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Research Article

Chronic quercetin exposure affects fatty acid catabolism in rat lung

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Abstract. Dietary quercetin intake is suggested to be health promoting, but this assumption is mainly based on mechanistic studies performed *in vitro*. Previously, we identified rat lung as a quercetin target tissue. To assess relevant *in vivo* health effects of quercetin, we analyzed mechanisms of effect in rat lungs of a chronic (41 weeks) 1% quercetin diet using whole genome microarrays. We show here that fatty acid catabolism pathways, like beta-oxidation and ketogenesis, are up-regulated by the long-term quercetin intervention. Up-regulation of genes (*Hmgcs2*, *Ech1*, *Acox1*, *Pcca*, *Lpl* and *Acaa2*) was verified and confirmed by quantitative real time PCR. In addition, free fatty acid levels were decreased in rats fed the quercetin diet, confirming that quercetin affects fatty acid catabolism. This *in vivo* study demonstrates for the first time that fatty acid catabolism is a relevant process that is affected in rats by chronic dietary quercetin.

Keywords. Quercetin, microarray, fatty acid metabolism, nutrigenomics, flavonoids, polyphenols.

Introduction

Flavonoids are polyphenolic compounds of plant origin that are present in our diet. Epidemiological studies suggest that a diet rich in flavonoids is protective against cardiovascular diseases and possibly lung cancer [1]. Mechanistic studies show that flavonoids have a wide range of properties that can contribute to the potential beneficial health effects of flavonoids. For example, they are strong antioxidants [2, 3], inhibit tumor formation [4], improve endothelial function [5, 6] and affect energy metabolism [7]. To elucidate the molecular mechanisms explaining the beneficial effects of flavonoids observed *in vivo*, several studies have employed a systems biology approach with transcriptomics and proteomics techniques, using cell culture systems [8–18]. However, these *in vitro* studies may have generated misleading findings due to the non-physiological exposure conditions mostly used. In the human body, flavonoids are rapidly metabolized and therefore flavonoid aglycones (flavonoid molecules without conjugates), frequently used *in vitro*, are hardly present in plasma and tissues [19]. In addition, flavonoids are rapidly oxidized under cell culture conditions giving rise to oxidation products and H_2O_2 [20]. This, and the observation that flavonoid metabolites have profoundly different bioactivities and pharmacokinetics than flavonoid aglycones [21–23], suggests that reported mechanisms of action of flavonoids based solely on *in vitro* experiments are of limited value.

Quercetin is a flavonol abundant in onions, apples, tea and red wine and one of the most studied flavonoids. Quercetin is taken up in the intestine and immediately

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metabolized by phase II enzymes in the intestine and liver to methoxy, glucuronic acid and sulfate conjugates [24, 25]. Chronic treatment of rats with a 0.1% and 1% quercetin diet for 11 weeks resulted in appearance of quercetin metabolites in all analyzed tissues. The highest levels of quercetin metabolites were found in the lungs [26]. For that reason, lung is a relevant *in vivo* target tissue for studying molecular mechanisms underlying the quercetin-mediated beneficial health effects using a transcriptomics approach. Only one study on quercetin has used an animal model in combination with large scale gene expression techniques. Mutch et al. [27] showed that exposure of hepatic cytochrome-P450 oxidoreductase-knockout and wild-type mice to an acute high dose of quercetin (7 mg/mouse), induced alterations in several biological pathways in jejunum, colon and liver. Although metabolism of quercetin did not differ between wild-type and knockout mice, tissue-specific effects on amino acid metabolism, lipid metabolism, glutathione metabolism and antigen presentation were found [27]. This study used an acute dose in combination with a genetic intervention.

The aim of our study was to identify, in chronically quercetin administered rats, the relevant *in vivo* biological processes that are regulated by long-term quercetin intervention, mimicking the effects of chronic daily intake of quercetin in the diet. We chose lung tissue as a target tissue of quercetin action, because the highest concentrations of quercetin metabolites were found in lung tissue after chronic dietary supplementation of quercetin [26]. In addition, lung tissue seems to be an important target based on epidemiological studies indicating that a high flavonoid diet is protective against lung cancer [1]. We describe the analysis of large-scale gene expression changes induced by quercetin in lungs of rats fed a 1% quercetin diet (~500 mg quercetin/kg body weight per day) for 41 weeks. We identified pathways affected by chronic quercetin intervention and used quantitative real time PCR (qRT-PCR) and plasma analysis to confirm the effects in these pathways.

Methods

Animal experiment. The animal experiment was performed as previously described [26]. In short, inbred male Fisher 344 rats (Charles River Laboratories, Inc., Sulzfeld, Germany) were housed in pairs in cages, and were fed for 41 weeks a RM3[E]FG SQC breeding diet (Special Diets Services, Witham, UK) supplemented with 0% (control), 0.1% (w/w) quercetin or 1% (w/w) quercetin (six rats per group). Calculated quercetin intake for rats fed a 1% quercetin diet were ~500 mg quercetin/kg body weight per day and for rats fed a 0.1% quercetin diet: ~50 mg quercetin/kg body weight per day. The control diet did not contain quercetin or quercetin glycosides (data not shown). Animal weight and food intake did not differ between control and quercetin intake groups during supplementation. After 41 weeks, rats were anaesthetized in the morning, without overnight fasting, by inhalation of 5% isoflurane, using N_2O/O_2 (1:1, v/v) as carrier. Animals were fully bled via the abdominal aorta. Blood (8–12 ml) was collected in EDTA-tubes and plasma was subsequently prepared in Leucosep Centrifuge tubes (Greiner Bio-one, Longwood, FL, USA) by centrifuging for 20 min at 1000 *g* and 4 °C. Plasma samples were stabilized with ascorbic acid (1 mg/ml plasma final concentration) before storage at –80 °C. After blood collection, lungs were dissected and the left and right lungs were separately snap frozen in liquid nitrogen and stored at –80 °C. The left lung was used for RNA isolation. For the microarray experiment, lungs of rats fed a 1% quercetin diet and control diet were used. For plasma parameter and qRT-PCR analysis all treatment groups (control, 0.1% quercetin and 1% quercetin) were used. The experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands.

RNA isolation. Freeze dried lung tissue was homogenized in liquid nitrogen and total RNA was isolated with TRIzol (Invitrogen), according to the instructions from the manufacturer, with an additional phenol/chloroform/isoamylalcohol (25:24:1, v:v:v) purification step, followed by a second chloroform purification. Integrity and quality of RNA samples were checked by gel electrophoresis and spectrophotometric analysis using a Nanodrop (Isogen Life Science). All RNA samples had $OD_{260/280}$ ratios between 1.9 and 2.1, $OD_{260/230}$ ratios higher than 1.8, and displayed excellent visual integrity.

Microarray. A pooled reference design was used to analyze differential lung gene expression in 1% quercetin-treated rats vs control rats. All samples were labeled individually in duplicate. The protocol described in [28] was used with adjustments. In brief, RNA samples (35 µg total RNA per sample) from control and 1% quercetin-treated rats were reverse transcribed and directly labeled with Cy5 (Amersham Biosciences) using SuperScript II Reverse Transcriptase kit (Invitrogen). A reference pool was prepared by mixing equal quantities of total RNA from all samples (control group and 1% quercetin group). The reference pool was complemented with additional total RNA isolated from lungs of rats treated with a 1% quercetin diet for 1 week to yield a sufficiently large reference pool. Reference RNA samples (35 µg total RNA per sample) were reverse transcribed and labeled with Cy3 (Amersham Biosciences). Labeled cDNA samples were purified using QIAquick PCR Purification Kit (Qiagen) and ethanol precipitation steps. After purification, samples were denatured and Cy3-labeled cDNA samples

were pooled. Equal volumes of Cy5 target sample and Cy3 reference sample were combined, mixed with 2× hybridization buffer (Agilent Technologies) and 10× control targets (Agilent Technologies) and hybridized to a Whole Rat Genome oligo array, containing 44 290 60-mer oligo spots (including ~3000 control spots, G4131A, Agilent Technologies), for 17 h at 60 °C in Agilent hybridization chambers in an Agilent hybridization oven with rotation (Agilent Technologies). After hybridization, microarrays were washed with an SSPE wash procedure (Agilent Technologies) according to manufacturers protocol and scanned with a Scanarray Express HT scanner (Perkin Elmer). Signal intensities for each spot were quantified using ArrayVision 8.0 (GE Healthcare life sciences). Saturated spots and spots with signal intensity lower than two times the background were discarded, leaving 33 241 transcripts for normalization and analysis of differential expression. Quality check was performed for each microarray using both the LimmaGUI package in R [29] from the Bioconductor project and Microsoft Excel. The best microarray from two duplicates, based on MA plot, normal probability plot and signal intensity distribution [30, 31], was selected for further analysis. Data normalization was performed according to Pellis et al. [32] using GeneMaths XT 1.5 (Applied Maths). Pathway analysis was performed using MetaCore (GeneGo). Cluster analysis was performed using GeneMaths XT 1.5. Annotation from Agilent (Version 20050601) and TIGR annotations from Resourcer [33] were used for gene identification. Genes were classified according to gene ontology terminology and literature mining.

Quantitative RT-PCR. Differential expression for individual genes was assessed by qRT-PCR. cDNA was synthesized from 1 μg total RNA for each sample using the iScript cDNA Synthesis kit (Bio-Rad). Primers were designed for Sybr Green probes with Beacon Designer 4.0 (Premier Biosoft International) (Table 1). PCR amplification and detection was performed with the iQ SYBR Green Supermix and the MyIQ single-color real-time PCR detection system (Bio-Rad). A standard curve for all genes including reference genes was generated using serial dilutions of a pool prepared from all cDNA samples. The level of mRNA for each gene was normalized using *Hmbs* and *Rps26* as reference genes, chosen on the basis of microarray data, which showed similar expression levels for all microarrays. All samples were analyzed within-run in duplicate. PCR amplification and detection for all samples and genes was performed in triplicate on 3 separate days.

Plasma parameters. Plasma levels of free fatty acids, triglycerides and β-hydroxybutyrate were analyzed using commercial kits (WAKO chemicals). Assays were adjusted so that small sample volumes could be used and analyzed with a microplate reader (BioTec Synergy HT). Free fatty acids and triglyceride assays contained ascorbate oxidase. Therefore, added vitamin C, up to 1 mg/ml, in plasma samples did not interfere in the assays. Vitamin C (1 mg/ml) did not interfere in the assay for β-hydroxybutyrate (data not shown).

Statistical methods and software. For microarray data analysis, p values for differential expression between the 1% quercetin group ($n = 6$) and control group ($n = 6$) were calculated using t-test statistics on log intensity values. Fold changes were calculated using median linear signal intensity values. The p value was used to prioritize data based on highest significance between the six control animals and six quercetin animals. t-test statistics were used to test for significant differences in the quantitative RT-PCR experiment and plasma parameter analysis, and $p < 0.05$ was considered significant.

Results

Quercetin induced global gene expression changes in lungs of rats. We analyzed global gene expression changes in lungs of rats fed a quercetin diet for 41 weeks using microarrays. From the 33 421 transcripts with signal intensities above two times the background, 384 genes

Table 1. Primers used for quantitative real time PCR. All primers were intron-spanning.

Gene symbol	Sequence ID	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product length (bp)
Lpl	NM_012598	TCTGTCACACGTCTAACACATCAC	ACAATAGAAGGCTCCTCACTTTGC	191
Ech1	NM_022594	TGTGGTCTCTGGTGCAGGAAAG	GGTATCGGCTGATGAGGTCTCG	134
Hmgcs2	NM_173094	GCCTTGGACCGATGCTATGC	CTAGGGATTTCTGGACCATCTTGC	145
Acaa2	NM_130433	GGAACACAGGCGACCTTTGAG	GTGGTGGCTGCTGACAATGAC	166
Acox1	NM_017340	ACAGTTCTGAGAGCACAGCATC	CATTCCAGGAGAAAGGTTAAGGC	108
Pcca	XM_341383	AATGGGCAAGGTGAAACTGGTG	GTGGTGACTGAAGGGCTACAAG	104
Hmbs	NM_013168	TCGCTGCATTGCTGAAAGGG	CATCCTCTGGACCATCTTCTTGC	200
Rps26	NM_013224	TCATAGCAAGGTTGTCAGGAATCG	CTTTGGTGGAGGTCGTGGTG	103

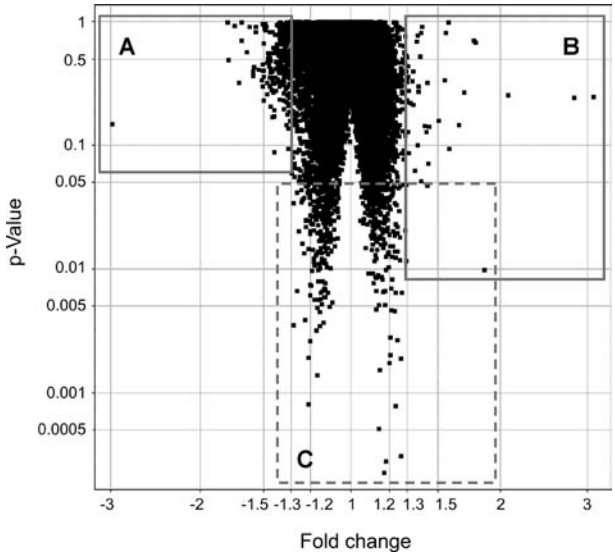

Figure 1. Volcano plot of 33 421 transcripts showing the *t*-test statistics *p* value plotted against the fold change of each transcript (1% quercetin vs control diet). (*a*) Genes that are down-regulated (fold change < –1.3), (*b*) genes that are up-regulated (fold change >1.3) and (*c*) genes with a $p < 0.05$.

were differentially expressed with a fold change higher than 1.3-fold. Of these, 34 genes were expressed with a fold change higher than 1.5-fold (Fig. 1). Essentially all of these genes showed a high variation in expression level in both the control and quercetin-treated group (Fig. 1a, b), and were therefore not significantly different between the two groups. Among the 34 genes with differential expression higher than 1.5-fold, 13 coded for immunoglobulins, whereas the remaining 21 genes did not belong to a common functional category (supplemental Table S1; supplemental data are available at www.foodbioactives.nl). Analysis of the similarities in expression profile of the 384 genes using hierarchical clustering resulted in grouping of 20 immunoglobulin genes up-regulated in the 1% quercetin-treated group as compared with the control diet group (Table 2). Three additional immunoglobulin genes were present in the 384 gene data set, of which 2 were down-regulated and 1 did not have a comparable profile. The average expression profile of the 20 up-regulated immunoglobulins (Fig. 2) illustrated that four animals in the control group showed a relatively low expression of immunoglobulin genes, whereas two animals showed a relatively high immunoglobulin gene expression. In contrast, in the quercetin diet group, five animals showed a relatively high immunoglobulin gene expression, whereas the remaining animal did not. This indicates that the immunoglobulin genes were coordinately regulated.

Genes were also ranked on the basis of highest significance instead of fold change (Fig. 1c). The 35 genes that were most significantly modulated ($p < 0.01$, Table 3) belonged to various functional categories. However, a group of 5

Table 2. Twenty up-regulated immunoglobulin genes (1% quercetin vs control) that grouped in one cluster. Identified using UPGMA clustering with Pearson's correlation coefficient as distance measure (384 genes were used with a fold change higher than 1.3). Genes are sorted based on cluster order.

Sequence ID	Gene name
Z75902[a]	Immunoglobulin, epsilon chain, variable region (clone Hg32)
XM_578308[a]	Similar to monoclonal antibody kappa light chain
XM_345754	Similar to Ig heavy chain v region VH558 A1/A4 precursor
AY331040[a]	Anti-SPE7 immunoglobulin E heavy chain variable region
Z93363[a]	Immunoglobulin variable region (clone ERF2.37)
XM_234745[a]	Similar to Ig heavy chain V-I region HG3 precursor
BC092586	Similar to immunoglobulin heavy chain 6 (Igh-6)
XM_345756[a]	Similar to BWK3 (predicted)
XM_234749	Similar to Ig heavy chain precursor V region (IdB5.7)
XM_234686[a]	Similar to immunoglobulin heavy chain
XM_345750	Similar to Ig H-chain V-JH3-region
XM_575534	Similar to NGF-binding Ig light chain
S81289	IgM kappa chain variable region {CDR1 to CDR3 region}
XM_341195	Immunoglobulin joining chain (predicted)
Z93359	Immunoglobulin variable region (clone ERF2.13)
AF217591[a]	Immunoglobulin kappa light chain variable region
X55180[a]	Monoclonal antibody Y13-259 Vk
XM_575533[a]	Similar to IG light chain Vk region Y13-259
M84148[a]	Rat IgK chain VJ1 region
XM_578339	Similar to immunoglobulin kappa-chain

[a] Immunoglobulin genes from supplemental Table S1 with fold change higher than 1.5.

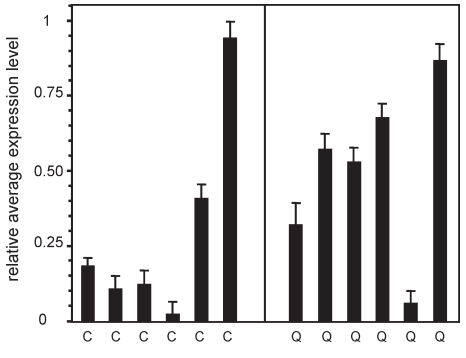

Figure 2. Relative average expression profile (mean ± SEM) of the 20 immunoglobulin genes from Table 2. Expression level for each gene was normalized using the mean expression level of that gene of all arrays ($n = 12$) (C: rat treated with a control diet for 41 weeks; Q: rat treated with a 1% quercetin diet for 41 weeks).

Table 3. Differentially expressed annotated genes with $p < 0.01$ in lungs of rats treated with a 1% quercetin diet ($n = 6$) as compared with rats treated with a control diet ($n = 6$). Genes are ordered based on function and within functional category based on p value.

Gene symbol	Gene name	Sequence ID	p value	Fold change
Energy metabolism and mitochondrial transport				
Atp5j2	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit f, isoform 2	Rn.3543	0.0008	–1.22
Ant2	ADP, ATP carrier protein, fibroblast isoform	NM_057102	0.0050	–1.14
Aco1	Aconitase 1	NM_017321	0.0069	1.18
Ech1	Enoyl CoA hydratase 1, peroxisomal	NM_022594	0.0082	1.16
Hmgcs2	3-Hydroxy-3-methylglutaryl-CoA synthase 2	NM_173094	0.0085	1.16
Nucleotide metabolism				
Dpysl3	Dihydropyrimidinase-like 3	Rn.93365	0.0019	1.26
Tyms	Thymidylate synthase	NM_019179	0.0039	–1.24
Cell adhesion				
Krt2-8	Keratin complex 2, basic, gene 8	BF281337	0.0059	–1.20
Igsf4a_predicted	Immunoglobulin superfamily, member 4A (predicted)	Rn.19928	0.0087	1.13
Asb16_predicted	Ankyrin repeat and SOCS box-containing 16 (predicted)	AW530584	0.0098	–1.16
Cell proliferation/differentiation				
Igfbp3	Insulin-like growth factor binding protein 3	NM_012588	0.0027	1.24
Fhl1	Four and a half LIM domains 1	BC061782	0.0080	1.21
Apoptosis				
Casp14_predicted	Caspase 14 (predicted)	XM_234878	0.0042	–1.15
Nme6	Expressed in non-metastatic cells 6, protein	AI014074	0.0063	–1.12
DNA repair				
Pnkp	Polynucleotide kinase 3′ -phosphatase	NM_001004259	0.0006	1.14
Mbd4_predicted	Methyl-CpG binding domain protein 4 (predicted)	XM_342742	0.0035	–1.30
Xpc_predicted	Xeroderma pigmentosum, complementation group C (predicted)	XM_232194	0.0067	1.13
Immune response				
LOC361454	Chemokine C-C motif receptor-like 1 adjacent	CB546044	0.0014	–1.17
Ccl12_predicted	Chemokine (C-C motif) ligand 12 (predicted)	XM_213425	0.0065	–1.28
LOC366755	Similar to immunoglobulin heavy chain	XM_345742	0.0097	1.86
Protein modification				
Gypc_predicted	Glycophorin C (Gerbich blood group) (predicted)	AY234182	0.0015	1.15
BRAP2	BRCA1-associated protein	BF284618	0.0066	–1.15
Hspcb	Heat shock 90 kDa protein 1, beta	AI236795	0.0094	–1.20
Pja2	Praja 2, RING-H2 motif containing	NM_138896	0.0096	1.09
Receptor				
Olr1350	Olfactory receptor 1350 (predicted)	NM_001000752	0.0039	–1.14
Olr197	Olfactory receptor 197 (predicted)	NM_001000188	0.0050	–1.10
Signal transduction				
Map2k4_predicted	Mitogen-activated protein kinase kinase 4 (predicted)	CA510796	0.0046	1.21
Rit1_predicted	Ras-like without CAAX 1 (predicted)	BF406174	0.0057	–1.13
Sorbs1_predicted	Sorbin and SH3 domain containing 1 (predicted)	AW917667	0.0068	1.21
Protein transport				
Vps39_predicted	Vacuolar protein sorting 39 (yeast) (predicted)	XM_342500	0.0054	1.14
Calcium transport				
Cacng6	Calcium channel, voltage-dependent, gamma subunit 6	NM_080694	0.0065	1.23
Cytoplasmic metabolism				
Ephx2	Epoxide hydrolase 2, cytoplasmic	BM986667	0.0078	–1.20
Miscellaneous				
Tm7sf1_predicted	Transmembrane 7 superfamily member 1 (predicted)	XM_237907	0.0003	1.26
Otos	Otospiralin	NM_139188	0.0020	–1.21
Tcte1_predicted	T-complex-associated testis expressed 1 (predicted)	XM_236941	0.0077	–1.12

genes (*Ech1*, *Hmgcs2*, *Ant2*, *Atp5j2*, *Aco1*) was involved in mitochondrial transport and energy metabolism. To get more insight whether energy metabolism was affected by quercetin intervention, we used a wider criterion for selection of genes ($p < 0.05$). Mapping this selection of differentially expressed genes (see supplemental Table S2) to the Metacore pathway database, containing over 400 curated pathway maps, showed an over-representation of genes involved in lipid metabolism pathways (data not shown). This indicated that energy metabolism is indeed a major process affected by quercetin.

We identified all genes involved in energy metabolism that were regulated by quercetin, and grouped them into the pathway diagram shown in Figure 3. Most of the regulated genes were shown to be involved in the catabolism of fatty acids. Quercetin induced both peroxisomal and

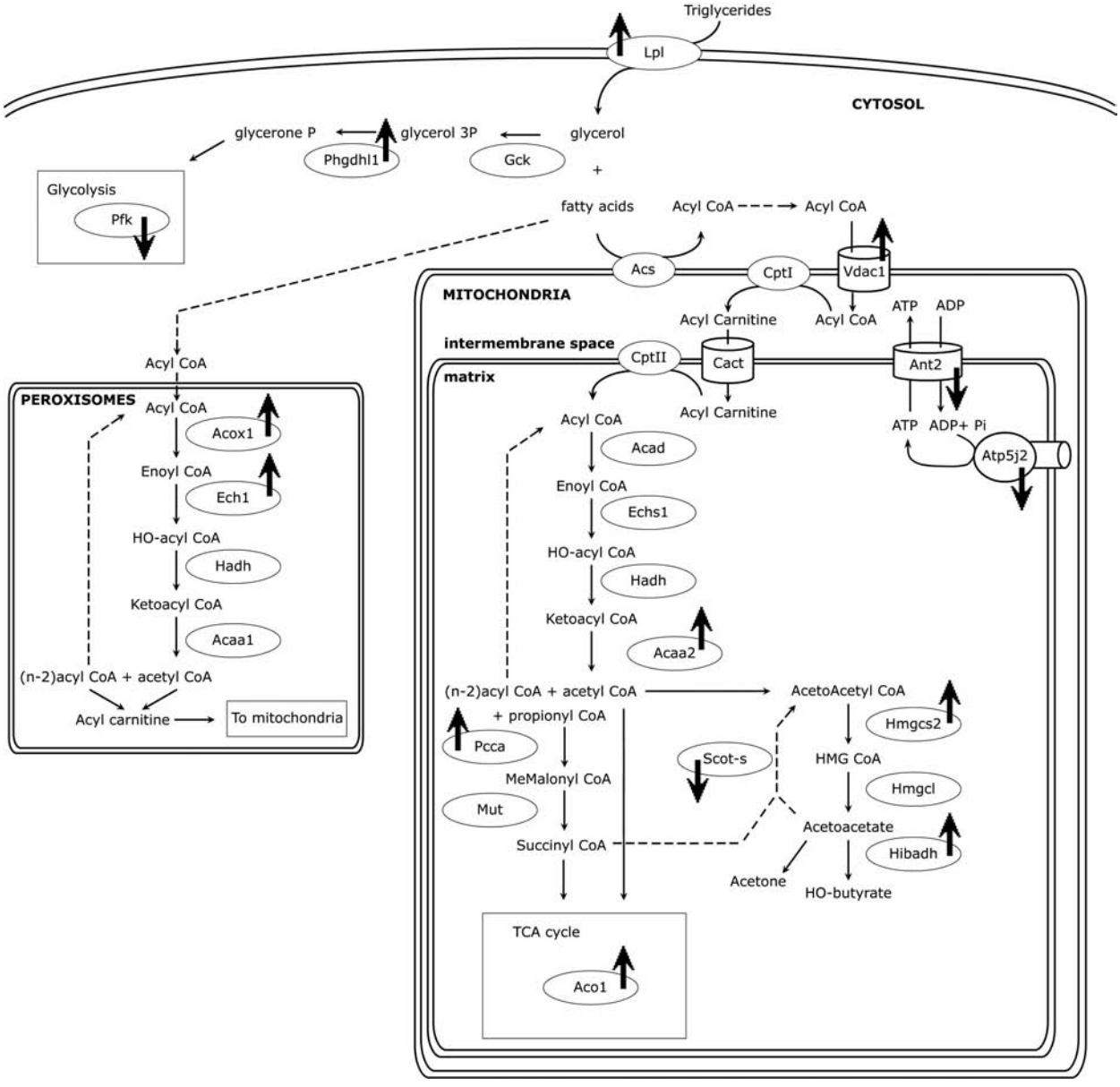

Figure 3. Diagram of fatty acid catabolism. Large arrows indicate up- or down-regulation by treatment of rats with a 1% quercetin diet for 41 weeks. Genes without an arrow did not change significantly. Ech1 together with Hadh are part of the bifunctional protein. Hadh was up-regulated as well, but did not pass the significance filtering criteria (data not shown). Acaa1: acetyl-CoA acyltransferase 1; Acaa2: acetyl-CoA acyltransferase 2; Acad: acetyl-CoA dehydrogenase; Aco1: aconitase 1; Acox1: acyl-CoA oxidase 1; Acs: acyl-CoA synthetase; Ant2: adenine nucleotide translocator 2; Atp5j2: ATP synthase (H^+ transporting, mitochondrial F0 complex, subunit f, isoform 2); Cact: carnitine-acylcarnitine translocase; CptI: carnitine palmitoyltransferase 1; CptII: carnitine palmitoyltransferase 2; Ech1: enoyl CoA hydratase 1 (peroxisomal); Echs1: enoyl CoA hydratase (short chain, 1, mitochondrial); Gck: glucokinase; Hadh: hydroxyacyl-CoA dehydrogenase; Hibadh: 3-hydroxyisobutyrate dehydrogenase; Hmgcl: 3-hydroxy-3-methylglutaryl-CoA lyase; Hmgcs2: 3-hydroxy-3-methylglutaryl-CoA synthase 2; Lpl: lipoprotein lipase; Mut: methylmalonyl CoA mutase; Pcca; propionyl CoA carboxylase; Pfk: phosphofructokinase; Phgdhl1: phosphoglycerate dehydrogenase like 1; Scot-s: succinyl-CoA:3-ketoacid-CoA transferase 1.

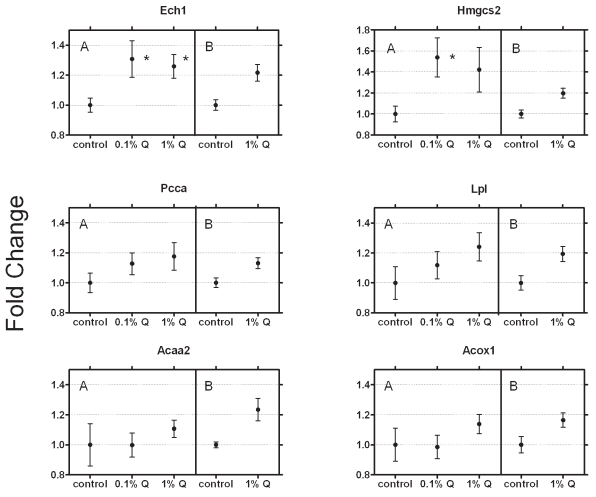

Figure 4. Expression of genes in lungs of rats treated with a control diet, 0.1% quercetin diet (0.1% Q) and 1% quercetin diet (1% Q) for 41 weeks. (*a*) Expression of genes analyzed with qRT-PCR, using *Hmbs* as reference gene [using Rps26 as reference gene showed similar results (data not shown)]. (*b*) Expression of genes analyzed with microarray. Data represent relative mean ± SEM ($n = 6$), control diet is set to 1.0. Significance is shown for qRT-PCR data only. Differences between expression levels in the control and 1% quercetin group in the microarray experiment were all significant (see supplemental Table S2 for p values). *$p < 0.05$ as compared with control group.

mitochondrial beta-oxidation genes (*Acaa2*, *Ech1*, *Acox1* and *Pcca*). In addition, the ketogenic pathway was clearly up-regulated: *Hmgcs2* and *Hibadh* expression levels were mutually increased in quercetin-treated rats, whereas *Scot-S*, the enzyme responsible for the reverse reaction, was down-regulated. Upstream of beta-oxidation, two important genes were also modulated by quercetin. Lipoprotein lipase (*Lpl*) and the gene coding for the channel importing acyl CoA into mitochondria, *Vdac1*, were both up-regulated by quercetin intervention. Another prominent mitochondrial transporter, *Ant2*, was down-regulated together with one of the subunits of the ATP synthase protein (*Atp5j2*). These two genes are both part of the last step in the oxidative phosphorylation. Besides regulation of genes involved in the catabolism of fatty acids, a key regulator of glycolysis, phosphofructokinase (*Pfk*) was down-regulated and tricarboxylic acid (TCA) cycle gene acotinase (*Aco1*) was up-regulated by chronic quercetin treatment. Of the two other adenine nucleotide translocators (*Ant1* and *Ant3*), only Ant1 was expressed in the lung, but did not change significantly. In addition, *Ppara*, *Ppard*, *Pparg*, *Pgc1a* and *Sirt1-7* were expressed in the lung but did not pass the significance filtering criteria (data not shown).

Fatty acid catabolism gene expression. To confirm microarray gene expression results, we analyzed quercetin-regulated genes related to the fatty acid catabolism pathways with quantitative real time PCR. We selected six genes (*Lpl*, *Hmgcs2*, *Ech1*, *Acaa2*, *Acox1*, *Pcca*) on the basis of regulation by quercetin and expression level in the microarray experiment. In addition, RNA isolated from lungs of rats fed a 0.1% quercetin diet for 41 weeks was used for qRT-PCR experiments. The genes with lowest variation within groups, and therefore the lowest p value in the microarray experiment (*Ech1* and *Hmgcs2*), were both significantly up-regulated in the 0.1% quercetin group and/or the 1% quercetin group ($p < 0.05$) (Fig. 4). *Ech1* was significantly up-regulated in both the 0.1% and 1% quercetin diet group, whereas *Hmgcs2* was significantly up-regulated only in the 0.1% quercetin diet group. Differential expression of *Acaa2*, *Acox1*, *Pcca* and *Lpl* using qRT-PCR was consistent with differential expression found in the microarray experiment. All genes were up-regulated by the 1% quercetin treatment; however, sample size (n = 6) was too small to reach significance using qRT-PCR ($p = 0.06$–0.1).

Fatty acid catabolism plasma parameters. To further investigate the role of quercetin in fatty acid catabolism, analysis of fatty acid catabolism parameters was performed in rat plasma. Consistent with microarray data and qRT-PCR results, chronic quercetin intervention in rats affected fatty acid catabolism plasma parameters. Plasma free fatty acid levels were significantly lower in the 1% quercetin group (Fig. 5a). The plasma levels of ketone bodies and triglycerides decreased with increasing quercetin dose, but were not significantly different between control and quercetin groups (Fig. 5b, c).

Discussion

In this study we established for the first time *in vivo* mechanisms of action of quercetin in lungs of rats chronically exposed to dietary quercetin. The effects of the long-term

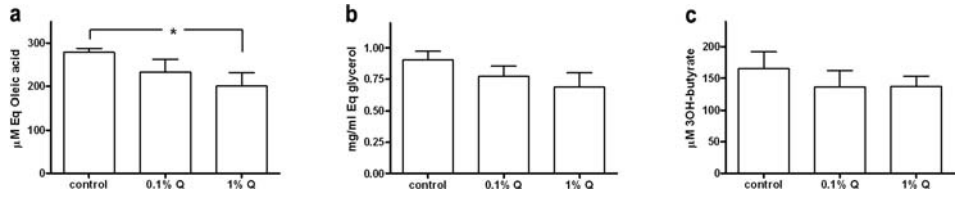

Figure 5. Plasma levels of fatty acid catabolism parameters in rats treated with a control diet, 0.1% quercetin diet (0.1% Q) or 1% quercetin diet (1% Q) for 41 weeks. Free fatty acids (*a*), triglycerides (*b*) and ketone bodies (*c*). Data represent mean ± SEM ($n = 6$). *$p < 0.05$.

nutritional polyphenolic intervention were explored on a whole genome level allowing for unbiased exploration. Quercetin coordinately up-regulated genes involved in fatty acid catabolism pathways in lungs, and lowered fatty acid levels in plasma. This indicates that chronic dietary intake of quercetin led to a modulation of fatty acid metabolism in rats.

Quercetin was shown to regulate genes in every step of the breakdown of triglycerides to short chain carbon molecules (Fig. 3). The first step in degradation of triglycerides is regulated by lipases that cleave fatty acids from glycerol for further processing in the cell. LPL is an extrahepatic lipase that is expressed in lungs [34], and is responsible for breakdown of plasma triglycerides from chylomicrons or lipoproteins. Entering the cell, fatty acids are converted to acyl CoA, and either degraded by peroxisomal beta-oxidation or transported into mitochondria, possibly by VDAC1 [35], for further degradation. VDAC1 is an outer mitochondrial membrane channel that permits passage of all metabolites, except for small membrane permeable compounds like oxygen, acetaldehyde and short chain fatty acids [36, 37]. After transportation into the mitochondrial matrix, acyl CoAs are degraded by a large set of beta-oxidation enzymes to yield acetyl CoA. Acetyl CoA can be utilized in the TCA cycle for energy production or converted to water-soluble transportable forms of acetyl CoA: acetoacetate and hydroxybutyrate by ketogenic enzymes HMGCS2, HMGL and HIBADH. Quercetin up-regulated *Lpl*, *Vdac1*, peroxisomal beta-oxidation enzymes (*Acox1* and *Ech1*), mitochondrial beta-oxidation enzymes (*Acaa2* and *Pcca*) and ketogenic enzymes (*Hmgcs2* and *Hibadh*). Peroxisomal *Ech1* together with *Hadh* are part of the bifunctional protein. Hadh was up-regulated as well, but did not pass the significance filtering criteria (data not shown). In contrast to the up-regulation of fatty acid catabolism pathways, adenine nucleotide translocator 2 (*Ant2*), together with one of the F0 subunits of ATP synthase, was down-regulated by quercetin. ATP synthase and ANT2 are responsible for the last steps of the oxidative phosphorylation, generating ATP by utilizing the inner membrane proton gradient and transporting ATP out of the mitochondrial matrix in exchange for ADP [38]. The fatty acid catabolism pathways will mostly be affected by quercetin when rate limiting steps are modulated. Quercetin regulated the rate limiting steps in peroxisomal beta-oxidation (*Acox1* [39]) and ketogenesis (*Hmgcs2* [40]); however, key regulators of mitochondrial beta-oxidation, like *Cpt1* [41] and *Acad* [39], were not affected. *Vdac1* was up-regulated by quercetin, but whether the up-regulation of this highly abundant porin in the outer mitochondrial membrane is important in controlling mitochondrial beta-oxidation is largely unknown. *Vdac1* was shown to be up-regulated by acetyl-L-carnitine in rat brain [42], and *Vdac1* gene expression was also shown to be important in regulation of ATP and ADP fluxes into and out of mitochondria [43], indicating that regulation of *Vdac1* on the gene expression level is of physiological relevance. Similarly, transcriptional regulation of *Lpl* by pharmacological and physiological stimuli resulted in enhanced LPL enzyme activities [44].

The down-regulation of genes coding for ANT2 and ATP synthase in combination with the up-regulation of substrate usage by quercetin may suggest an uncoupling of oxidative phosphorylation. Classical uncoupling in brown adipose tissue is typically regulated by uncoupling protein UCP1, leading to heat production instead of ATP [45, 46]. Uncoupling can also function to control the NAD^+/NADH ratio, thereby regulating metabolic processes dependent on these cofactors like ketogenesis [47, 48]. However, UCP1 was not significantly modified by the quercetin intervention, nor were UCP2 and UCP3. Quercetin-mediated down-regulation of ATP production and translocation in combination with an increase in ketone body formation indicates that oxidation of fatty acids was not needed for energy production. Instead, this process seems to be taking care of an excess of fatty acids in the lungs, consequently producing ketone bodies that might be exhaled immediately via the pulmonary system and not being absorbed in plasma, explaining the fact that the level of plasma ketone bodies was not altered by quercetin. The increased expression of ketogenic enzymes can also be explained by the fact that glycolysis was decreased, as indicated by the down-regulation of one of the key glycolytic enzymes, *Pfk*. Therefore, TCA cycle intermediates are not available from glycolysis and, therefore, the excess of acetyl CoA can only be converted into ketone bodies. This is supported by the fact that succinyl CoA seems to be available as an intermediate for the TCA cycle, because *Pcca*, the enzyme involved in the formation of succinyl CoA from odd-chain fatty acids, was up-regulated, and *Scot-S*, the enzyme that consumes succinyl CoA to produce, together with acetoacetate, acetoacetyl CoA, was down-regulated. As a result, succinyl CoA probably complements for the decreased levels of intermediates from glycolysis.

The decrease in plasma free fatty acid levels in quercetin-treated rats is in line with other studies showing that dietary quercetin can lower free fatty acid levels in rabbits [49]. The major site for metabolism of fatty acids is the liver. Therefore, the contribution of lung fatty acid catabolism to systemic fatty acid levels is probably low. However, the decrease in plasma fatty acid levels is consistent with the fact that quercetin modulates fatty acid metabolism in rats. Effects of quercetin on metabolism of energetic substrates were also reported by Mutch et al. [27]. Using affymetrix gene chips and cytochrome P450 reductase knockout mice, they showed that after a short, 4-h exposure to a 0.62% quercetin diet, amino acid metabolism, lipid metabolism and glutathione metabolism pathways were altered in liver and intestines. Moreover,

genetically eliminating the expression of the phase I enzyme did not alter quercetin metabolism itself, but altered the tissue-specific location of differentially regulated major metabolism pathways by quercetin [27].

Several other flavonoids have also been shown to interfere with the homeostasis of fat storage and fatty acid metabolism. The strongest evidence for modulation of lipid metabolism by a flavonoid was found for epigallocatechin galate (EGCg), a polyphenol found in high levels in green tea. Dietary supplementation of EGCg or extracts of green tea reduced the increase in body weight and adipose tissue mass induced by feeding a high fat diet in rodents (reviewed in [7, 50]). The anti-lipidemic effect of EGCg could be explained by a decrease in energy uptake in the intestines [51, 52] and an increase in fatty acid oxidation rates [53, 54]. The physiological effects on energy expenditure and body weight by tea catechins, like EGCg, were accompanied by modulation of genes involved in fatty acid oxidation and fatty acid synthesis. Green tea extracts increased the expression of fatty acid oxidation genes, medium chain acyl-CoA dehydrogenase (*Mcad*) and acyl-CoA oxidase (*Acox1*), in liver and muscle in mice [54, 55]. In addition, tea catechins were shown to influence gene expression of important fatty acid synthesis genes; fatty acid synthase (*Fas*) and stearoyl-CoA dehydrogenase (*Scd*) were both down-regulated by EGCg treatment in mice [52, 54]. Although in our study we did not challenge the animals with a high fat diet, we detected a similar up-regulation of fatty acid oxidation genes *Acox1*, *Acaa2*, *Ech1* and *Pcca* by dietary quercetin in the lungs of rats.

Mechanistic insights on how quercetin can modulate fatty acid metabolism come from studies using models that show direct actions of quercetin on enzyme activities or indirect activation of regulating factors. Firstly, quercetin was shown to inhibit ATP synthase resulting in decreased mitochondrial and cellular ATP levels [56–59]. NAD^+/NADH ratios were also affected by quercetin in isolated perfused liver [60, 61]. Secondly, quercetin and other flavonoids are known to interact with several transcription factors and other regulatory proteins [62, 63]. Among these, PPAR nuclear transcription factors or sirtuins may be targets for quercetin action [21, 64–66] in lungs of rats, mediating the observed effect of quercetin on fatty acid metabolism. Interpretation of studies illustrating *in vitro* activation of regulatory proteins by quercetin, however, is hampered by the fact that quercetin aglycone is normally not present in plasma and tissues of subjects [19, 26], unless local deconjugation of quercetin metabolites results in the formation of quercetin aglycone by enzymes with glucuronidase or sulfatase activity. Under certain physiological conditions, like inflammation and neoplastic growth, deconjugation of flavonoid glucuronides can occur, liberating the flavonoid aglycone [67, 68].

An additional biological response of rat lungs to quercetin supplementation seemed to be related to immune function. The highest fold changes of differential expression were found for genes coding for immunoglobulins. However, the variation between animals was large in both the control and quercetin groups. Cluster analysis of genes with highest fold change resulted in grouping of 20 immunoglobulin genes coordinately regulated in each animal. In two control animals and five quercetin animals immunoglobulin gene expression was relatively higher than in the remaining animals, demonstrating that immune response, possibly by an increase in lymphocyte infiltration in the lung, was enhanced in these animals. This may be a bona-fide response, but the power of the present study is insufficient to conclude this. This shows the importance of using individual hybridization of samples on microarrays. It also shows that pooling of samples before hybridization or labeling would have resulted in identifying immune response as the most important process affected by quercetin treatment, and would lead to the fatty acid catabolism being unidentified.

Microarrays have evolved from a promising technique with a wide range of possibilities [69, 70] to a valuable and reproducible tool for assessing gene expression changes [71]. Application of microarrays to nutritional studies can give insights into how organisms react to dietary compounds [72]. A limitation of studying effects of dietary compounds on gene expression is that the changes induced by these compounds are most of the times small [73]. Unlike pharmaceutical interventions specifically directed at one high-affinity molecular target, dietary intervention is less selective and generally results in small effects on gene expression. Using microarrays this problem can be overcome by studying a large number of genes at the same time. In this way, a combined effect of several small changes in the expression of genes belonging to a similar biological pathway can be easily detected. Other techniques, like RT-PCR and Northern blotting, would fail to identify such effects, because of the relatively low number of genes tested at the same time. Pathway analysis software is in most cases indispensable in obtaining differentially modulated biological processes. A notable difficulty of assessing gene expression changes on a genome level is the problem of multiple testing. To obtain significant changes in gene expression the sample size should be sufficiently large, else correcting for multiple comparisons within the same experiment (*e.g.* 44 000 comparisons on one microarray) does not yield significant data. In other words, the proportion of false positives in the dataset is large when thousands of genes are analyzed simultaneously. Methods for correcting for false discovery rate, like Benjamini-Hochberg, Holm and Bonferroni methods [74], are commonly used. Also Bayesian approaches are used that take into account the expression level of the genes for statistical analysis, assuming simi-

lar variances in genes with similar expression levels [75]. However, the costs for microarray experiments are most of the time still too high to allow for a high number of biological replicates. We prioritized the data on the basis of p value, and used the genes with the lowest p values ($p < 0.05$) for further analysis, without using statistical multiple correction techniques. When we combined the small effects of the most significantly changed genes into common biological pathways, we were able to identify effects of quercetin on fatty acid catabolism pathways. Individual gene expression changes were confirmed with qRT-PCR, and a decrease in plasma fatty acid levels indicated that quercetin altered fatty acid metabolism in rats. This biological validation of microarray data with independent techniques is the most powerful way to eliminate the possible misinterpretation of processes being false positively assigned as being regulated [76].

In conclusion, this study shows that quercetin induces fatty acid catabolism pathways in rat lung. In addition, quercetin showed an effect similar to uncoupling of oxidative phosphorylation combined with an up-regulation of genes involved in the formation of ketone bodies. Quercetin is, therefore, newly identified as another flavonoid that can regulate fatty acid metabolism *in vivo*.

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