at OSU, and Daskal Foundation (O.Z., Q.S., C. L., LX). The project described was supported by Award Number R21OD017244 (O.Z.) and UL1RR025755 (OSUCCC) from the National Center for Research Resources, funded by the Office of the Director, National Institutes of Health (OD) and supported by the NIH Roadmap for Medical Research. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.

Abbreviations used

APCI-MS	atmospheric pressure chemical ionization-mass spectrometry
CCL	CC chemokine ligands
CXC	chemokines
GFP	green fluorescent protein
Cxcl1	The chemokine (C-X-C motif) ligand 1, alias: GRO1, GRO α , KC, NAP-3;
Cxcl5	The chemokine (C-X-C motif) ligand 1, alias: ENA78
EY	egg yolk
EY-P	egg yolk polar fraction
EY-NP	egg yolk non-polar fraction
HPLC	High-performance liquid chromatography
Ip10	interferon gamma-induced protein 10
IRF	interferon
Ly96	lymphocyte antigen 96
MCP1	monocyte chemotactic protein-1
Mig	mitogene-inducible gene
NF-κB	nuclear factor kappa B
Nfkb-RE	nuclear factor κ B response element

NRF2	nuclear factor erythroid 2 related factor
PPARγ	peroxisome proliferator-activated receptor gamma
PUFA	Polyunsaturated fatty acid
RAR	retinoic acid receptor
ROS	reactive oxygen species
SREBP	sterol regulatory binding protein
STAT	signal transducers and activators of transcription family
TLR	Toll-like receptors
Tollip	toll interacting protein
VDR	vitamin D receptor

References

1. Baker RG, Hayden MS, Ghosh S. NF-kappaB, inflammation, and metabolic disease. *Cell metabolism*. 2011; 13:11–22. [PubMed: 21195345]
2. Alison MR, Nicholson LJ, Lin WR. Chronic inflammation and hepatocellular carcinoma. Recent results in cancer research. *Fortschritte der Krebsforschung Progres dans les recherches sur le cancer*. 2011; 185:135–48.
3. Jaruga B, Hong F, Kim WH, Gao B. IFN-gamma/STAT1 acts as a proinflammatory signal in T cell-mediated hepatitis via induction of multiple chemokines and adhesion molecules: a critical role of IRF-1. *Am J Physiol Gastrointest Liver Physiol*. 2004; 287:G1044–52. [PubMed: 15246962]
4. Kim S, Kim HS, Chung KW, Oh SH, Yun JW, Im SH, Lee MK, Kim KW, Lee MS. Essential role for signal transducer and activator of transcription-1 in pancreatic beta-cell death and autoimmune type 1 diabetes of nonobese diabetic mice. *Diabetes*. 2007; 56:2561–8. [PubMed: 17620422]
5. Chavey C, Lazennec G, Lagarrigue S, Clape C, Iankova I, Teyssier J, Annicotte JS, Schmidt J, Matakis C, Yamamoto H, Sanches R, Guma A, Stich V, Vitkova M, Jardin-Watelet B, Renard E, Strieter R, Tuthill A, Hotamisligil GS, Vidal-Puig A, Zorzano A, Langin D, Fajas L. CXC ligand 5 is an adipose-tissue derived factor that links obesity to insulin resistance. *Cell metabolism*. 2009; 9:339–49. [PubMed: 19356715]
6. Berg AH, Lin Y, Lisanti MP, Scherer PE. Adipocyte differentiation induces dynamic changes in NF-kappaB expression and activity. *Am J Physiol Endocrinol Metab*. 2004; 287:E1178–88. [PubMed: 15251865]
7. Shaw OM, Pool B, Dalbeth N, Harper JL. The effect of diet-induced obesity on the inflammatory phenotype of non-adipose-resident macrophages in an in vivo model of gout. *Rheumatology (Oxford)*. 2014
8. Nov O, Shapiro H, Ovadia H, Tarnovscki T, Dvir I, Shemesh E, Kovsan J, Shelef I, Carmi Y, Voronov E, Apte RN, Lewis E, Haim Y, Konrad D, Bashan N, Rudich A. Interleukin-1beta regulates fat-liver crosstalk in obesity by auto-paracrine modulation of adipose tissue inflammation and expandability. *PLoS One*. 2013; 8:e53626. [PubMed: 23341960]
9. Ridker PM. Moving beyond JUPITER: will inhibiting inflammation reduce vascular event rates? *Curr Atheroscler Rep*. 2013; 15:295. [PubMed: 23225175]
10. Jialal I, Kaur H, Devaraj S. Toll-like receptor status in obesity and metabolic syndrome: a translational perspective. *J Clin Endocrinol Metab*. 2014; 99:39–48. [PubMed: 24187406]
11. Majumder S, Zhou LZ, Chaturvedi P, Babcock G, Aras S, Ransohoff RM. p48/STAT-1alpha-containing complexes play a predominant role in induction of IFN-gamma-inducible protein, 10 kDa (IP-10) by IFN-gamma alone or in synergy with TNF-alpha. *J Immunol*. 1998; 161:4736–44. [PubMed: 9794404]

12. Jin C, Flavell RA. Innate sensors of pathogen and stress: linking inflammation to obesity. *J Allergy Clin Immunol*. 2013; 132:287–94. [PubMed: 23905917]
13. Yan X, Zhu MJ, Xu W, Tong JF, Ford SP, Nathanielsz PW, Du M. Up-regulation of Toll-like receptor 4/nuclear factor-kappaB signaling is associated with enhanced adipogenesis and insulin resistance in fetal skeletal muscle of obese sheep at late gestation. *Endocrinology*. 2010; 151:380–7. [PubMed: 19887565]
14. Burke SJ, Lu D, Sparer TE, Masi T, Goff MR, Karlstad MD, Collier JJ. NF-kappaB and STAT1 control CXCL1 and CXCL2 gene transcription. *Am J Physiol Endocrinol Metab*. 2014; 306:E131–49. [PubMed: 24280128]
15. Taniguchi T, Ogasawara K, Takaoka A, Tanaka N. IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol*. 2001; 19:623–55. [PubMed: 11244049]
16. Brasier AR. The NF-kappaB regulatory network. *Cardiovasc Toxicol*. 2006; 6:111–30. [PubMed: 17303919]
17. Thach CT, Finkelstein JN. Cationic nanoparticles disrupt cellular signaling in a cholesterol dependent manner. *Toxicol In Vitro*. 2013; 27:1277–86. [PubMed: 23274767]
18. D'Amore S, Vacca M, Graziano G, D'Orazio A, Cariello M, Martelli N, Di Tullio G, Salvia R, Grandaliano G, Belfiore A, Pellegrini F, Palasciano G, Moschetta A. Nuclear receptors expression chart in peripheral blood mononuclear cells identifies patients with Metabolic Syndrome. *Biochim Biophys Acta*. 2013; 1832:2289–301. [PubMed: 24060638]
19. Siriwardhana N, Kalupahana NS, Cekanova M, LeMieux M, Greer B, Moustaid-Moussa N. Modulation of adipose tissue inflammation by bioactive food compounds. *J Nutr Biochem*. 2013; 24:613–23. [PubMed: 23498665]
20. Ziouzenkova O, Perrey S, Asatryan L, Hwang J, MacNaul KL, Moller DE, Rader DJ, Sevanian A, Zechner R, Hoefler G, Plutzky J. Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an antiinflammatory role for lipoprotein lipase. *Proc Natl Acad Sci U S A*. 2003; 100:2730–5. [PubMed: 12606719]
21. Chinetti G, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. *Inflamm Res*. 2000; 49:497–505. [PubMed: 11089900]
22. Glauert HP. Vitamin E and NF-kappaB activation: a review. *Vitam Horm*. 2007; 76:135–53. [PubMed: 17628174]
23. Carlsen H, Alexander G, Austenaa LM, Ebiara K, Blomhoff R. Molecular imaging of the transcription factor NF-kappaB, a primary regulator of stress response. *Mutat Res*. 2004; 551:199–211. [PubMed: 15225593]
24. Ma J, Wang H, Wang Y, Zhang S. Endotoxin-neutralizing activity of hen egg phosvitin. *Mol Immunol*. 2013; 53:355–62. [PubMed: 23079731]
25. Mahmoudi M, Ebrahimzadeh MA, Pourmorad F, Rezaie N, Mahmoudi MA. Anti-inflammatory and analgesic effects of egg yolk: a comparison between organic and machine made. *Eur Rev Med Pharmacol Sci*. 2013; 17:472–6. [PubMed: 23467945]
26. Anderson KE. Comparison of fatty acid, cholesterol, vitamin A and E composition, and trans fats in eggs from brown and white egg strains that were molted or nonmolted. *Poult Sci*. 2013; 92:3259–65. [PubMed: 24235237]
27. Golzar Adabi SH, Ahbab M, Fani AR, Hajbabaie A, Ceylan N, Cooper RG. Egg yolk fatty acid profile of avian species--influence on human nutrition. *J Anim Physiol Anim Nutr (Berl)*. 2013; 97:27–38. [PubMed: 22035443]
28. Shen Q, Chitchumroonchokchai C, Thomas JL, Gushchina LV, Disilvestro D, Failla ML, Ziouzenkova O. Adipocyte reporter assays: application for identification of anti-inflammatory and antioxidant properties of mangosteen xanthones. *Mol Nutr Food Res*. 2014; 58:239–47. [PubMed: 24039005]
29. Yasmeen R, Reichert B, Deiluiis J, Yang F, Lynch A, Meyers J, Sharlach M, Shin S, Volz KS, Green KB, Lee K, Alder H, Duester G, Zechner R, Rajagopalan S, Ziouzenkova O. Autocrine function of aldehyde dehydrogenase 1 as a determinant of diet- and sex-specific differences in visceral adiposity. *Diabetes*. 2013; 62:124–36. [PubMed: 22933113]

30. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of biological chemistry*. 1957; 226:497–509. [PubMed: 13428781]
31. Stoffel W, Chu F, Ahrens EH. Analysis of Long-Chain Fatty Acids by Gas-Liquid Chromatography. *Analytical Chemistry*. 1959; 31:307–308.
32. Belury MA, Kempa-Steczko A. Conjugated linoleic acid modulates hepatic lipid composition in mice. *Lipids*. 1997; 32:199–204. [PubMed: 9075211]
33. Kopec RE, Schweiggert RM, Riedl KM, Carle R, Schwartz SJ. Comparison of high-performance liquid chromatography/tandem mass spectrometry and high-performance liquid chromatography/photo-diode array detection for the quantitation of carotenoids, retinyl esters, alpha-tocopherol and phylloquinone in chylomicron-rich fractions of human plasma. *Rapid Commun Mass Spectrom*. 2013; 27:1393–402. [PubMed: 23681818]
34. Krinninger P, Brunner C, Ruiz PA, Schneider E, Marx N, Foryst-Ludwig A, Kintscher U, Haller D, Laumen H, Hauner H. Role of the adipocyte-specific NF-kappaB activity in the regulation of IP-10 and T cell migration. *Am J Physiol Endocrinol Metab*. 2011; 300:E304–11. [PubMed: 21062959]
35. Moriuchi H, Moriuchi M, Fauci AS. Nuclear factor-kappa B potently up-regulates the promoter activity of RANTES, a chemokine that blocks HIV infection. *J Immunol*. 1997; 158:3483–91. [PubMed: 9120310]
36. Matsuzaki S, Ishizuka T, Hisada T, Aoki H, Komachi M, Ichimonji I, Utsugi M, Ono A, Koga Y, Dobashi K, Kurose H, Tomura H, Mori M, Okajima F. Lysophosphatidic acid inhibits CC chemokine ligand 5/RANTES production by blocking IRF-1-mediated gene transcription in human bronchial epithelial cells. *J Immunol*. 2010; 185:4863–72. [PubMed: 20861350]
37. Ohmori Y, Schreiber RD, Hamilton TA. Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. *J Biol Chem*. 1997; 272:14899–907. [PubMed: 9169460]
38. Sun H, Chung WC, Ryu SH, Ju Z, Tran HT, Kim E, Kurie JM, Koo JS. Cyclic AMP-responsive element binding protein- and nuclear factor-kappaB-regulated CXC chemokine gene expression in lung carcinogenesis. *Cancer Prev Res (Phila)*. 2008; 1:316–28. [PubMed: 19138976]
39. Ito M, Nagasawa M, Omae N, Tsunoda M, Ishiyama J, Ide T, Akasaka Y, Murakami K. A novel JNK2/SREBP-1c pathway involved in insulin-induced fatty acid synthesis in human adipocytes. *J Lipid Res*. 2013; 54:1531–40. [PubMed: 23515281]
40. Kamisuki S, Mao Q, Abu-Elheiga L, Gu Z, Kugimiya A, Kwon Y, Shinohara T, Kawazoe Y, Sato S, Asakura K, Choo HY, Sakai J, Wakil SJ, Uesugi M. A small molecule that blocks fat synthesis by inhibiting the activation of SREBP. *Chem Biol*. 2009; 16:882–92. [PubMed: 19716478]
41. Pearce KL, Clifton PM, Noakes M. Egg consumption as part of an energy-restricted high-protein diet improves blood lipid and blood glucose profiles in individuals with type 2 diabetes. *The British journal of nutrition*. 2011; 105:584–92. [PubMed: 21134328]
42. Goodrow EF, Wilson TA, Houde SC, Vishwanathan R, Scollin PA, Handelman G, Nicolosi RJ. Consumption of one egg per day increases serum lutein and zeaxanthin concentrations in older adults without altering serum lipid and lipoprotein cholesterol concentrations. *The Journal of nutrition*. 2006; 136:2519–24. [PubMed: 16988120]
43. Zhu X, Owen JS, Wilson MD, Li H, Griffiths GL, Thomas MJ, Hiltbold EM, Fessler MB, Parks JS. Macrophage ABCA1 reduces MyD88-dependent Toll-like receptor trafficking to lipid rafts by reduction of lipid raft cholesterol. *J Lipid Res*. 2010; 51:3196–206. [PubMed: 20650929]
44. Law RE, Stimmel JB, Damore MA, Carter C, Clarke S, Wall R. Lipopolysaccharide-induced NF-kappa B activation in mouse 70Z/3 pre-B lymphocytes is inhibited by mevinolin and 5'-methylthioadenosine: roles of protein isoprenylation and carboxyl methylation reactions. *Mol Cell Biol*. 1992; 12:103–11. [PubMed: 1729592]
45. Guijarro C, Kim Y, Schoonover CM, Massy ZA, O'Donnell MP, Kasiske BL, Keane WF, Kashtan CE. Lovastatin inhibits lipopolysaccharide-induced NF-kappaB activation in human mesangial cells. *Nephrol Dial Transplant*. 1996; 11:990–6. [PubMed: 8671958]

46. van der Meij E, Koning GG, Vriens PW, Peeters MF, Meijer CA, Kortekaas KE, Dalman RL, van Bockel JH, Hanemaaijer R, Kooistra T, Kleemann R, Lindeman JH. A clinical evaluation of statin pleiotropy: statins selectively and dose-dependently reduce vascular inflammation. *PLoS One*. 2013; 8:e53882. [PubMed: 23349755]
47. Malik S, Sharma AK, Bharti S, Nepal S, Bhatia J, Nag TC, Narang R, Arya DS. In vivo cardioprotection by pitavastatin from ischemic-reperfusion injury through suppression of IKK/NF-kappaB and upregulation of pAkt-e-NOS. *J Cardiovasc Pharmacol*. 2011; 58:199–206. [PubMed: 21654332]
48. Guillet-Deniau I, Pichard AL, Kone A, Esnous C, Nieruchalski M, Girard J, Prip-Buus C. Glucose induces de novo lipogenesis in rat muscle satellite cells through a sterol-regulatory-element-binding-protein-1c-dependent pathway. *J Cell Sci*. 2004; 117:1937–44. [PubMed: 15039461]
49. Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, Solanes G, Lowell BB, Spiegelman BM. Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest*. 1998; 101:1–9. [PubMed: 9421459]
50. Walker AK, Jacobs RL, Watts JL, Rottiers V, Jiang K, Finnegan DM, Shioda T, Hansen M, Yang F, Niebergall LJ, Vance DE, Tzoneva M, Hart AC, Naar AM. A conserved SREBP-1/ phosphatidylcholine feedback circuit regulates lipogenesis in metazoans. *Cell*. 2011; 147:840–52. [PubMed: 22035958]
51. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*. 1994; 264:1415–21. [PubMed: 8197455]
52. Moore F, Naamane N, Colli ML, Bouckennooghe T, Ortis F, Gurzov EN, Igoillo-Esteve M, Mathieu C, Bontempi G, Thykjaer T, Orntoft TF, Eizirik DL. STAT1 is a master regulator of pancreatic {beta}-cell apoptosis and islet inflammation. *J Biol Chem*. 2011; 286:929–41. [PubMed: 20980260]
53. Zhang H, Yang R, Wang Z, Lin G, Lue TF, Lin CS. Adipose tissue-derived stem cells secrete CXCL5 cytokine with neurotrophic effects on cavernous nerve regeneration. *J Sex Med*. 2011; 8:437–46. [PubMed: 21114767]

Figure 1. Delipidation of EY with hexane extraction moderately reduces its cholesterol and triglyceride content

(A) Schematic of EY extraction procedure. EY (~15mL) was diluted with PBS to obtain 50mL whole EY solution (#1 tube). 1mL of EY (black area in #2 tube) was extracted with hexane in a 10mL glass tube. The remaining 1mL fraction was termed polar 'EY-P' (hatched area in #3 tube). Hexane phase was collected to another glass tube (#4 tube), evaporated by nitrogen gas, and then reconstituted in 1mL ethanol. This phase was termed nonpolar 'EY-NP' (white area in #5 tube). For analysis of fatty acids and vitamins, EY-P fraction was additionally extracted as described in methods.

(B) Cholesterol content in EY (black bars), EY-P (hatched bars), and EY-NP (white bars) fractions was measured in EY. Here and throughout study we used EY obtained from different suppliers. Data (n=5, mean±SD) are shown as percent of esterified and non-esterified cholesterol in EY fractions (100%, horizontal dashed line). (B, low insert) Triglyceride content is shown as % of EY content (100%, n=3, dashed line).

Figure 2. Low variability in fatty acid composition across EY fractions from different suppliers Fatty acid composition was measured in EY (black bars), EY-P (hatched bars), and EY-NP (white bars) fractions (n=4). Data are shown as mean $\pm$ SD % of distribution in whole FA in each fraction. P values were determined using Mann–Whitney U test. **(A)** Saturated fatty acids: C16:0, palmitic acid; C18:0, stearic acid; **(A, upper insert)** C14:0, myristic acid; **(A, lower insert)** C20:0, arachidic acid. **(B)** Monounsaturated fatty acids: vaccenic acid; C18:1n9, **(B, upper insert)** C16:1n7, palmitoleic acid; C18:1n7, oleic acid; **(B, lower insert)** C20:1n9, cetoleic acid. **(C)** Polyunsaturated ω 6 fatty acids: C18:2n6, linoleic acid; **(C, upper insert)** C18:3n6, gamma linoleic acid; C20:2n6, eicosadienoic acid. **(C, lower insert)** Conjugated linoleic acid, C9t11CLA. **(D)** Polyunsaturated ω 3 fatty acids: C22:6n3, docosahexaenoic acid; C18:3n3, alpha linolenic acid; **(D, upper insert)** C22:5n3, docosapentaenoic acid;

Figure 3. Vitamin and carotenoids are present in EY-P and absent in EY-NP fractions after hexane extraction

Same EY fractions (n=4) that were examined for fatty acid composition (Fig. 2) were analyzed for vitamin and carotenoids content using HPLC/MS/MS. **(A)** Concentrations of lutein/zeaxanthin in EY-P and EY-NP fractions. **Inserts** show one representative chromatogram for EY-NP (**upper**) and EY-P (**lower**) fractions. **(B)** Concentrations of retinol in EY-P and EY-NP fractions. **Inserts** show one representative chromatogram for EY-NP (**upper**) and EY-P (**lower**) fractions. **(C)** Representative chromatograms of EY-NP (**upper insert**) and EY-P (**lower insert**) fractions showed absence vitamins A, E, and D in EY-NP fraction.

Figure 4. EY-P fractions inhibit NF-κB activity in *NfκB*-RE/GFP and 3T3-L1 adipocytes

(A) *NfκB*-RE/GFP preadipocytes were differentiated 3 days and stimulated with identical concentrations of EY (black bar), EY-P (hatched bar), and EY-NP (white bar) EY fractions (5μL EY fraction/mL DMII) for 24h. Control, *NfκB*-RE/GFP cells were maintained in DMII (Contr, gridded bar). Data is shown as mean±SD, n=12 for Control and EY or n=11 for EY-P and EY-NP treated cells. The fluorescence/protein ratio was obtained from total cell lysates in RIPA buffer. Data are shown as a % of control fluorescence/protein ratio (100%, dashed line).

(B) *NfκB*-RE/GFP preadipocytes were differentiated for 3 days and stimulated with 2, 4, 5, 8μL EY-P/mL DMII. Data are shown as mean±SD of fluorescence/protein ratio (n=4). Correlation was examined by Pearson test, groups were compared using Mann–Whitney U test.

(C) 3T3-L1 preadipocytes were differentiated for 4 days. The expression of *Mcp* gene family was measured using the nCounter data analysis system from NanoString Technologies. *Mcp* expression in 3T3-L1 adipocytes are shown as mean±SD (n=3).

(D–F) 3T3-L1 preadipocytes were differentiated for 3 days and stimulated with EY (black bar), EY-P (hatched bar), EY-NP (white bar) fractions (5μL EY fraction/mL DMII) for 24 h. The expression of *Mcp1*, *Mcp2*, and *Mcp3* was shown as % of expression compared to in control adipocytes (gridded bar, 100% - dashed line) (mean±SD, n=3). Significance was examined using Mann–Whitney U test.

Figure 5. EY-P fractions suppress expression of NF- κ B regulated downstream genes in 3T3-L1 adipocytes

(A) The relative expression of *Cxcl5*, *Cxcl1*, *Mig*, *Ip10*, *Rantes* and *Stat1* was measured by the nCounter data analysis system from NanoString Technologies using same samples that were described in Fig. 4C. (B–F) Expression of *Cxcl5*, *Cxcl1*, *Mig*, *Ip10*, *Rantes* and *Stat1* genes in same 3T3-L1 adipocytes described in Fig. 4 D–F.

Figure 6. EY-P fractions inhibit expression of specific Toll-like (Tlr) receptors

(A) The relative expression of *Tlr 1-9* was measured by NanoString using same samples that were described in Fig. 4C. (B–D) Expression of *Tlr2*, *Tlr3*, *Tlr4* genes as well as Ly96 (E) and Tollip (F) in same 3T3-L1 adipocytes described in Fig. 4 D–F.

Figure 7. Fatostatin, retinol and lutein suppressed EY-NP-induced NF-κB activation in *Nfκb*-RE/GFP adipocytes

(A) Control *Nfκb*-RE/GFP preadipocytes were differentiated for 4 days (Contr, grid bar). *Nfκb*-RE/GFP preadipocytes were differentiated for 3 days and stimulated for 24 h with 20nM fatostatin (Fatost, dotted bars), EY (black bar), EY-NP (white bar), EY-NP with fatostatin (20nM, EY-NP+Fatost, bar with horizontal lines). Data are shown as % of control (100%, red dashed line). Data (mean±SD, n=3) are shown as % of expression compared to in control adipocytes. *P*, Mann–Whitney U test.

Different concentrations of cholesterol (B), α-tocopherol (C), all trans retinol (D), and lutein (E) were added during the induction of *Nfκb*-RE/GFP preadipocyte differentiation and were replaced every 48 h. Differentiation continued for 5 days. Data are shown as GFP/mCherry ratio (n=3). Correlation was examined by Pearson test. Groups were compared using Mann–Whitney U test. An asterisk indicates significant difference between control and cholesterol stimulated adipocytes.

Figure 8. Hypothetical mechanism for EY-P-suppressed NF-κB activation in adipocytes
(A) The major TLR on adipocytes are TLR2, TLR3, and TLR4. The inhibition of TLRs was mediated via inhibitory adaptor protein Tollip, while Ly96 inhibited TLR4 activation. TLR activation induces signaling cascade leading to translocation of NF-κB complex, for example, p50/p65 into nucleus and activation of NF-κB-dependent genes (*MCP* family, *CXC* family, *Mig*, *Ip10*, *Rantes*, etc.). Cholesterol to phospholipid ratio in the endoplasmic reticulum (ER) membranes control activation of SREBP transcription factor, which co-regulate genes involved in NF-κB activation (*Tlr2*, *Tollip*, *Ly96*, *Stat1*, etc.). EY and EY-NP fractions activate NF-κB through SREBP-dependent pathways and could be inhibited by SREBP inhibitor fatostatin. Partial delipidation EY (such as EY-P fraction, which is not shown in the schematics) alters phospholipid to cholesterol ratio and might prevent SREBP activation, resulted in the suppression of TLR2 and elevated expression of Tollip and Ly96 inhibitory proteins in adipocytes. Thus, partially delipidated EY-P fraction leads to the inhibition of NF-κB activation and its downstream inflammatory targets.