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Effects of Coenzyme Q_{10} on Gene Expression and Inflammation: Results from *In silico*, *In vitro* and *In vivo* Studies

Dissertation

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„Gehe nicht, wohin der Weg gehen mag,

sondern dorthin, wo kein Weg ist,

und hinterlasse eine Spur.“

(*Jean Paul*)

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Summary

CoQ_{10} acts as an obligatory cofactor in the electron transport in the respiratory chain. Additionally, CoQ_{10} is required for the biosynthesis of pyrimidine nucleotides and the function of uncoupling proteins (UCPs). The reduced form of CoQ_{10} ($Q_{10}H_2$) serves as a potent antioxidant of lipid membranes. More recently, CoQ_{10} has been identified as a modulator of gene expression *in vitro*. This established function of CoQ_{10} was investigated in the present thesis on the molecular, cellular and physiological level by the use of bioinformatics and cell culture models as well as animal and human studies.

Based on text mining analysis, a functional connection of CoQ_{10}-sensitive genes was performed. Through signalling pathways of G-protein coupled receptors, JAK/STAT and integrin, 17 genes were functionally connected as previously published in Caco-2 cells. Moreover, promoter regions of genes related to inflammation revealed binding sites for the pivotal inflammatory transcription factor NFκB. To evaluate the data from the *in silico* analysis in an experimental context, monocytic cells were either treated with the oxidized (Q_{10}) or reduced ($Q_{10}H_2$) form of CoQ_{10}. Subsequently, the LPS-induced release of NFκB-dependent cytokines and chemokines was determined in cell-free supernatants. Finally, both for Q_{10}- and $Q_{10}H_2$-incubated cells reduced secretion levels of the pro-inflammatory mediators TNFα, RANTES and MIP-1α have been observed.

On the basis of the *in vitro* results, indicating slight differences in the anti-inflammatory properties of Q_{10} and $Q_{10}H_2$, redox-dependent gene expression patterns were hypothesized. To test this assumption *in vivo*, a genome-wide expression profiling was performed in various tissues (liver, kidney, heart and brain) of SAMP1 mice. Animals were either supplemented with Q_{10} or $Q_{10}H_2$ (500 mg/kg BW/d) for 6 (6 M) or 14 (14 M) months, respectively. In doing so, liver seemed to be the main target tissue of CoQ_{10} intervention, followed by kidney, heart and brain. In comparison to Q_{10}, $Q_{10}H_2$ supplementation was more effective to increase total CoQ_{10} levels in liver tissues of SAMP1 mice. Evaluation of the array data also indicated a stronger impact on gene expression by $Q_{10}H_2$ when compared to Q_{10}. Gene expression analysis in the liver of 14 M SAMP1 mice identified 11 $Q_{10}H_2$-sensitive genes primarily involved in cholesterol and lipid metabolism as well as inflammation and cell differentiation. Results from text mining revealed a functional connection of these genes in PPARα signalling pathways. Interestingly, these genes were not regulated in liver tissues of

Q_{10}-treated mice. Moreover, a key regulator gene in cholesterol metabolism, CYP51, was significantly down-regulated in the $Q_{10}H_2$-treated group, but became up-regulated in Q_{10}-supplemented animals. Hence, the redox sensitivity of the identified genes might be a possible explanation for the observed differences in liver cholesterol levels of $Q_{10}H_2$- and Q_{10}-supplemented mice.

For further verification of the results obtained from *in vitro* experiments, 53 healthy male volunteers were supplemented with $Q_{10}H_2$ (150 mg/d) for 2 weeks. Based on microarray data and stringent selection criteria, 7 $Q_{10}H_2$-sensitive genes related to inflammatory and apoptotic processes were identified in isolated monocytes. For the identified $Q_{10}H_2$-sensitive genes, text mining analysis revealed a functional connection in NFκB and PPAR signalling pathways. As PPARs are known key players in lipid metabolism and cell differentiation, in addition to the transcriptional effects, a putative impact on physiological parameters such as LDL cholesterol and blood cell count was determined. Thereby, $Q_{10}H_2$ supplementation showed a significant reduction of LDL serum cholesterol levels. Additionally, due to the significant differences in the count of maturated red blood cells (erythrocytes) and immature reticulocytes, effects on cell differentiation processes were hypothesized.

In summary, the results from the *in silico*, *in vitro* and *in vivo* studies show anti-inflammatory properties of $Q_{10}H_2$ as well as a regulatory role in cholesterol metabolism and cell differentiation processes. These effects could be explained, at least in part, by a modulatory impact of $Q_{10}H_2$ on redox-sensitive NFκB/PPARα dependent gene expression.

Zusammenfassung

Coenzym Q_{10} (CoQ_{10}) ist ein essentieller Cofaktor bei der Übertragung von Elektronen in der mitochondrialen Atmungskette. CoQ_{10} ist außerdem notwendig für die Pyrimidinbiosynthese und für die Funktion von Entkopplungsproteinen. Die reduzierte Form von CoQ_{10} ($Q_{10}H_2$) wirkt zusätzlich als Antioxidanz in biologischen Membranen. Aus zurückliegenden *in-vitro*-Untersuchungen kann auch ein Einfluss von CoQ_{10} auf Ebene der Genexpression geltend gemacht werden. Diese neue Funktion von CoQ_{10} wurde in dieser Arbeit mit Hilfe von bioinformatischen Ansätzen, Zellkulturexperimenten sowie Tier- und Humanstudien auf molekularer, zellulärer und physiologischer Ebene analysiert.

Mit Hilfe einer Text-Mining-Methode wurde eine funktionelle Verknüpfung von CoQ_{10}-sensitiven Genen abgebildet. Unter Einbezug von publizierten Genexpressionsdaten aus humanen Caco-2-Zellen wurden 17 Gene identifiziert, die durch gemeinsame Signalkaskadewege (G-Protein-gekoppelter Rezeptor, JAK/STAT und Integrin) miteinander verbunden sind. Zusätzlich wurden in den Promotoren von einigen CoQ_{10}-sensitiven Genen Bindungsstellen für den zentralen proinflammatorischen Transkriptionsfaktor NFκB identifiziert. Zur Validierung dieser *in-silico*-Analyse auf experimenteller Ebene wurden monozytäre Zellen mit oxidiertem (Q_{10}) oder reduziertem ($Q_{10}H_2$) CoQ_{10} präinkubiert. Anschließend wurde die LPS-induzierte Freisetzung von NFκB-abhängigen Zytokinen und Chemokinen im Zellkulturmedium untersucht. Die $Q_{10}H_2$- und Q_{10}-Inkubation führte zu einer signifikant verminderten Sekretion der proinflammatorischen Marker TNFα, RANTES und MIP-1α.

Auf Grundlage der *in-vitro*-Untersuchungen, die unterschiedliche antiinflammatorische Potentiale zwischen $Q_{10}H_2$ und Q_{10} erkennen lassen, wurde zusätzlich auf redox-sensitive Genexpressionsmuster geschlossen. Um dieser Annahme unter *in-vivo*-Bedingungen nachzugehen, wurde ein genomweites Expressionsprofil in Leber, Niere, Herz und Gehirn von SAMP1-Mäusen erstellt. Hierzu wurden die Tiere entweder mit $Q_{10}H_2$ oder Q_{10} (500 mg/kg BW/d) bzw. einer entsprechenden Kontrolldiät über einen Zeitraum von 6 (6 M) bzw. 14 (14 M) Monaten supplementiert. Hinsichtlich der untersuchten Gewebekonzentrationen wurde gezeigt, dass die Leber das Hauptzielorgan für die orale CoQ_{10}-Aufnahme darstellt, gefolgt von Niere, Herz und Gehirn. Im Vergleich zu Q_{10} führte die Aufnahme an $Q_{10}H_2$ zu einer signifikant höheren Akkumulation an Gesamt-CoQ_{10} in der Leber. In Übereinstimmung dazu konnte durch $Q_{10}H_2$-Supplementation auch ein

verstärkter Einfluss auf Genexpressionsebene in den untersuchten Geweben geltend gemacht werden. Aus genomweiten Expressionsanalysen in der Leber als Hauptzielorgan der 14 M SAMP1-Mäuse wurden 11 $Q_{10}H_2$-sensitive Gene identifiziert, die primär dem Cholesterolstoffwechsel, dem Lipidstoffwechsel, der Inflammation und der Zelldifferenzierung zuzuordnen waren. Unter Einbezug von Text-Mining-Analysen wurde für die identifizierten $Q_{10}H_2$-sensitiven Gene eine funktionelle Verbindung im PPARα-Signalweg postuliert. Diese Gene wurden nicht in den Lebergeweben der Q_{10}-supplementierten Tiere reguliert. Außerdem zeigte CYP51 – ein Schlüsselgen der Cholesterolbiosynthese – eine gegenläufige Regulation in Lebergeweben von $Q_{10}H_2$- und Q_{10}-supplementierten Tieren. Die Redox-Sensitivität der identifizierten Gene könnte eine mögliche Erklärung für die beobachteten unterschiedlichen Cholesterolkonzentrationen in der Leber von $Q_{10}H_2$- und Q_{10}-gefütterten Tieren liefern.

Zur weiteren Verifikation der *in-vitro*-Daten wurden 53 gesunde männliche Probanden über einen zweiwöchigen Zeitraum mit $Q_{10}H_2$ (150 mg/d) supplementiert. Mittels genomweiter Expressionsanalysen konnten 7 $Q_{10}H_2$-sensitive Gene in Monozyten identifiziert werden, die im Bereich Inflammation und Apoptose eine relevante Rolle spielen. Auf Grundlage von Text-Mining-Analysen wurden für die identifizierten $Q_{10}H_2$-sensitiven Gene funktionelle Verknüpfungen in PPAR- und NFκB-Signalwegen postuliert. Aufgrund der bekannten Funktion von PPARs im Lipidstoffwechsel und bei Zelldifferenzierungsprozessen wurden zusätzlich zu den transkriptionellen Veränderungen auch physiologische Parameter, wie LDL-Cholesterolspiegel und Blutzellenzahl miterfasst. Die Supplementation mit $Q_{10}H_2$ führte zu einer signifikanten Abnahme der LDL-Cholesterolkonzentration im Serum. Darüber hinaus konnten signifikante Veränderungen im Differenzierungsgrad der roten Blutzellen (Erythrozyten- und Retikulozytenzahl) festgestellt werden.

Zusammenfassend zeigen die Ergebnisse aus den *in-silico*-, *in-vitro*- und *in-vivo*-Studien, dass $Q_{10}H_2$ anti-inflammatorische Eigenschaften hat und an der Regulation des Cholesterolstoffwechsels und an Zelldifferenzierungsprozessen beteiligt ist. Die beobachteten Effekte sind zumindest teilweise durch einen Einfluss von $Q_{10}H_2$ auf die redox-sensitive und NFκB-/PPARα-abhängige Genexpression zu erklären.

Abbreviations

AACS	acetoacetyl-CoA synthetase
BRE	brain and reproductive organ-expressed
CCL3	chemokine (C-C motif) ligand 3
cDNA/RNA	complementary DNA/RNA
CFLAR	caspase 8 and FADD-like apoptosis regulator gene
CoQ_{10}	coenzyme Q_{10}
CRP	C-reactive protein
CXCL2	chemokine (CXC motif) ligand 2
CYP51	lanosterol 14α-demethylase
FABP5	fatty acid binding protein 5
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GIMAP7	GTPase, IMAP family member 7
HDL	high density lipoprotein
HMGCL	3-hydroxy-3-methylglutaryl-coenzyme A lyase
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HMGCS1	3-hydroxy-3-methylglutaryl-coenzyme A synthase 1
LPS	lipopolysaccharide
LXR	liver X receptor
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCP-1	monocyte chemoattractant protein-1
MIP	macrophage inflammatory protein
MMD	monocyte to macrophage differentiation factor
MPA2L	macrophage activation-2 like
MPEG1	macrophage-expressed gene 1
mRNA	messenger RNA
NFκB	nuclear factor kappa B
NMR	nuclear magnetic resonance
NR4A2	nuclear receptor subfamily 4, group A, member 2
oxLDL	oxidized LDL
PLTP	phospholipid transfer protein
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1
PPAR	peroxisome proliferator-activated receptor
Q_{10}	oxidized form of CoQ_{10}
$Q_{10}H_2$	reduced form of CoQ_{10}
qRT-PCR	quantitative real-time PCR
RANTES	regulated upon activation, normal T cell expressed and secreted
RNA	ribonucleic acid
ROS	reactive oxygen species
RXR	retinoid x receptor
SAMP1	senescence-accelerated mice prone 1
SREBF1	sterol regulatory element binding factor-1
STAT1	signal transducer and activator of transcription 1
TG	triglycerides
TNFα	tumor necrosis factor alpha
VLDL	very low density lipoprotein

General Introduction

Structure and biosynthesis of Coenzyme Q_{10}

Coenzyme Q or ubiquinone (CoQ) contains a benzoquinone ring and a lipid side chain constructed with several isoprenoid units. The number of isoprenoid units is species-specific. In humans, CoQ_{10} is the dominant form of CoQ (Figure 1).

Figure 1 Biochemical structure of CoQ_{10}

Coenzyme Q is synthesized in most mammalian cells. The endogenous synthesis of CoQ_{10} in humans occurs via the mevalonate pathway. This pathway includes a sequence of cellular reactions leading to the synthesis of farnesyl-pyrophosphate (Farnesyl-PP), a common substrate for the synthesis of cholesterol, dolichol, dolichyl-phosphate, coenzyme Q_{10} (ubiquinone) and the prenylation of proteins [1]. In comparison to other isoprenoid lipids, CoQ_{10} contains a benzoate ring as a non-isoprenoid moiety, which is originated from tyrosine. After condensation of the ring with the isoprenoid side chain, multiple enzymatic modifications and substitutions of the benzoate ring occur. Although the chronology of enzymatic steps is not totally disclosed yet, it is obvious that only one enzyme, the Farnesyl-PP synthase mediates the formation of dimethylallyl-PP and isopentenyl-PP in the mevalonate pathway [2].

Uptake and distribution

CoQ_{10} in the diet

The average daily dietary intake of CoQ_{10} is between 3-5 mg [3]. Normal plasma CoQ_{10} levels are around 1 µg/ml [4-6]. For a significant increase of blood plasma concentrations to 2 µg/ml, CoQ_{10} intake amounts of at least 100 mg/day are required. As shown in table 1, even with a diet containing large amounts of CoQ_{10}-rich foods, supplementation is necessary to increase CoQ_{10} concentrations in blood plasma about baseline levels.

Table 1 Coenzyme Q_{10} in the diet

Food	CoQ_{10} content [µg/g]	Daily Portion [g/day]	CoQ_{10} Intake [mg/day]
Chicken leg	17	120	2.0
Beef heart	41	120	4.8
Beef liver	19	120	2.3
Lamb leg	2.9	120	3.5
Herring	27	26	0.7
Trout	11	100	1.1
Orange	2.2	200	0.44
Cauliflower	0.6	200	0.12
Spinach	2.3	200	0.46
Potato	0.24	200	0.05

Data are based on [3,7]

Animal studies

For determination of CoQ_{10}-dependent effects, an uptake of exogenously administered CoQ_{10} by peripheral cells and tissues is an essential precondition. Early studies in rats and mice [8-12] at doses ranging from 10 to 123 mg/kg/day show increases of CoQ_{10} in liver and plasma only. One of the earliest reports studying the systemic distribution and uptake of [14C]-CoQ_{10} after intravenous application (0.6 mg/kg) in guinea pigs indicated that uptake from blood to peripheral tissues occurred for as long as 24 h. Concentrations in the heart, adrenal gland and brain peaked at approximately 24 h [13]. The observed differences between these animal studies have been linked to CoQ_9 concentration, which is the predominant endogenous form in rats and mice, whereas CoQ_{10} is in guinea pigs. However, studies utilizing higher doses of CoQ_{10} ranging from 150 to 650 mg/kg/day reported increased CoQ_{10} levels in tissues of rats and mice [14,15]. The increase of CoQ (CoQ_9 and CoQ_{10}) content was primarily affected by extension of supplementation period when compared to short-term applications of CoQ_{10} [15].

Human studies

In view of its biochemical structure, CoQ_{10} is nearly insoluble in aqueous solutions. Hence, after oral ingestion, exogenous CoQ_{10} is incorporated into chylomicrons for transport in the lymph to the peripheral blood [16]. Pharmacokinetics studies in humans indicate an increase of CoQ_{10} plasma levels about baseline – after fasting – after 1-2 h of oral administration [17-19]. Data from a recent study indicate a dose-dependent increase in CoQ_{10} plasma concentrations after supplementation with 90 mg, 150 mg or 300 mg of the reduced form of CoQ_{10} ($Q_{10}H_2$, ubiquinol) [17]. Most important, the increase of plasma levels per 100 mg values was remarkably higher when compared to the results obtained with the oxidized form (Q_{10}, ubiquinone) [20]. However, with regard to the administration of $Q_{10}H_2$, only slight differences have been observed between 90 mg, 150 mg or 300 mg $Q_{10}H_2$ with respect to increase per 100 mg (2.926, 2.457 and 2.550, respectively). Hence, the efficiency of absorption was not remarkable affected with increasing doses of $Q_{10}H_2$ in the described range. In general, in these studies plasma ubiquinol concentrations accounted for 96-98.5 % of total plasma CoQ_{10} [17]. This is comparable with natural occurring CoQ_{10} levels in healthy subjects [21-23]. However, in terms of increase over baseline values (fold change) and net increase per 100 mg, a more efficient absorption of CoQ_{10} in the reduced form ($Q_{10}H_2$) is assumed.

Biological functions of CoQ_{10}

Coenzyme Q_{10} has been identified as a crucial cofactor in several biological processes including its electron carrier function in the respiratory chain, as proton carrier in membranes other than mitochondria and its cofactor function of uncoupling proteins (UCPs). Moreover, the reduced form of CoQ_{10} serves as a potent antioxidant in mitochondria and lipid membranes, as well as a regenerator of other lipid-soluble antioxidants. More recently, an impact of CoQ_{10} on gene expression has been identified. The main biological functions of CoQ_{10} are summarized in table 2.

Table 2 Overview of main biological functions of CoQ_{10} and their original publication dates

Biological Function	Publication
Electron carrier in the respiratory chain	Crane et al, 1957
Proton carrier in other membranes than mitochondria	Sastry et al., 1961
Antioxidant of lipid membranes	Sastry et al., 1961
Cofactor of dihydroorotate dehydrogenase	Jones, 1980
Regeneration of antioxidants	Kagan et al., 1990
Regulator of transition pore opening	Fontaine et al., 1998
Cofactor of uncoupling proteins (UCPs)	Echtay et al., 2000
Mediator of gene expression	Groneberg et al., 2005

Cofactor functions of CoQ_{10}

CoQ_{10} functions as a cofactor in the transport of electrons from mitochondrial respiratory chain complexes I and II to complex III [24,25]. Additionally, CoQ_{10} is required as a cofactor for the biosynthesis of pyrimidine nucleotides (dihydroorotate dehydrogenase) [26], as well as for the permeability transition pore opening [27] and uncoupling protein function (UCPs) [28].

UCPs are located in the inner mitochondrial membrane and can translocate protons (H^+) from the outside to the inside of mitochondria [28]. Five UCPs (1-5) are known in humans and CoQ_{10} has been identified as an obligatory cofactor for UCP function. The oxidized form (Q_{10}) is able to mediate this essential function of CoQ_{10}, however, the reduced form ($Q_{10}H_2$) is not [28].

The inner membrane of mitochondria has a low permeability to ions so that the trans-membraneous transport mechanisms are dependent on the presence of macromolecule transporters and ion channels. The permeabilization of the membrane is due to the opening of an inner mitochondrial complex, the permeability transition pore (PTP) [29]. CoQ_{10} has been shown to prevent PTP opening by counteracting mitochondrial membrane potential depolarization [30].

Moreover, CoQ_{10} is involved in the biosynthesis of pyrimidine nucleotides. The de novo biosynthesis pathway contains six enzymes including dihydroorotate dehydrogenase (DHOD) [31,32]. CoQ_{10} has been identified as an essential compound in the biosynthesis of pyrimidine nucleotides as a cofactor for DHOD [26].

Antioxidant action of CoQ_{10}

In general there are four major groups of naturally occurring lipid soluble antioxidants including carotenoids, tocopherols, estrogens and coenzyme Q. The redox functions of CoQ_{10} are due to its redox-dependent ability to exchange two electrons between the oxidized (ubiquinone) and reduced form (ubiquinol) (Figure 2). This redox-dependent step can both be driven by simultaneous transfer of two electrons in a single step, or by two sequential steps of one electron transfer through a partially reduced semiquinone intermediate [33].

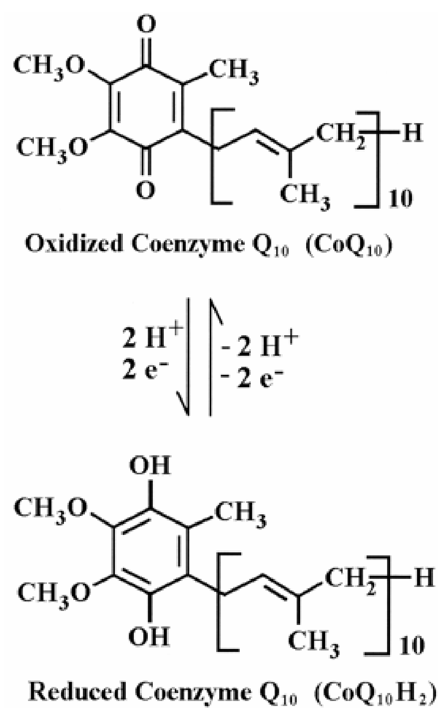

Figure 2 Schematic overview of the CoQ_{10} redox regulatory system

A large amount of data collected by experiments on e.g. liposomes, mitochondria and lipoproteins of the blood demonstrated that CoQ_{10} acts in its reduced form ($Q_{10}H_2$, ubiquinol) as a potent antioxidant and inhibits lipid peroxidation [34]. The effectiveness of CoQ_{10} as an inhibitor of lipid peroxidation is based on its complex interaction during the peroxidation process. As shown in figure 3, its primary action is the prevention of lipid peroxyl radicals (LOO•) production during the initiation process. Thus, $Q_{10}H_2$ reduces the initiating perferryl radical with the formation of ubisemiquinone and H_2O_2. Additionally, $Q_{10}H_2$ might eliminate LOO• directly. It has also been demonstrated that $Q_{10}H_2$ regenerates the antioxidant vitamin E from α-

tocopheroxyl radical [35]. Furthermore, CoQ_{10} has been shown to reduce protein oxidation [36], although not against all types of oxidative damage [37]. In general, the close spatial relationship of CoQ_{10} to the neighbouring membrane proteins seems to be a main factor for the observed protective effects of CoQ_{10} against lipid and protein oxidation, and moreover, DNA damage [38].

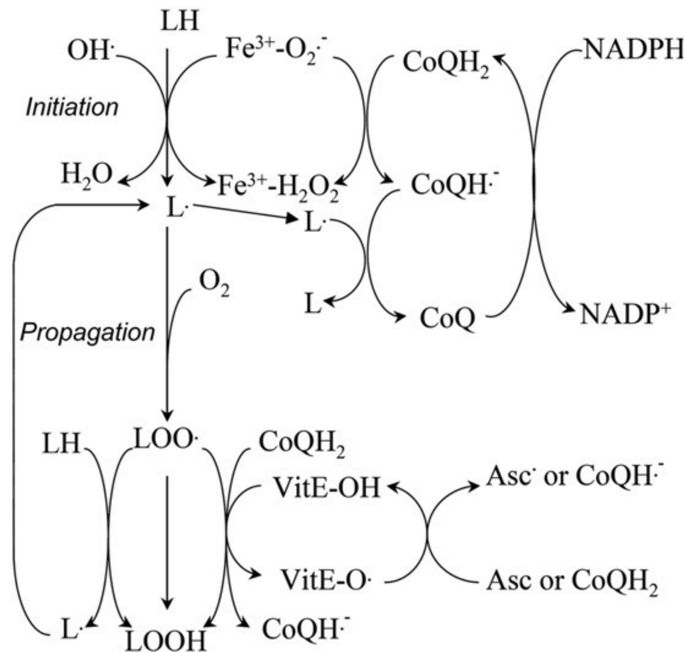

Figure 3 Ubiquinol-mediated action on lipid peroxidation

LH, polyunsaturated fatty acid; OH•, hydroxyl radical; Fe^{3+}-$O_2^{\bullet -}$, perferryl radical; $CoQH_2$, reduced CoQ_{10}; $CoQH^{\bullet -}$, ubisemiquinone; L•, carbon-centered radical; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide; Vit-O•, α-tocopheroxyl radical; asc•, ascorbyl radical, (taken from Bentinger et al., 2007)

Antioxidant role during aging

It is widely recognized that during aging there is a pro-oxidizing shift in the cellular redox state [39]. This process is accompanied by oxidatively damaged molecules, which in turn play a causal role in senescence processes [40-43]. Generally, the CoQ_{10} content as well as the ratios of CoQ_{10} and CoQ_9 vary in different organelles, tissues and species [44]. In mice, a 100-fold variation has been observed between different tissue homogenates [8,45] in the rank order kidney > heart > skeleton > muscle > brain > liver. However, it has to be taken into account that CoQ (CoQ_9 and CoQ_{10}) content of mitochondria was 6, 3, 4 and 23 times higher in liver, kidney, heart and skeletal muscle, respectively. Finally it seems that during aging CoQ concentrations primarily decrease in mitochondria [46-48] when compared to plasma

or tissue homogenates [47,49,50]. This assumption is also supported by further reports in mammalian species [51,52] demonstrating that the species-specific maximum life span is inversely correlated with mitochondrial rates of $O_2^{\bullet -}$ and H_2O_2 generation. Moreover, the CoQ_9 and CoQ_{10} content, as well as the ratio of these two are in turn correlated to $O_2^{\bullet -}$ generation [53]. For instance, CoQ_9 content of cardiac mitochondria correlates directly and CoQ_{10} inversely with the rate of $O_2^{\bullet -}$ generation.

Antioxidant role in the blood

As indicated for most of the tissues, the majority of CoQ_{10} in blood plasma occurs in the reduced form, which is finally due to the activity of the membrane-bound NAD(P)H oxidoreductases [33,54-57] and FAD-containing homodimeric enzymes including e.g. lipoamide dehydrogenase or glutathione reductase [58]. In plasma, CoQ_{10} is mainly transported by low-density lipoprotein cholesterol (LDL) and other apolipoprotein B-containing proteins, as well as high-density lipoprotein (HDL) [59,60]. The basal content of CoQ_{10} in LDL, VLDL and HDL is approximately 1.0, 1.2 and 0.1 nmol/mg protein, respectively. Following a single oral dose of 200 mg CoQ_{10}, the mean amounts increased to 3.5, 3.2 and 0.3 nmol/mg protein [61]. Furthermore, in comparison to mean tissue antioxidant concentrations where CoQ_{10} is 6-10 times higher than vitamin E, its amount in the blood is only one-tenth of vitamin E. Due to the efficient reductase mechanisms, however, CoQ_{10} remains in its active form ($Q_{10}H_2$) and plays an important role in preventing oxidation of LDL particles and other lipid soluble antioxidants in the blood [62]. Since oxidized LDL is considered to play an important role in the development of atherosclerosis, the intervention with CoQ_{10} is of great interest [63-65]. In contrast to red blood cells, which contain only minor levels of intracellular CoQ_{10}, mononuclear (monocytes, lymphocytes) and polynuclear (granulocytes) white blood cells contain considerable amounts of CoQ_{10}. Mononuclear cells were reported to have CoQ_{10} concentrations of 65 ± 24 pmol/mg protein [66]. Moreover, it has been shown that dietary administration *in vivo* increased CoQ_{10} content of monocytes and lymphocytes but not of polymorphic cells [67]. In general, not all observed effects on blood parameters can be explained by antioxidant mechanisms of CoQ_{10}, as several oxidative stress markers have not been shown to decrease after supplementation [68]. Hence, it appears that other mechanisms than the antioxidant role of CoQ_{10} – probably mediated by gene expression [67,69] – might be relevant in blood cell metabolism.

Gene expression and CoQ_{10}

Previous and recent *in vitro* and *in vivo* studies indicate effects of Coenzyme on gene expression (Table 3). These effects might be mediated by interacting with a transcription factor. Alternatively, the autoxidation of the semiquinone radical formed in various membranes during electron transport activity is a primary basis for the generation of hydrogen peroxide (H_2O_2) [70]. H_2O_2 in turn can activate transcription factors such as nuclear factor kappa B (NFκB) [71,72]. In general there are distinct hints that reactive oxygen species (ROS) mediate modulatory effects on gene expression [71,73-77]. In addition to its well known function as an antioxidant [35,78], the impact of Coenzyme Q_{10} on gene expression needs to be further evaluated as indicated by the inadequate number of previous and recently published studies (Table 3). Especially studies reflecting effects of CoQ_{10} on gene expression in humans are lacking.

Table 3 Overview about previous and recently published studies reflecting CoQ_{10} effects on gene expression

Cell line/Species	Cell/Tissue model	References
In vitro		
HeLa cells	cervix	Gorelick et al., 2004
Caco-2 cells	intestinum	Groneberg et al., 2005
LT97 and SW480 cells	colon	Nohl et al., 2005
THP-1 cells	monocytes	Schmelzer et al., 2009
In vivo		
Humans	skeletal muscle	Linnane et al., 2002
C57BL6 mice	heart	Lee et al., 2004
C57BL6J mice	liver	Schmelzer et al., 2009
C57BL6 mice	liver	Sohet et al., 2009
SAMP1 mice	liver, heart, brain, kidney	Schmelzer et al., 2009, *in press*

General Introduction

Aim of the thesis

The present thesis was focused on effects of Coenzyme Q_{10} on inflammatory processes and lipid metabolism by targeting gene expression.

To support the hypothesis of a functional role of CoQ_{10} on gene expression, a text mining analysis was initially performed on data from a previous cell culture experiment in human Caco-2 cells [69].

Chapter I

C. Schmelzer, I. Lindner, C. Vock, K. Fujii, F. Döring

"Functional connections and pathways of coenzyme Q_{10}-inducible genes: an *in-silico* study". printed in *IUBMB Life*

For evaluation of the postulated effects obtained by *in silico* analysis, anti-inflammatory effects of the reduced form of CoQ_{10} ($Q_{10}H_2$) were determined in the monocytic cell line THP-1.

Chapter II

C. Schmelzer, G. Lorenz, G. Rimbach, F. Döring

„*In-vitro* effects of the reduced form of Coenzyme Q_{10} on secretion levels of TNFα and chemokines in response to LPS in the human monocytic cell line THP-1". printed in the *Journal of Clinical Biochemistry and Nutrition*

To evaluate putative redox-dependent differences of CoQ_{10} on gene expression, microarray data from different tissues of SAMP1 mice were analyzed after supplementation of mice with the oxidized (Q_{10}) or reduced form ($Q_{10}H_2$) of CoQ_{10} for 6 or 14 months.

Chapter III

C. Schmelzer, H. Kubo, M. Mori, J. Sawashita, M. Kitano, K. Hosoe, I. Boomgaarden, F. Döring, K. Higuchi

"Supplementation with the reduced form of Coenzyme Q_{10} decelerates phenotypic characteristics of senescence and induces a peroxisome proliferator-activated receptor-α gene expression signature in SAMP1 mice". in press in *Molecular Nutrition and Food Research*

To determine putative anti-inflammatory and anti-apoptotic effects of $Q_{10}H_2$ *in vivo*, expression levels of genes and proteins related to inflammatory and apoptotic processes were examined in isolated monocytes and serum samples of $Q_{10}H_2$-supplemented subjects.

Chapter IV

C. Schmelzer, P. Niklowitz, J. Okun, T. Menke, F. Döring

“Effects of the reduced form of Coenzyme Q_{10} on gene expression, inflammation and cell differentiation in humans”. drafted manuscript

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General Discussion

Coenzyme Q_{10} (CoQ_{10}) is a lipid-soluble compound functioning as an essential cofactor in the respiratory chain [1,2] and serves as a potent antioxidant in mitochondria and lipid membranes [3,4]. Furthermore, CoQ_{10} acts as an obligatory cofactor for the dihydroorotate dehydrogenase [5] and for the function of uncoupling proteins (UCPs) [6]. More recently, CoQ_{10} has been identified *in vitro* as a modulator of genes involved in cell signalling, metabolism and transport [7]. In view of the antioxidant properties of the reduced form of CoQ_{10} ($Q_{10}H_2$), anti-inflammatory effects were hypothesized. In order to unravel putative effects of $Q_{10}H_2$ on inflammation and lipid metabolism on the transcriptional and physiological level, cell culture experiments in human monocytes as well as studies in SAMP1 mice and humans were performed in this thesis.

To identify CoQ_{10}-sensitive genes with putative functional connections in inflammatory processes, text-mining analysis was initially performed with gene expression data from a previous *in vitro* study [7] (Chapter I). For this purpose, intestinal Caco-2 cells were incubated with 50 µM CoQ_{10} for 24 h. Gene array technology revealed changes in steady-state levels of 464 genes at a threshold-factor of at least 2.0 in three independent experiments. To decipher functional and regulatory connections between the identified CoQ_{10}-inducible genes and more precisely, to define a putative role of CoQ_{10} in inflammatory processes, BiblioSphere PathwayEdition (BSPE) software package was used. To our knowledge, BSPE is the only currently available system combining literature analysis and promoter analysis. The primary source of BSPE is NCBI Pubmed, where about 15 million scientific abstracts are available for analysis of co-citations of uploaded gene names, synonyms and relation concepts. For transcription factor binding site analysis in promoter regions of co-citated genes, Genomatix MatInspector was additionally applied. From the combined literature and transcriptional factor binding site analysis with the concomitant inclusion of stringent criteria (GFG level B3), several CoQ_{10}-inducible genes were functionally connected with each other by signalling pathways of G-protein coupled receptors, JAK/STAT or integrin (Figure 1). Moreover, promoter analysis of genes which are related to inflammation revealed binding sites for the pivotal inflammatory transcription factor nuclear factor kappa B (NFκB) [8]. In summary, text mining analysis supported our hypothesis of CoQ_{10}-modulating effects on the expression of inflammatory genes. A general impact of CoQ_{10} on gene

expression was also confirmed by other *in vitro* and *in vivo* studies [9-15]. However, with respect to these data, several side effects including e.g. autoxidation of the semiquinone intermediate ($Q_{10}H\bullet$) may also contribute to the observed effects of CoQ_{10} on gene expression. This is due to the fact that the $Q_{10}H\bullet$-mediated generation of hydrogen peroxides (H_2O_2) and reactive oxygen species (ROS) may also have a distinct impact on gene expression [16,17].

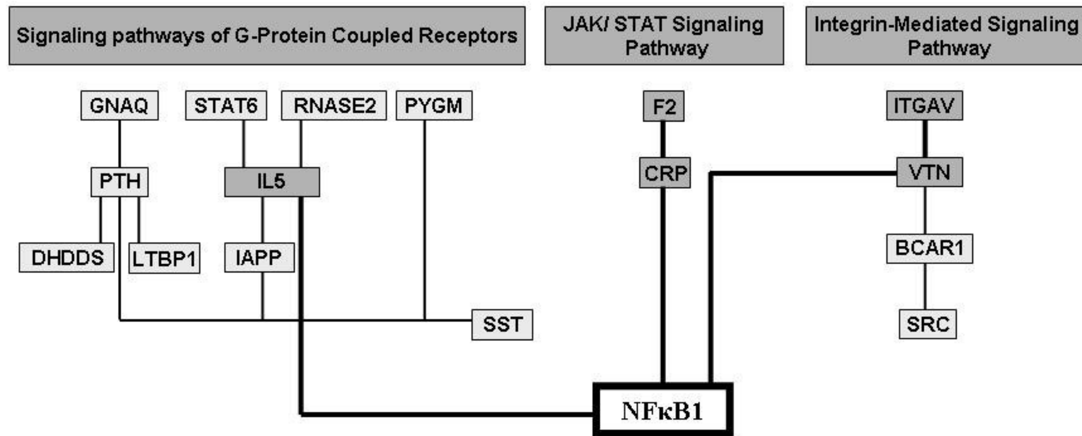

Figure 1 Identification of putative connections and pathways of CoQ_{10}-sensitive genes by BiblioSphere PathwayEdition Software

The indicated pathways have been identified by analysis of 464 CoQ_{10}-inducible genes with the BiblioSpherePathwayEdition software package based on co-citations with transcription factors, functional co-citations, and co-citations with other genes in the network. The figure was modified according to Schmelzer et al. [18] and emphasizes genes with promoter binding sites for the transcription factor NFκB. BCAR1, Breast cancer anti-estrogen resistance 1; CRP, C-reactive protein; DHDDS, Dehydrodolichyl diphosphate synthase; F2, Thrombin; GNAQ, Guanine nucleotide binding protein; IAPP, Islet amyloide polypeptide; IL5, Interleukin 5; LTBP1, Latent transforming growth factor beta binding protein 1; ITGAV, vitronectin receptor; PTH, Parathyroid hormone; PYGM, Glycogen phosphorylase; RNASE2, Ribonuclease; SRC, V-src sarcoma; SST, Somatostatin; STAT6, Signal transducer and activator of transcription 6; VTN, Vitronectin

General Discussion

With regard to the results from the *in silico* analysis (Chapter I), indicating modulatory effects of CoQ_{10} on inflammatory gene expression, the impact of pre-incubation with the reduced form of CoQ_{10} ($Q_{10}H_2$) on LPS-induced release of various key pro-inflammatory mediators (TNFα, MIP-1α, RANTES and MCP-1) was studied in the monocytic cell line THP-1 (Chapter II). With respect to the literature data [19,20] and our own published results [21,22], most *in vitro* studies used oxidized CoQ_{10} (Q_{10}). Although Q_{10} can be enzymatically converted into its reduced form [23], this step may be accompanied by the generation of reactive oxygen species (ROS) [24] with an additional impact on gene expression [16,17]. In general THP-1 cells were used as a model of human monocytes, because monocytic cells are relevant key players in a variety of inflammatory processes [25,26]. Triggering of cells with lipopolysaccharide (LPS), the major outer membrane component of gram-negative bacteria, induces downstream signalling cascades of the transcription factor NFκB [27]. This in turn leads to the translocation of NFκB from the cytoplasm to the nucleus, where it specifically binds to promoter regions of inflammatory genes [25,27]. Our working model suggests that, due to its antioxidant properties, $Q_{10}H_2$ may be able to reduce LPS-induced ROS generation with subsequent NFκB translocation into the nucleus and inflammatory gene expression (Figure 2). In this context, NFκB activity has been shown to be inhibited by various compounds with antioxidant properties [28,29].

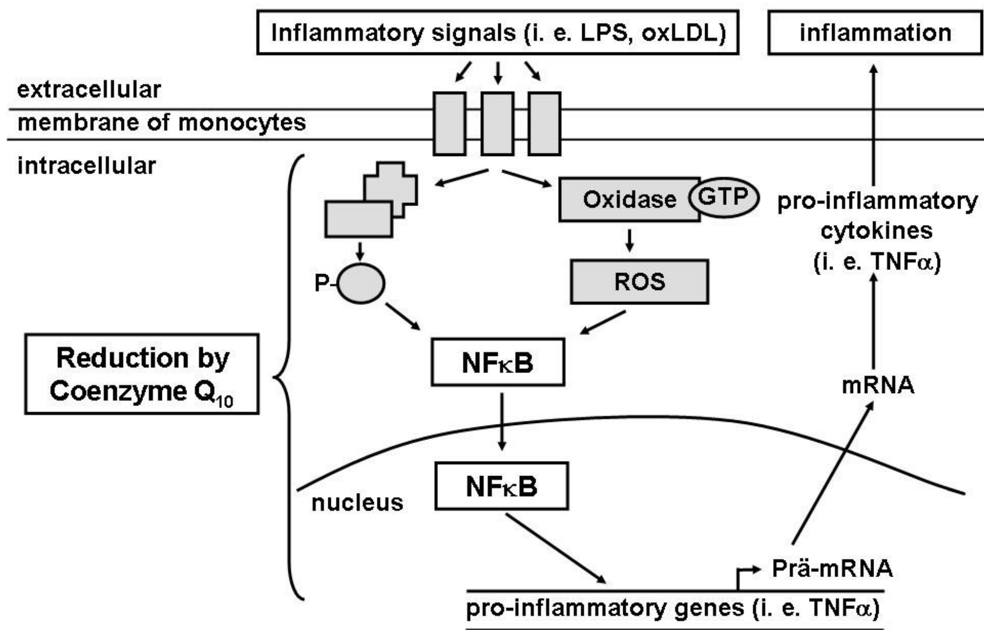

Figure 2 A current working model illustrating the anti-inflammatory role of $Q_{10}H_2$ by targeting gene expression

The *in vitro* results in the monocytic cell line THP-1 suggest distinct reducing effects of $Q_{10}H_2$ on the release of key pro-inflammatory cytokines (TNFα) and chemokines (RANTES, MIP-1α) (Chapter II). In contrast, no effects were observed for MCP-1. These effects were comparable to a previous study in THP-1 cells, where the oxidized form of CoQ_{10} (Q_{10}) was used [21]. However, with regard to this study, anti-inflammatory effects on the LPS-induced release of inflammatory mediators were slightly more pronounced with $Q_{10}H_2$ when compared to Q_{10}. Although it has been shown that Q_{10} can be efficiently converted into its reduced form by various enzymes [30-33], the localization of Q_{10} reductases differs considerably between cell types and compartiments. Thus, a compartmentalization of reductase activities in the cell, and moreover, the clarification of mechanisms underlying the incorporation of CoQ_{10} in the plasma membrane may give a further hint for the efficiency of Q_{10} reduction. With regard to the current knowledge on membrane dependent reductase mechanisms, a direct incubation with $Q_{10}H_2$ seems to be most effective. In this respect, dose-dependent intracellular increases of total CoQ_{10} as well as relative levels of $Q_{10}H_2$ have been observed after $Q_{10}H_2$ incubation (0.1 – 100 µM) in THP-1 cells [34].

In view of the *in vitro* experiments indicating stronger anti-inflammatory effects of $Q_{10}H_2$ when compared to Q_{10} [21,35], redox-dependent gene expression patterns were hypothesized. To determine this assumption under physiological conditions, a

genome-wide expression profiling in various tissues (liver, kidney, heart and brain) of SAMP1 mice supplemented with $Q_{10}H_2$ or Q_{10} (500 mg/kg BW/d) for 6 or 14 months, respectively, was performed (Chapter III). SAMP1 mice were used because besides other manifestations of the aging process, they also show a higher oxidative stress status [36,37]. Hence, they are a suitable model to study putative effects of antioxidants on physiological and molecular readouts. Based on gene expression data, liver has been shown to be the main target tissue of CoQ_{10} intervention, followed by kidney, heart and brain. A prior accumulation of oral and/or intra-peritoneal administered CoQ_{10} in the liver of rodents has been already described before [38-41]. One possible reason for this effect could be the primary incorporation of CoQ_{10} in LDL particles for its transport in the blood and subsequent accumulation in liver tissues [42]. CoQ_{10}-mediated preventions of LDL oxidation processes have been already described *in vitro* and *in vivo* [43-46]. Moreover, stringent evaluation of array data revealed a stronger impact on gene expression by $Q_{10}H_2$ when compared to Q_{10}. This might be due to differences in the bioavailability. In fact, $Q_{10}H_2$ supplementation was more effective than Q_{10} to increase CoQ_{10} levels in the liver of SAMP1 mice. This was also indicated by plasma levels of a previous study in SAMP1 mice [47] and humans [48,49]. To explore long-term effects of $Q_{10}H_2$-supplementation on the transcriptional level, a more detailed analysis of the array data was performed in liver tissues of SAMP1 mice supplemented with $Q_{10}H_2$ for 14 months. Finally, 11 $Q_{10}H_2$-sensitive genes have been identified, primarily related to cholesterol (HMGCS1, HMGCL, HMGCR) and lipid/lipoprotein metabolism (AACS, SREBF1, FABP5, PLTP) as well as inflammation (STAT1) and cell differentiation processes (MMD2, MPEG1, MPA2L). Results from text mining revealed an involvement of these genes in PPARα signalling pathways. Interestingly, these genes, with the exception of SREBF1, were only regulated in liver tissues of $Q_{10}H_2$-supplemented animals when compared to Q_{10}-treated mice. This was also shown by the subsequent results from qRT-PCR verification experiments for these genes and additional genes playing also a key role in the cholesterol biosynthesis pathway (*unpublished results*). Moreover, CYP51, was significantly down-regulated in the $Q_{10}H_2$-treated group, but became up-regulated in Q_{10}-supplemented animals. The encoded protein of this gene plays a key role in the demethylation process of lanosterol, a relevant metabolite in the cholesterol biosynthetic pathway. These data show, to our knowledge for the first time, redox-dependent gene regulatory effects of

CoQ_{10} *in vivo*. Furthermore, our data from liver tissues of SAMP1 mice also show pronounced differences in cholesterol content of $Q_{10}H_2$ and Q_{10}-supplemented mice with distinct higher levels in the $Q_{10}H_2$-group (*unpublished results*). As the key regulator genes of cholesterol biosynthesis were simultaneously down-regulated in the $Q_{10}H_2$-treated group, a negative feedback mechanism of liver cholesterol concentration on the transcription of cholesterol biosynthetic genes is suggested. A negative feedback loop in liver cholesterol metabolism has been already described previously [50-53] and was reported to be mediated by lanosterol 14alpha-demethylase (CYP51) through binding of sterol regulatory element binding proteins (SREBPs) [54-57]. It would be interesting to know under future studies whether effects on various up- and downstream metabolites of lanosterol demethylase (CYP51) including e.g. lanosterol, lathosterol, 7-dehydro-cholesterol and desmosterol (Figure 3) are also existent. Potential differences in the bioavailability of $Q_{10}H_2$ and Q_{10} with an additional impact on cholesterol absorption could also be determined by major plant sterol concentrations of e.g. beta sitosterol, stigmastanol and campesterol in the respective liver samples. This is due to the fact that plant sterols are chemical homologues of cholesterol, which have been shown to interfere with cholesterol in the intestine, and thus reduce the efficiency of its absorption [58].

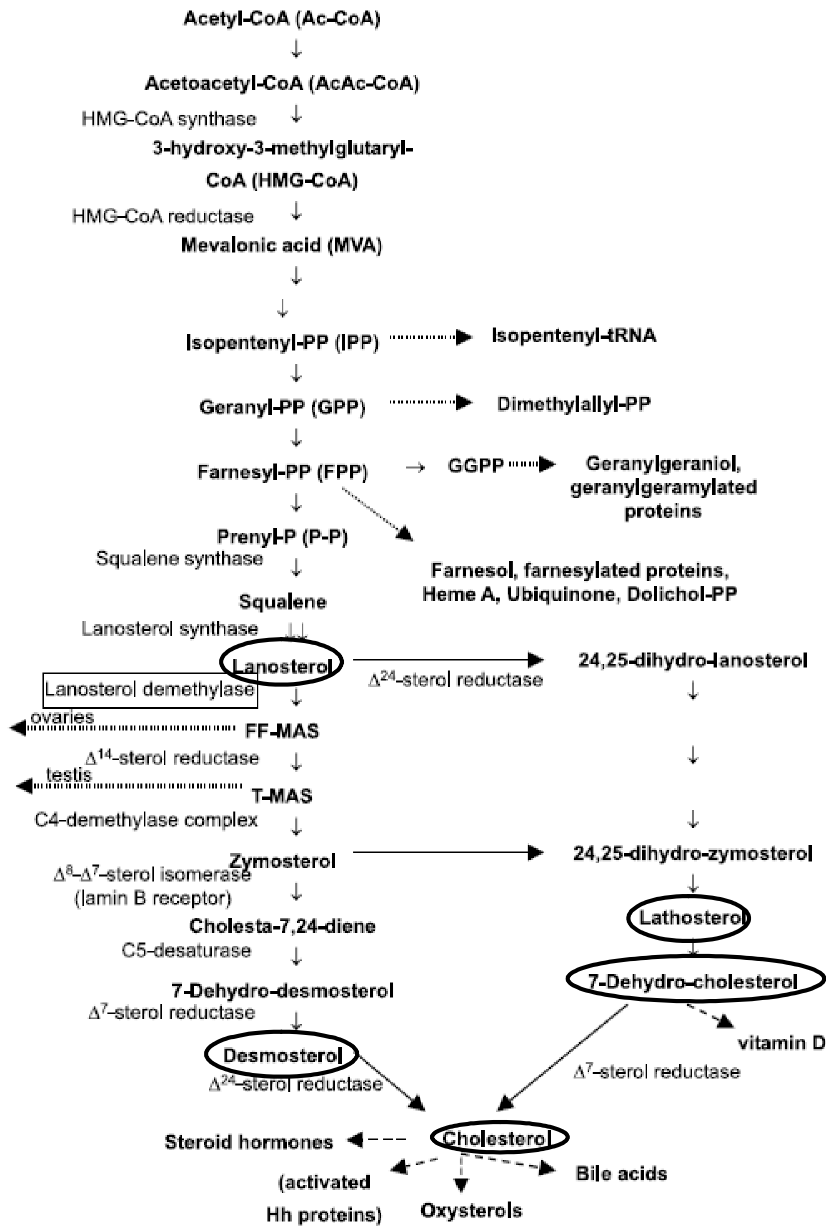

Figure 3 Scheme of the cholesterol biosynthetic pathway including the intermediates assumed to be differently affected by $Q_{10}H_2$ or Q_{10} treatment through lanosterol demethylase (CYP51) expression

Dotted arrows depict intermediates of the cholesterol biosynthetic pathway that accumulate in a tissue-specific manner and have other physiological roles than related to cholesterol metabolism. Cholesterol metabolites are indicated by dashed arrows. The lanosterol demethylase is boxed. Encircled terms reflect intermediates with a putative different accumulation in liver tissues of $Q_{10}H_2$ and Q_{10}-supplemented SAMP1 mice. (modified after Debeljak et al., 2003)

General Discussion

As described in chapter III, the observed regulatory effects of $Q_{10}H_2$ on genes involved in cholesterol and lipid metabolism, cell differentiation/proliferation processes and inflammation are hypothesized to be mediated through PPARα signalling. PPARα-mediated effects on lipid metabolism as well as inflammatory and differentiation processes have been already described previously [59-64]. Finally, three putative functions of $Q_{10}H_2$ were proposed for PPARα-mediated signalling processes (Chapter III): 1. as an antioxidant, leading to decreased levels of LXR agonists 2. as an antagonist of LXR, leading to PPARα/RXR heterodimers and PPRE activation and 3. as an agonist of PPARα, leading to PPARα/RXR heterodimers and PPRE activation. As the identified gene pattern was not found in liver samples of Q_{10}-supplemented mice, a $Q_{10}H_2$-specific effect due to its antioxidant properties and/or bioavailability was suggested. In this context, dietary effects on PPARα signalling pathways have already been described in mice for other lipophilic antioxidants such as α-tocopherol [65]. Although the exact mechanism of $Q_{10}H_2$-PPARα interaction cascades has not been definitely clarified, our results support a regulatory role of $Q_{10}H_2$ in PPARα signalling processes.

As the data from the *in vitro* experiments indicated slightly stronger anti-inflammatory effects of $Q_{10}H_2$ when compared to Q_{10} [21,35], a 2-week human intervention study was performed with $Q_{10}H_2$ (150 mg/d) in 53 healthy male volunteers (Chapter IV). Monocytes were isolated from volunteers before (T_0) and after (T_{14}) supplementation with $Q_{10}H_2$ to obtain expression changes of genes related to inflammatory and apoptotic processes. In this context anti-inflammatory [21,22,35] and anti-apoptotic [66-70] effects of CoQ_{10} have been already described *in vitro*. However, most published studies examined effects of the oxidized form of CoQ_{10} [19,20,71,72]. To our knowledge, this is the first study investigating anti-inflammatory and anti-apoptotic effects of the reduced form of CoQ_{10} ($Q_{10}H_2$) in a healthy study population. As all determined effects in this study were related to a simple one-group pre-post analysis, additional criteria were implemented to evaluate the compliance of $Q_{10}H_2$ supplementation and its subsequent effects on gene expression in isolated monocytes of study subjects. Thus, the involvement of additional monocyte and plasma samples from volunteers after a 4-week washout period was considered as an alternative approach for the reliability of data from non-placebo controlled intervention studies and has been already described in the literature [73]. Moreover,

the reliability of the microarray data was additionally confirmed by using quantitative real-time PCR (qRT-PCR) for selected target genes. In view of the consistent data obtained from microarray as well as technical and biological verification experiments, 7 $Q_{10}H_2$-sensitive genes (TNFα, CXCL2, CCL3, GIMAP7, NR4A2, BRE and PMAIP1) related to inflammatory and apoptotic responses were identified in isolated monocytes of the study subjects. To verify these effects also on the protein level, relevant key inflammatory mediators in monocytes (TNFα, CXCL2, MCP-1, CRP) were additionally determined in serum samples of study subjects. However, neither in native nor in *ex vivo* LPS-stimulated whole blood samples significant changes were found for the selected parameters. The observed discrepancies between the *in vitro* results (Chapter II) and the present data may be due to the heterogeneous cell populations in whole blood from which serum or plasma samples were separated for subsequent experiments. In fact, differences in gene expression were already indicated by studies in different leukocyte subpopulations, where more stable expression profiles were achieved by positive selection of certain cell types [74]. Furthermore, post-transcriptional mechanisms including mRNA degradation or transrepression by complementary micro RNAs [75] as well as posttranslational mechanisms [76] may also account for the unaltered effects on protein levels. Based on gene expression results, text mining application (BibliosSphere Pathway Edition) revealed an involvement of these genes in NFκB and PPAR-signalling pathways. As our previous study in liver tissues of $Q_{10}H_2$-supplemented SAMP1 mice revealed PPARα gene expression patterns related to lipid metabolism and cell differentiation/proliferation processes (Chapter III), the present study was additionally focused on the alteration of respective physiological parameters. Finally, significant reductions of LDL cholesterol levels have been obtained in serum samples of $Q_{10}H_2$-supplemented subjects with two independent methods (routine laboratory detection vs. NMR-based technology). Although the absolute amount of LDL cholesterol differed with regard to the applied method of measurement, the observed effects were in qualitative agreement. With regard to array data from SAMP1 mice [15] and human study experiments, common regulated genes related to cell differentiation processes (e.g. MMD2) have been identified. Accordingly, significant effects on haematological parameters including e.g. the number of erythrocytes and reticulocytes have been additionally observed in the human study. These physiological alterations are a first hint for directly mediated effects of $Q_{10}H_2$ on cell

differentiation/proliferation processes *in vivo*, or more explicitly, on the process of erythropoiesis [77]. However, the observed effects might also be ascribed to the radical scavenging activity of $Q_{10}H_2$ [78,79], finally leading to a decreased ROS concentration. This is due to the fact that ROS may also function as signalling intermediates, finally contributing to cellular functions such as proliferation and differentiation [80]. In this context, the antioxidant scavenger enzyme peroxiredoxin 1 (PRDX1) has been described as a key regulator of neuronal cell proliferation processes *in vitro*. Although the suggested mechanisms in monocytic cells could only in part explain the observed effects on haematological parameters, a modulatory role for $Q_{10}H_2$ in the process of erythropoiesis was suggested.
In summary, it is hypothesized that the reduced form of CoQ_{10} ($Q_{10}H_2$) induces the NFκB- and/or PPARα-mediated signalling cascade through its antioxidant properties. This may in turn also explain the observed anti-inflammatory and anti-apoptotic effects on gene expression.

In conclusion, the results from the *in silico*, *in vitro* and *in vivo* studies show anti-inflammatory properties of $Q_{10}H_2$ as well as a regulatory role in cholesterol metabolism and cell differentiation processes. These effects might be explained, at least in part, by a modulatory impact of $Q_{10}H_2$ on redox-sensitive NFκB/PPARα dependent gene expression.

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Publications and Drafted Manuscript

Chapter I

Functional connections and pathways of coenzyme Q_{10}-inducible genes: an *in-silico* study. printed in *IUBMB Life*

Chapter II

***In-vitro* effects of the reduced form of Coenzyme Q_{10} on secretion levels of TNFα and chemokines in response to LPS in the human monocytic cell line THP-1.** printed in the *Journal of Clinical Biochemistry and Nutrition*

Chapter III

Supplementation with the reduced form of Coenzyme Q_{10} decelerates phenotypic characteristics of senescence and induces a peroxisome proliferator-activated receptor-α gene expression signature in SAMP1 mice.
in press in *Molecular Nutrition and Food Research*

Chapter IV

Effects of the reduced form of Coenzyme Q_{10} on gene expression, inflammation and cell differentiation in humans. drafted manuscript

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Hypothesis

Functional Connections and Pathways of Coenzyme Q_{10}-inducible Genes: An *In-silico* Study

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Summary

Coenzyme Q_{10} (CoQ_{10}, ubiquinone) is an essential cofactor in the electron transport chain, serves as a potent antioxidant in mitochondria and lipid membranes, and is often used as a dietary supplement for a number of diseases including cardiovascular diseases. Recently, we obtained evidence that CoQ_{10} (Kaneka Q_{10}^{TM}) affects the expression of hundreds of human genes. To decipher the functional and regulatory connections of these genes, a literature search combined with transcription factor binding site analysis was performed using Genomatix BiblioSphere and MatInspector. This *in-silico* analysis revealed 17 CoQ_{10}-inducible genes which are functionally connected by signalling pathways of G-protein coupled receptors, JAK/STAT, integrin, and beta-arrestin. Promoter analysis of these CoQ_{10}-inducible genes showed one group of NFκB-regulated genes, namely IL5, thrombin, vitronectin receptor and C-reactive protein (CRP). Furthermore, a common promoter framework containing binding sites of the transcription factor families EVI1, HOXF, HOXC, and CLOX was identified in the promoters of IL5, CRP, and vitronectin receptor. The identified CoQ_{10}-inducible genes and pathways play an important role in inflammatory response. Since these effects are based on an *in-vitro* study, the effect of CoQ_{10} on vascular health *in vivo* needs to be addressed in further animal and/or human intervention studies.

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Keywords Coenzyme Q_{10}; *in-silico* analysis; cardiovascular diseases.

INTRODUCTION

Coenzyme Q_{10} (CoQ_{10}) is an essential electron carrier and proton translocator in the mitochondrial respiratory chain (*1*). CoQ_{10} is also an obligatory cofactor of the dihydroorotate dehydrogenase (*2*) and serves as a potent antioxidant in membranes by directly scavenging radicals (*3, 4*) and regenerating α-tocopherol (*5–7*). More recently, the role of CoQ_{10} in the function of uncoupling proteins was discussed (*8–10*). The functional diversity of CoQ_{10} reflects its suitability for applications in clinical studies as an dietary supplement for a number of diseases (*11*). These include Parkinson's disease (*1, 12–15*), mitochondrial myopathies (*16, 17*), age-related macular degeneration (*18*), migraine (*19*), idiopathic asthenozoospermia (*20, 21*), and cardiovascular diseases (*22–24*). The molecular mechanisms by which CoQ_{10} mediates these beneficial effects are uncertain. We (*25*) and others (*26, 27*) obtained evidence that CoQ_{10} influences the expression of hundreds of genes involved in different cellular pathways. To decipher the functional and regulatory connections of these genes we employed bioinformatic techniques to access the actions of CoQ_{10} in detail. This *in-silico* approach revealed that CoQ_{10} modulates inflammatory pathways via gene expression. Thus, some of the effects of CoQ_{10} on vascular health may be due to this property.

MATERIAL AND METHODS

In-Silico Analyses

We used the freely-available part of Genomatix Software 2006 (www.genomatix.de). The CoQ_{10}-regulated genes were taken from our recent publication (*25*). In that study, we incubated intestinal Caco-2 cells with 50 μM CoQ_{10} of a liposomal preparation for 24 h. After exposition, gene array technology revealed changes in steady-state mRNA levels for hundred of human genes. The accession numbers of these CoQ_{10}-regulated genes (*25*) were uploaded to *BiblioSphere-PathwayEdition* (BSPE). This text mining tool identifies functional connections based on co-citations of gene names and synonyms (*28*). The co-citation filter 'gene ... function word ... gene' (GFG level B3) was applied. The accession numbers of filtered genes were then uploaded to

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Gene2Promoter software which allowed the identification of promoter regions based on individual transcripts (*29*). The obtained promoter sequences were adjusted to 600 bp, 500 bp upstream and 100 bp downstream of transcriptional start sites, and deposited in *MatInspector* to identify functional and common modules in input promoters (*30, 31*). A *common sites analysis* was performed. We chose only models common to at least three input sequences (60%). The minimum and maximum distance between two elements was chosen 5 and 50 bp, respectively.

RESULTS

The Text-mining System BiblioSpherePathwayEdition (BSPE) Revealed 17 CoQ_{10}-senstive Genes which are Functionally Connected by Four Different Pathways

Recently, we identified 464 differentially regulated genes in the intestinal Caco-2 cell line after CoQ_{10}-treatment at a threshold-factor of at least 2.0 in three independent experiments (*25*). These genes were used to identify their putative functional connections by using the text-mining system BSPE. Of 464 uploaded transcripts, 413 were recognized by the program. Transcripts which showed co-citations with transcription factors, functional co-citations (GFG level B3) and co-citations with other genes of the input list were selected. Based on these stringent criteria, we identified 19 CoQ_{10}-inducible genes whereby 17 genes are functionally connected by signalling pathways of G-protein coupled receptors, JAK/STAT, integrin, and beta-arrestin (Fig. 1, Table 1). Since five of these genes code for proteins involved in inflammation (IL5, thrombin, vitronectin, vitronectin receptor, C-reactive protein), a sub-analysis was performed. As shown in Fig. 2, these genes are connected by the transcription factor NFκB1.

The MatInspector-based Promoter Analysis of 17 Connected CoQ_{10}-senstive Genes Revealed Common Regulatory Modules in Three Inflammatory Genes

To identify putative functional and common frameworks in the regulatory regions of the 17 identified CoQ_{10}-inducible genes, their promoter sequences were extracted from NCBI GenBank using Genomatix Gene2Promoter software and were deposited in *MatInspector*. We searched for common frameworks containing at least two transcription factor binding sites (TFBS) at a distance between 5 and 50 bp, and the quorum constraint was adjusted to 60%. The search was done with combinations of five promoter sequences. A framework common to all input promoters or common frameworks with five elements was not found. Whereas frameworks with two or three elements are common in input genes, we identified only one framework with four elements. As shown in Fig. 3, a common framework containing the binding sites of the transcription factor families EVI1 (ecotropic viral integration site 1 encoded factor), HOX (homeodomain transcription factor) and CLOX (cut-like homeo box) were found in the promoters of IL5, C-reactive protein, and vitronectin receptor.

DISCUSSION

In this study we have performed an *in-silico* approach to decipher the functional and regulatory connections of 464 human genes which were recently identified (*25*) as 'CoQ_{10}-inducible'. To obtain convincing connections we combined a literature analysis with a transcriptional factor binding site search. A recent analysis of genes encoding small leucine rich proteoglycans showed indeed, that this combined analysis seems to be more predictive than sole searches for transcription factor binding sites (*32, 33*). Although the analysed CoQ_{10}-inducible gene can be grouped according to for example mitochondrial respiration or plasma membrane redox component, our strategy with rigorous criteria revealed that 17 CoQ_{10} inducible genes are connected by four different cellular signalling pathways. Whereby, the genes of IL5, thrombin, vitronectin, vitronectin receptor, and C-reactive protein (CRP) seem to be regulated by NFκB1 and promoter frameworks containing the transcription factors EVI, HOX, and CLOX. Although the precise roles of these transcriptional factors are not completely unravelled, they are essentially involved in different aspects of development and are linked to several human diseases including inflammation (*34–37*). Accordingly, IL5, thrombin, vitronectin and its receptor as well as CRP are key components in similar steps of inflammation processes. Although the *in-vivo* relevance of these effects has to be clarified, an up-regulation of these genes could for example sensitize the inflammatory responses of monocytes.

Whereas IL5 is mainly responsible for the tissue damage observed in allergic disorders (*38*), the other identified genes seem to be important in the development of atherosclerosis. CRP has been reported as a potent peptide that causes platelet adhesion to epithelial cells, thereby regulating atherothrombosis (*39*). This step is also regulated by vitronectin and its receptor, since they interact with thrombin and antithrombin III (*40*). The finding that expression of vitronectin and its receptor is modulated by CoQ_{10} is particularly interesting for several reasons. First, plasma vitronectin levels are increased in patients with coronary atherosclerosis (*41*). Second, it has been shown that vitronectin-mediated cell survival also includes regulation of NFκB-activity (*42*). Third, vitronectin is essential for monocyte adhesion to endothelium (*43*). Finally, dose-, and time-dependent inhibitory properties of CoQ_{10} on platelet aggregability have been already shown in a previous study with swine (*44*). Another study indicated a significant inhibition of vitronectin-receptor expression in human

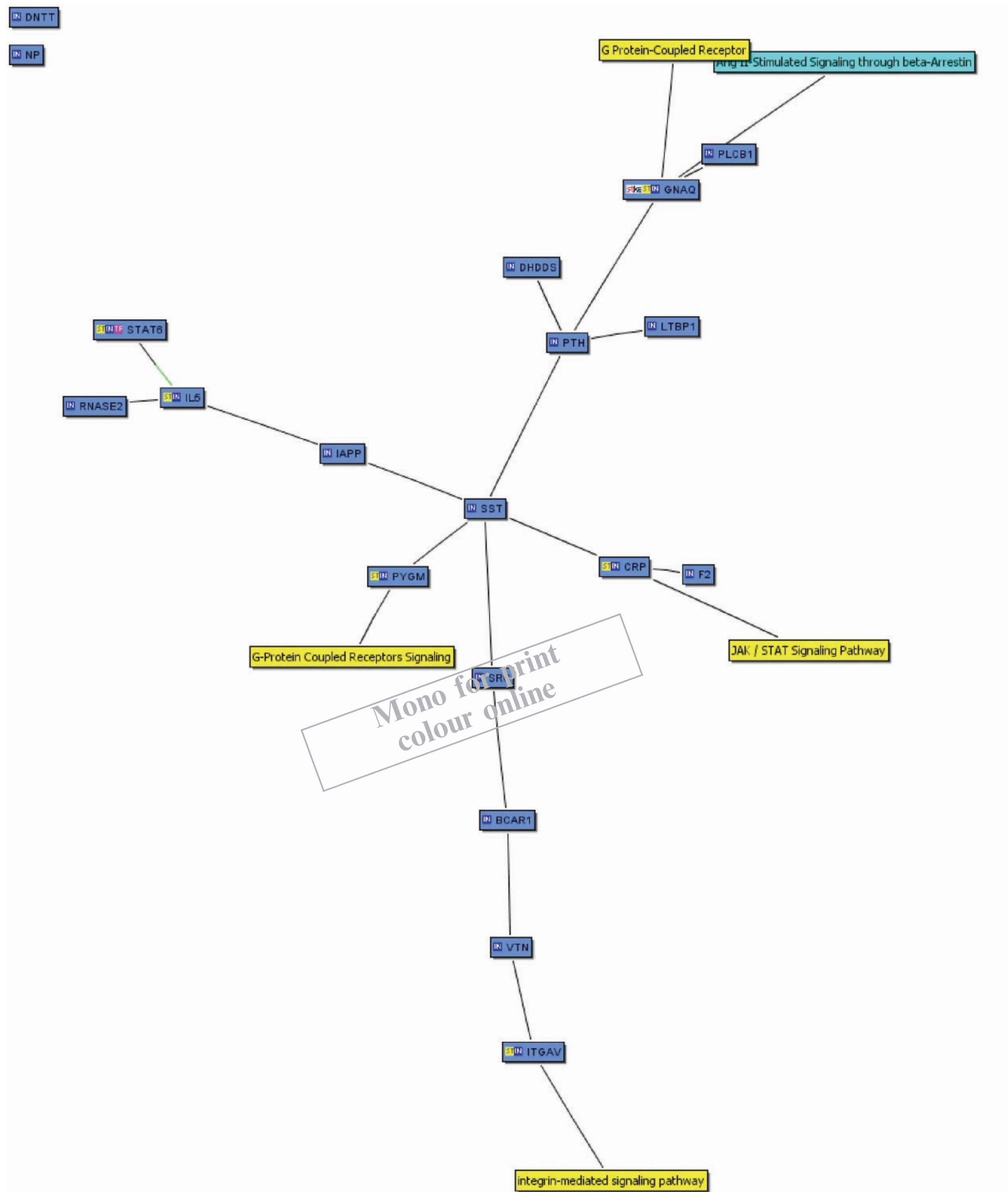

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Figure 1. BiblioSphere Pathway view network of input genes regulated by CoQ$_{10}$. A network of 17 genes was identified by analysis of 464 CoQ$_{10}$-inducible genes with the BiblioSpherePathwayEdition software package based on co-citations with transcription factors, functional co-citations, and co-citations with other genes in the network. The abbreviations and descriptions are listed in Table 1. The genes DNTT and NP are not part of the network. IN, input gene; ST, gene product is part of a Genomatix signal transduction pathway; STKE, gene is part of a SignalTransductionKnowledgeEnviromental connection map.

Table 1
CoQ_{10}-inducible genes in the identified network (see Fig. 1)

Symbol	Transcript[a]	Description	Fold change by CoQ_{10}[b]
SST	NM_001048	Somatostatin	+2.3
IL5	NM_000879	Interleukin 5, colony-stimulating factor, eosinophil	+5.6
F2	NM_000506	Coagulation factor II, thrombin	+2.4
RNASE2	NM_002934	Ribonuclease, RNase A family	+16.0
PYGM	NM_005609	Glycogen phosphorylase	+3.5
ITGAV	NM_002210	Integrin alpha V, vitronectin receptor	+3.0
DNTT	NM_004088	Deoxynucleotidyltransferase, terminal	+3.3
STAT6	NM_003153	Signal transducer and activator of transcription 6, interleukin-4 induced	+2.6
LTBP1	NM_000627	Latent transforming growth factor beta binding protein 1	+2.7
CRP	NM_000567	C-reactive protein, pentraxin-related	+3.0
GNAQ	NM_002072	Guanine nucleotide binding protein, G protein, q polypeptide	+3.1
PTH	NM_000315	Parathyroid hormone	+2.8
IAPP	NM_000415	Islet amyloid polypeptide	+4.9
BCAR1	NM_014567	Breast cancer anti-estrogen resistance 1	+2.9
PLCB1	NM_015192	Phospholipase C, beta 1	+4.6
DHDDS	NM_024887	Dehydrodolichyl diphosphate synthase	+2.6
VTN	NM_000638	Vitronectin	+4.1
SRC	NM_005417	V-src sarcoma	+2.8
NP	NM_000270	Nucleoside phosphorylase	+3.9

[a]NCBI GenBank Accession number.
[b]Observed by array analysis as described (*25*): expression data were normalized to average expression levels of three housekeeping genes, namely, ß-actin, GAPDH and ubiquitin.

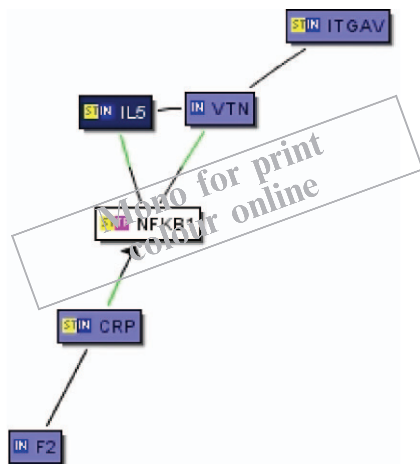

Figure 2. BiblioSphere Pathway view network of input genes which are involved in inflammation and regulated by CoQ_{10}. The network of five selected genes was obtained with BiblioSpherePathwayEdition software package based on co-citations with transcription factors, functional co-citations, and co-citations with other genes in the network. The abbreviations and descriptions of genes were listed in Table 1. IN, input gene; ST, gene product is part of a Genomatix signal transduction pathway; TF, transcription factor.

platelets throughout CoQ_{10} treatment (*45*). Therefore, one possible mechanism by which CoQ_{10} produces positive effects in cardiovascular diseases is through platelet inhibition. This putative beneficial mechanism is accompanied by other effects of CoQ_{10} on cardiovascular diseases. This includes protection of LDL from oxidation, prevention of free-radical damage caused by neutrophils and reduction of oxidative injury by endothelial cells (*11, 46, 47*). Of course, based on our *in-vitro* data the effect of CoQ_{10} on vascular health *in vivo* need to be studied in future animal and/or human intervention studies. In those studies, tissue specific effects have to be taken into account. Further, the applied CoQ_{10} concentration of 50 μM for 24 h *in vitro* is difficult to achieve in humans. On the other side, the exposure time *in vivo* is quite longer than in our *in-vitro* study. Again, *in-vivo* studies are necessary to evaluate the effect of CoQ_{10} on gene expression and vascular health. In addition, future analysis of the gene expression data on protein and metabolite level is necessary.

Taken together, based on our *in-silico* analysis of more than 400 CoQ_{10}-inducible genes, we obtained evidence that a part of the CoQ_{10} regulation plays an important role in inflammatory response. Since these effects are based on *in-vitro* study, the effect of CoQ_{10} on vascular health *in-vivo* needs to be addressed in further animal and/or human intervention studies.

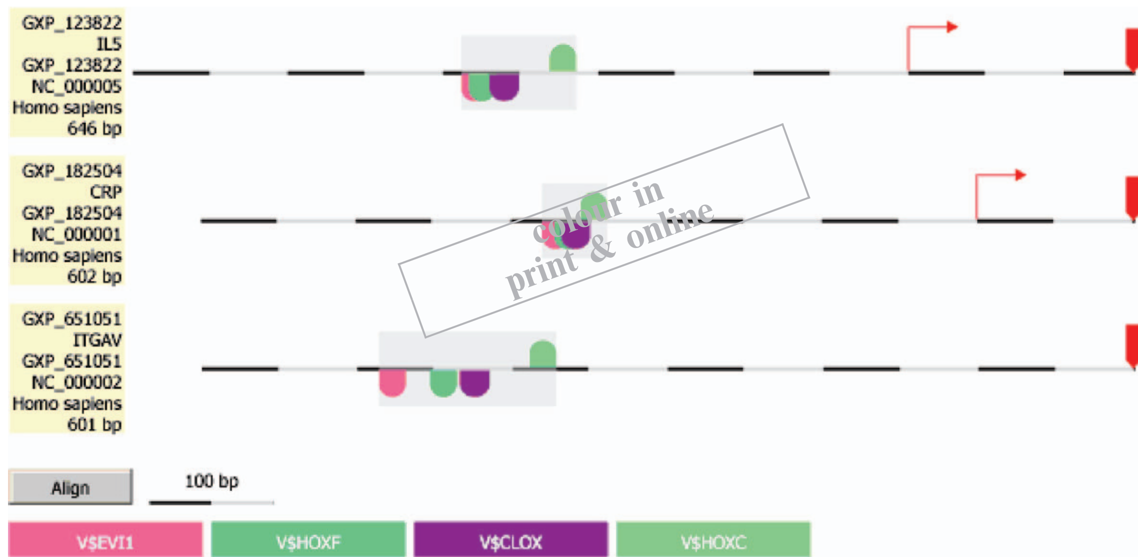

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Figure 3. Four element promoter frameworks shared by three CoQ$_{10}$-inducible genes. EVI1 (red), HOXF (green), CLOX (purple), and HOXC elements and combined frameworks (grey) location in promoter regions of CoQ$_{10}$-inducible genes IL5, CRP, and ITGAV relative to transcription start site (red arrow). EVI, ecotropic viral integration site 1 encoded factor; HOX, homeodomain transcription factor; CLOX, cut-like homeo box.

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In Vitro Effects of the Reduced Form of Coenzyme Q_{10} on Secretion Levels of TNF-α and Chemokines in Response to LPS in the Human Monocytic Cell Line THP-1

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Summary **Ubiquinol-10 (QH_2), the reduced form of Coenzyme Q_{10} (CoQ_{10}) serves as a potent antioxidant of lipid membranes. Because many antioxidants reveal potent anti-inflammatory effects, the influence of QH_2 on lipopolysaccharide (LPS)-induced pro-inflammatory cytokines and chemokines were determined in the human monocytic cell line THP-1. Stimulation of cells with LPS resulted in a distinct release of Tumour necrosis factor-alpha (TNF-α), Macrophage inflammatory protein-1 alpha (MIP-1α), Regulated upon activation, normal T cell expressed and secreted (RANTES) and Monocyte chemotattractant protein-1 (MCP-1). The LPS-induced responses were significantly decreased by pre-incubation of cells with QH_2 to 60.27 ± 9.3% ($p = 0.0009$), 48.13 ± 6.93% ($p = 0.0007$) and 74.36 ± 7.25% ($p = 0.008$) for TNF-α, MIP-1α and RANTES, respectively. In conclusion, our results indicate anti-inflammatory effects of the reduced form of CoQ_{10} on various proinflammatory cytokines and chemokines *in vitro*.**

***Key Words*: coenzyme Q_{10}, ubiquinol-10, inflammation, monocytes**

Introduction

Exposure of cells to the pro-inflammatory lipopolysaccharide (LPS) triggers TLR4-dependent phosphorylation cascades which lead to activation of NFκB. This central transcription factor induces the expression and subsequent secretion of various pro-inflammatory cytokines and chemokines [*1–3*]. Reactive oxygen species (ROS) are important for the activity of the TLR4-signalling pathway [*4*]. Accordingly, antioxidants are described as anti-inflammatory agents [*5*, *6*]. Because Coenzyme Q_{10} (CoQ_{10}) is a potent antioxidant, we postulated that this molecule possesses anti-inflammatory properties. More recently was shown that CoQ_{10} supplementation minimizes oxidative stress during statin drug therapy [*7*]. Indeed, we found a reduction of LPS-induced cytokine release by CoQ_{10} in murine and human monocytic cell lines [*8*]. In the latter study, we treated cells with the oxidized form of CoQ_{10} (ubiquinone-10) which is converted intracellular to ubiquinol-10 (QH_2). As only the reduced form of CoQ_{10} can act as an antioxidant, here we studied effects on secretion of the cytokine TNF-α and different chemokines in LPS-stimulated THP-1 cells that were directly incubated with QH_2.

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Material and Methods

Reagents

Lipopolysaccharide (LPS, *E.coli* O55:B5) was obtained from Sigma-Aldrich (Taufkirchen, Germany). The aqueous solutions of ubiquinol-10 (PEG-60 hydrogenated castor oil, ubiquinol-10, glycerol, water) and the corresponding vehicle (no ubiquinol-10 supplement) were received from KANEKA Corporation (Osaka, Japan).

Table 1. Effect of ubiquinol-10 (QH_2) and the reference substances PDTC and NAC on viability of THP-1 cells.

	10% DMSO	Vehicle control	100 μM PDTC	100 μM NAC	1 μM QH_2	10 μM QH_2	100 μM QH_2
%	1.71	148.87	109.44	92.51	120.74	109.68	98.43
±SEM	±0.36	±15.09	±22.33	±21.38	±13.20	±15.70	±16.09

THP-1 cells were either treated with 1–100 μM QH_2 or 100 μM PDTC or NAC for 24 h. Medium was used as negative control (data not shown) and 10% dimethyl sulfoxide (DMSO) as positive control (poco, positive control). The applied amount of the vehicle (veco) was in accordance to 100 μM QH_2. The cell viability of the negative control was set to 100% and the other values (means ± SEM) were referenced to it. Three independent experiments were performed in triplicate.

Cell culture

Cultivation of THP-1 cells occurred routinely in RPMI medium 1640 supplemented with 10% FCS and 1% antibiotics (penicillin/streptomycin) in a humidified incubator containing 5% CO_2 at 37°C. For determination of TNF-α and chemokines, cells were plated at a density of 0.5×10^6 cells in a 12-well plate for 24 h before pre-incubation. Subsequently, cells were preincubated with either 10 μM ubiquinol-10 or the reference substances pyrrolidine-dithiocarbamate (PDTC) or N-acetyl-cysteine (NAC), or the respective vehicle control. After 24 h, cell culture medium was removed and fresh LPS-containing medium (1 μg/ ml) was added for 4 h. Finally, for cytokine determination via ELISA, supernatants were kept and stored at −80°C. For protein determination via the BRADFORD method, cells were collected into NET-buffer.

Cytotoxicity

For determination of cell viability, the Cell-Titer Glo® Luminescent Assay was used. Thus, total ATP levels were measured as an index of the viable cell number. The luminescence was detected on a GloMax® (Promega, Mannheim, Germany).

Determination of TNF-α and chemokines

Using TNF-α as an internal control, this cytokine was determined by DuoSet ELISA (R&D Systems, Wiesbaden, Germany) as well as multiplex suspension array technology (BioRad, Munich, Germany) according to the manufacturer's instructions. The chemokines MCP-1, MIP-1α and RANTES were determined by the multiplex suspension array system.

Protein concentration

Cells were collected into NET-buffer (50 mM TRIS [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 0.5% NP-40) and the cell suspension was treated with ultrasonics and then centrifugated by 14000 rpm at 4°C for 20 min. Determination of protein concentration occurred in the resulting supernatant by the Bradford method according to the manufacturer's instructions.

Statistics

All data are results of two (PDTC, NAC) or three (QH_2) independent biological experiments performed in duplicate and expressed as means ± standard error of the mean (SEM). Results were analyzed by an unpaired two-sided Student's *t*-test using SPSS 11.5 for Windows and GraphPad Prism 4.0 software. *p*-values less than or equal to 0.05 were considered statistically significant.

Results and Discussion

NFκB is a multisubunit transcription factor that is ubiquitously expressed in different cell types and can be activated by several agents such as LPS, TNF-α or the oxidant hydrogen peroxide (H_2O_2) [*9*]. This activation process includes phosphorylation of the IκB subunit and its dissociation from the inactive cytoplasmic complex. Thus, the active dimer of p50 and p65 translocates into the nucleus where specific target genes of pro-inflammatory mediators and cytokines become immediately up-regulated [*9*, *10*]. However, this NFκB-activating cascade was shown to be inhibited by antioxidants such as PDTC and NAC. This has led to the hypothesis that oxygen radicals are key players in the activation of NFκB through an redox-dependent mechanism [*9*, *11*, *12*]. Because many antioxidants reveal potent anti-inflammatory effects, the influence of QH_2 on LPS-induced pro-inflammatory cytokines and chemokines was determined in the human monocytic cell line THP-1. All experiments were performed with the well known radical scavengers PDTC and NAC to validate the putative anti-inflammatory effects of QH_2. To implement culture conditions that do not lead to unspecific side effects, cell vitality was measured at different medium concentrations of QH_2. As shown in Table 1, no cytotoxic effects were found for PDTC (100 μM), NAC (100 μM) and QH_2 (1, 10, 100 μM). For further experiments we used 10 μM QH_2, because this concentration leads to a significantly higher intracellular QH_2 content in THP-1 cells and is also achievable in human serum through QH_2 supplementation (unpublished results). As shown in Figure 1A-D, unstimulated THP-1 monocytes secrete low amounts of the pro-inflammatory cytokine TNF-α and chemokines MIP-1α, RANTES and MCP-1 into the medium. However, stimulation with LPS induces approximately 58-(TNF-α), 7-(MIP-1α), 2-(RANTES) and 4-(MCP-1) fold higher levels of these pro-inflammatory

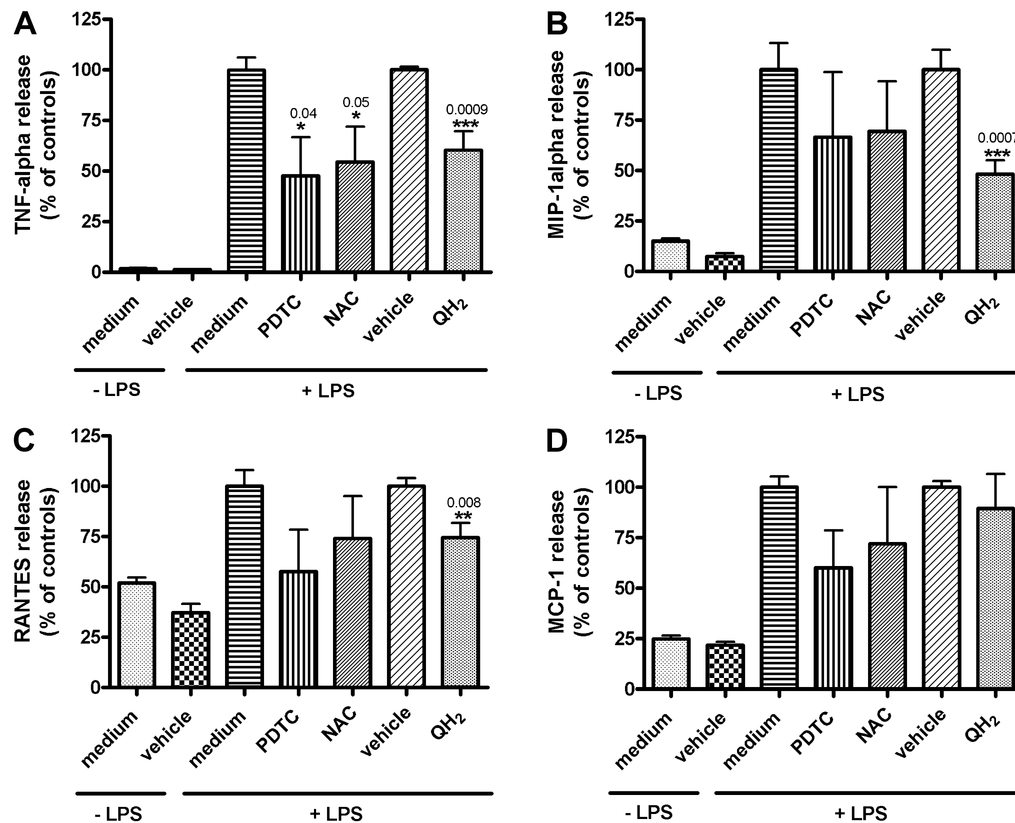

Fig. 1. Effects of pre-treatment of ubiquinol on LPS-induced release of TNF-α (A), MIP-1α (B), RANTES (C) and MCP-1 (D) in THP-1 cells.
Cells were either pre-treated with 10 µM QH_2 or the respective reference substances PDTC or NAC, or medium and vehicle for 24 h. Afterwards, media were removed and cells were treated with LPS (1 µg/ml medium) for 4 h. The resulting concentrations (pg/µg cellular protein) of TNF-α, MIP-1α, RANTES and MCP-1 of the vehicle controls (+LPS) were set to 100% for QH_2-pretreated cells and the other values were referenced to it. Values from PDTC- and NAC-pretreated cells were related to medium controls (+LPS) taken as 100%. Statistically significant data (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) are means ± SEM of four (ubiquinol, vehicle) or two (PDTC, NAC, medium) independent experiments performed in duplicate.

agents in the cell culture medium within 4 h, respectively. Next, we tested the effect of pre-incubation of cells with 10 µM QH_2 for 24 h. Thus, as shown in Figure 1A–C, the LPS-induced responses were significantly decreased to 60.27 ± 9.3%, 48.13 ± 6.93% and 74.36 ± 7.25% for TNF-α, MIP-1α and RANTES, respectively (Fig. 1A–C). No significant effect was found for MCP-1 (Fig. 1D). Pre-incubation of cells with 10 µM PDTC or 10 µM NAC decreased TNF-α levels significantly to 47.69 ± 19.07% and 54.43 ± 17.64%, respectively (Fig. 1A). No significant effects of PDTC and NAC were found on LPS-induced secretion levels of other pro-inflammatory mediators.

Inflammation has been related to the pathogenesis of various diseases, such as atherosclerosis [*13*]. Monocytes play an important role in the response to inflammatory agents, particularly to those derived from gut bacteria and are able to enter the circulation, such as bacterial endotoxins. Thus, endotoxins circulate at low concentrations in the blood of all healthy individuals, but are also increased after a high-fat meal [*14*]. However, elevated levels are associated with an increased risk of atherosclerosis or sepsis [*14–16*]. For our experiments we used LPS, a compound of gram-negative bacteria that is also relevant *in vivo* to trigger a serious medical inflammatory process *in vitro*. Finally, stimulation of monocytes with LPS induces production of ROS, which in turn activate the transcription factor NFκB [*4*, *9*] that triggers a large amount of genes encoding for inflammatory mediators and cytokines [*17*]. Numerous studies in monocytes revealed natural occurring antioxidants as compounds with anti-inflammatory effects [*18*, *19*]. Here we used CoQ_{10} in order to study its putative anti-inflammatory effect in the human monocytic cell line THP-1. For this purpose, we used the reduced form of CoQ_{10}, QH_2, which functions as an antioxidant. To our knowledge, effects of QH_2 on inflammatory markers have not been investigated so far, because this form of CoQ_{10} is not commonly available. Thus, only a few studies were published using QH_2 *in vivo*. These studies indicate effects

of QH_2 on safety and bioavailability [*20*], sperm kinetic features [*21*], oxidative imbalance in children with Trisomy 21 [*22*] and neuroprotection in an animal Parkinson model [*23*]. In THP-1 cells, we found that QH_2 reduces significantly the secretion of the pro-inflammatory agents TNF-α, MIP-1α, and RANTES in response to LPS. This putative anti-inflammatory effect of QH_2 could be due to its antioxidant property in cell membranes, because LPS-induced ROS production occurs very closely to the membrane [*4*]. One important membrane-associated complex that is relevant for generation of ROS in monocytic cells [*24*] is the NADPH oxidase. This complex is described to consist of four proteins, whereas Rac is the most critical component for a functional NADPH oxidase. Rac is regulated by small GTP-binding proteins [*25*]. In this context it was shown that LPS induced Rac activity and moreover, the NADPH oxidase-dependent ROS formation [*26*]. Thus it seems that LPS directly initiates the NADPH oxidase activity by downstream signalling pathways. Results from a further study indicate an up-regulation of the NADPH oxidase complex through a NFκB-dependent TNF-α activation process which finally leads to enhanced ROS production and further NFκB-activation [*24*]. This in turn might contribute to sustained releases of pro-inflammatory cytokines and mediators. In this context it was also shown that the well known antioxidant and radical scavenger NAC inhibited NFκB-activation via reduction of H_2O_2 [*9*], an important reactive oxygen intermediate (ROI) of the NADPH oxidase pathway. These strong radical scavenging effects are also described for PDTC [*27*], which we used as an additional internal control to describe the putative anti-inflammatory effects of the reduced form of CoQ_{10} (QH_2), a compound with strong antioxidant properties. In general it seems that QH_2 mediates stronger anti-inflammatory effects on the tested pro-inflammatory compounds than PDTC and NAC, two well known radical scavengers mediating its anti-inflammatory properties through a diminished NFκB activation. Thus it seems that the reduced form of CoQ_{10} (QH_2) mediates its anti-inflammatory effects at least in part through its strong antioxidant properties. However, these effects may be additionally mediated by gene expression. It has been shown in skeletal muscle of humans [*28*], heart of mice [*29*], CaCo-2 [*30*], and HeLa cells [*31*] that CoQ_{10} influences the expression of different genes. These hypotheses should be tested in future studies. In conclusion, our results indicate anti-inflammatory effects of the reduced form of CoQ_{10} on various proinflammatory cytokines and chemokines *in vitro*.

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Research Article

Supplementation with the reduced form of Coenzyme Q_{10} decelerates phenotypic characteristics of senescence and induces a peroxisome proliferator-activated receptor-α gene expression signature in SAMP1 mice

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Our present study reveals significant decelerating effects on senescence processes in middle-aged SAMP1 mice supplemented for 6 or 14 months with the reduced form ($Q_{10}H_2$, 500 mg/kg BW/day) of coenzyme Q_{10} (CoQ_{10}). To unravel molecular mechanisms of these CoQ_{10} effects, a genome-wide transcript profiling in liver, heart, brain and kidney of SAMP1 mice supplemented with the reduced ($Q_{10}H_2$) or oxidized form of CoQ_{10} (Q_{10}) was performed. Liver seems to be the main target tissue of CoQ_{10} intervention, followed by kidney, heart and brain. Stringent evaluation of the resulting data revealed that $Q_{10}H_2$ has a stronger impact on gene expression than Q_{10}, primarily due to differences in the bioavailability. Indeed, $Q_{10}H_2$ supplementation was more effective than Q_{10} to increase levels of CoQ_{10} in the liver of SAMP1 mice. To identify functional and regulatory connections of the "top 50" ($p<0.05$) $Q_{10}H_2$-sensitive transcripts in liver, text mining analysis was used. Hereby, we identified $Q_{10}H_2$-sensitive genes which are regulated by peroxisome proliferator-activated receptor-α and are primarily involved in cholesterol synthesis (*e.g.* HMGCS1, HMGCL and HMGCR), fat assimilation (FABP5), lipoprotein metabolism (PLTP) and inflammation (STAT-1). These data may explain, at least in part, the decelerating effects on degenerative processes observed in $Q_{10}H_2$-supplemented SAMP1 mice.

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Keywords:
CoQ_{10} / Gene expression / Inflammation / Lipid metabolism / peroxisome proliferator-activated receptor-α

1 Introduction

Coenzyme Q_{10} (CoQ_{10}) is an essential cofactor in the electron transport chain, serves as a potent antioxidant in lipid membranes and is a cofactor of uncoupling proteins. More recently, we identified CoQ_{10} as a compound with anti-inflammatory properties *in vitro* [1, 2]. These effects are thought to be mediated through gene expression and/or the radical scavenging activity of the reduced form of CoQ_{10} ($Q_{10}H_2$) [3, 4]. Moreover, $Q_{10}H_2$ also indicated stronger anti-inflammatory effects than the oxidized form (Q_{10}) *in vitro*. From these data, different effects of the oxidized and reduced form of CoQ_{10} on redox-dependent gene expression patterns were hypothesized. Very recently, we provide evidence *in vitro* and in mice that $Q_{10}H_2$ modulates the expression of the anti-inflammatory microRNA-146a [5].

The senescence-accelerated mice is a well-established model to study the aging process in higher organisms [6, 7]. SAMP

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Abbreviations: **LXR,** Liver X receptor; **PPAR-α,** peroxisome proliferator-activated receptor-α; **PPRE,** peroxisome proliferator response element; **RXR,** retinoid X receptor

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strains grow normally but show early signs of aging including, *e.g.* reduced physical activity, loss of hair glossiness and shorter life span [8]. Analysis of aging dynamics, based on survival curves, senescence scores and growth rate, shows that the aging pattern in SAMP strains is characterized by accelerated senescence after normal development [8, 9]. Because SAMP1 mice show also a high oxidative stress status [10, 11], they are a suitable model to study putative effects of antioxidants such as CoQ_{10} on physiological and molecular readouts. Our present study shows significant reducing effects on accelerated senescence processes in SAMP1 mice supplemented with $Q_{10}H_2$. To get insight into the molecular mechanisms of these effects, we studied the influence of Q_{10} as well as $Q_{10}H_2$ on gene expression in liver, heart, brain and kidney of SAMP1 mice.

2 Materials and methods

2.1 Animals

SAMP1 mice were reared in the Division of Laboratory Animal Research, Research Center for Human and Environmental Sciences, Shinshu University, under specific pathogen-free conditions at $24 \pm 2°C$ and a 12-h light–dark cycle. Water and food intake were available *ad libitum*. At the beginning of the long-term controlled study, 4-wk-old female SAMP1 mice were purchased from Japan SLC (Hamamatsu, Japan) and housed 3–6 *per* cage (20 cm wide, 30 cm long, 10 cm deep). Grouping of the animals remained unchanged throughout the study. Body weights, food intake and degree of senescence were calculated monthly while mice were inspected daily. Animals were sacrificed by cardiac puncture under anaesthesia with diethyl ether after 6 or 14 months of $Q_{10}H_2$ or Q_{10} supplementation. Organs (liver, heart, brain and kidney) were removed and stored at –80°C until RNA isolation. Study protocol and experimental procedures were approved by the ethics committee of Shinshu University.

2.2 Evaluation of degree of senescence

The degree of senescence was evaluated by a grading system [9]. Eleven categories of behavioral activity and gross appearances of the skin, eyes, and spine were considered to be associated with the aging process: each category was graded 0 to 4 according to the degree of change, and the grading score for each mouse was the sum of the grades of each category. Generally, the grading was done at a fixed time (from 2 pm to 4 pm) by an observer who was blinded to the treatment of the mice.

2.3 $Q_{10}H_2$ and Q_{10} supplementation and preparation of the diet

In the initial phase of the experiment, SAMP1 mice were randomly assigned to three groups: $Q_{10}H_2$ animals ($n = 22$), Q_{10} animals ($n = 11$) or control animals ($n = 20$).

The oxidized (Q_{10}) or reduced form ($Q_{10}H_2$) of CoQ_{10} was added to a standard laboratory mouse diet (powdered CE-2, CLEA Japan) using corn oil (1%, v/w) as a vehicle and to achieve a final concentration of Q_{10} or $Q_{10}H_2$ of 0.5%, respectively. The control diet was prepared using corn oil only. The mixture was incorporated in pellet-type chow by adding 30% v/v ethanol solution, pressure shaping and drying. Storage of the diet was conducted at –20°C for up to 4 wk before administration.

2.4 Determination of total CoQ_{10} and its redox state in liver samples of 14 M SAMP1 mice

Total levels and redox state of CoQ_{10} ($Q_{10}H_2$, Q_{10}) were determined in liver homogenates of 14 M intervention ($Q_{10}H_2$, Q_{10}) and control mice ($n = 3$ *per* each group). The method is based on HPLC analysis with electrochemical detection with minor modifications as described before [12]. In brief, 1.95 mL of 2-propanol was added to 50 mg liver sample and mixed with a Polytron homogenizer. Subsequently, the homogenate was diluted 15-fold with 2-propanol. After centrifugation ($9500 \times g$, 3 min, 4°C), 50 μL of the supernatant was injected into the HPLC system.

The mobile phase consisted of 0.05 M sodium perchlorate in methanol/hexane (88:12 v/v) at a flow rate of 1.0 mL/min. The oxidation potential of the ECD was 600 mV (*versus* Ag/AgCl). $Q_{10}H_2$ and Q_{10} levels were quantified by an external standard method based on peak area.

2.5 Isolation of mRNA from mice tissue for microarray analysis

Total RNA was extracted from mice tissues ($n = 3$ *per* each group) with the following kits (all from Qiagen, Japan) according to the manufacturer's instructions: RNeasy Kit (for liver and kidney), RNeasy Fibrous Tissue Mini Kit (for heart) and RNeasy Lipid Tissue Mini Kit (for brain).

2.6 Expression profiling

Microarray analysis was conducted on three samples for each group, respectively, by using GeneChip® Mouse Genome 430 2.0 Array (Affymetrix) containing 45 100 probe sets. Q1 The procedure was performed according to the manufacturer's instructions using Poly-A RNA Control Kit (Affymetrix) and One-Cycle cDNA Synthesis Kit (Affymetrix) for cDNA synthesis, Sample Cleanup Module (Affymetrix) for purification, and IVT Labeling Kit (Affymetrix) for synthesis of biotin-labeled cRNA. Fifteen micrograms of fragmented cRNA was hybridized to a Mouse Genome 430 2.0 Array for 16 h at 45°C at 60 rpm. After hybridization,

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arrays were washed on GeneChip® Fluidics station 450 (Affymetrix) and stained with streptavidin–phycoerythrin. Thereafter, microarrays were scanned with a GeneChip® Scanner 3000 7G (Affymetrix). Expression data were normalized with Affymetrix GeneChip Operating Software 1.4 using mean value (global normalization). Only probe sets showing present calls for all three arrays at one experimental group (intervention or control) were considered for further analysis. The accession number for the complete datasets submitted to NCBI Gene Expression Omnibus is GSE15129 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE15129).

2.7 Text-mining study

2.7.1 Genomatix bibliosphere

Genomatix Software 2008 (www.genomatix.de) was used to perform text-mining analysis. The probe set IDs of the $Q_{10}H_2$-regulated genes were uploaded to BibliospherePathwayEdition Software. This text-mining tool identifies putative functional connections based on co-citations of gene names and synonyms from NCBI Pubmed [13]. The co-citation filter "gene…function word…gene" (GFG level B3) was applied.

2.8 Statistics

Results were analyzed by an unpaired, two-sided Student's *t*-test using SPSS 11.5 for Windows and GraphPad Prism 4.0 software. *p*-Values less than or equal to 0.05 were considered statistically significant.

3 Results

3.1 Effects of $Q_{10}H_2$- and Q_{10}-supplementation on food intake and grading score of senescence in SAMP1 mice

Food intake of SAMP1 mice was calculated monthly and no difference in food consumption was apparent among the three groups (control, $Q_{10}H_2$ and Q_{10}). Based on mean food intake (3.4 g/d) and mean body weight (33 g), mice consumed 500 mg/kg BW/d $Q_{10}H_2$ or Q_{10}, respectively. No differences in body weight were found between $Q_{10}H_2$ and Q_{10} supplemented animals. Senescence grading scores increased from 16 or 20 wk of age in SAMP1 mice of all groups ($Q_{10}H_2$, Q_{10} and control). However, grading scores in the $Q_{10}H_2$ group were lower than in Q_{10}- and control-treated mice. There was a significant difference between control and $Q_{10}H_2$ mice from 16 to 68 wk of age ($p<0.05$) (Fig. 1). This result was also found in previous experiments [14].

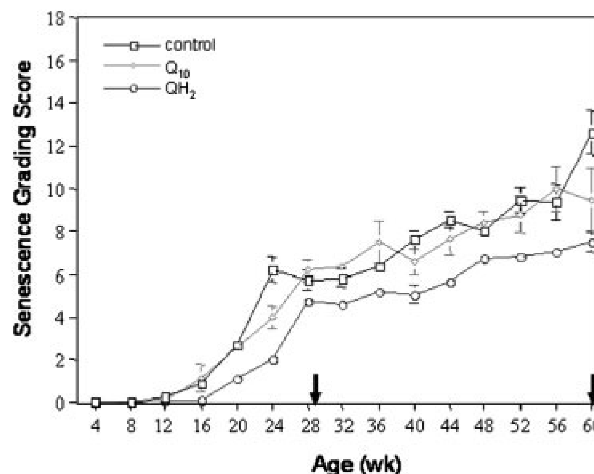

Figure 1. Age-related change on senescence grading scores in SAMP1 mice supplemented with $Q_{10}H_2$, Q_{10} or vehicle control. SAMP1 mice were supplemented with either $Q_{10}H_2$ or Q_{10} (~500 mg/kg BW/d), or a respective control diet for 14 months. Senescence grading scores increased from 16 or 20 wk of age in SAMP1 mice of all groups ($Q_{10}H_2$, Q_{10}, control). Grading scores in the $Q_{10}H_2$ group were lower than in Q_{10} and control mice. There was a significant difference ($p<0.05$) between control and $Q_{10}H_2$ mice from 16 to 60 wk of age ($p<0.05$). However, no significant differences were found for Q_{10}-treated animals when compared with controls.

3.2 Effects of $Q_{10}H_2$- and Q_{10}-supplementation on whole genome expression profiles in different tissues of SAMP1 mice

Microarray-based whole genome expression profiles were analyzed from liver, heart, brain and kidney of SAMP1 mice supplemented with $Q_{10}H_2$, Q_{10} or a control diet. From every experimental group, six mice *per* each group were sacrificed at 6 and 14 months (three mice at each time point, respectively) after supplementation, resulting in a total of 72 microarrays. Differentially expressed genes in tissues of SAMP1 mice treated with $Q_{10}H_2$ or Q_{10} were selected as follows. First, transcripts with at least three present calls in one group (intervention *versus* control) were chosen for further analysis. Second, transcripts showing at least a 1.5-fold increase or decrease in the $Q_{10}H_2$ or Q_{10} groups *versus* control animals at $p<0.05$ by Student's *t*-test, or alternatively, a 1.3-fold increase or decrease at $p<0.01$, were selected. Third, only those transcripts were referred to as $Q_{10}H_2$- and/or Q_{10}-sensitive having been significantly expressed in at least three different tissues, or alternatively, in two tissues at different time points or with a fold-change level of at least ≥I1.5I at $p<0.01$ (Tables 1 and 2). Based on these stringent criteria, 20 and 3 transcripts were identified as $Q_{10}H_2$- or Q_{10}-sensitive, respectively (Table 1). Most $Q_{10}H_2$-sensitive transcripts were differentially expressed in liver and kidney at 6 or 14 months after supplementation. Moreover, 17 transcripts were identified to be regulated by

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Table 1. Expression profiles of $Q_{10}H_2$- and Q_{10}-sensitive transcripts in different tissues

	Fold change $Q_{10}H_2$								
	Liver		Heart		Brain		Kidney		
Gene ID	6M	14M	6M	14M	6M	14M	6M	14M	Gene name
228880						1.74**		2.15**	RIKEN CDNA 2010005I16 GENE
217232	2.10**						1.86**	1.45**	CELL DIVISION CYCLE 27 HOMOLOG (S. CEREVISIAE)
17979	3.64*						2.16*	1.59**	NUCLEAR RECEPTOR COACTIVATOR 3
108962	2.21*	1.76*					1.68*		RIKEN CDNA 4833441D16 GENE
20481	3.67**						2.36**		SLOAN-KETTERING VIRAL ONCOGENE HOMOLOG
72949	3.21**						1.82*	1.82**	CYCLIN T2
170942	2.60**			1.91*			1.96**	1.56*	ERYTHROID DIFFERENTIATION REGULATOR 1
100910	2.10**			2.18**			1.70*		RIKEN CDNA 2010209O12 GENE
66277		−1.70**	−1.59**						KRUPPEL-LIKE FACTOR 15
21413	3.19*						1.75*	1.77*	TRANSCRIPTION FACTOR 4
84092	2.28*	2.97*					1.56*		PUTATIVE DEUBIQUITINATING ENZYME
56490	3.53**							1.82**	ZINC FINGER AND BTB DOMAIN CONTAINING 20
NA	2.31*	1.69*					1.72**		NA
94112	2.93*						2.17**	1.69*	POSITIVE COFACTOR 2, MULTIPROTEIN COMPLEX, GLUTAMINE/Q-RICH-ASSOCIATED PROTEIN
170942	2.26**						2.02**	1.51*	ERYTHROID DIFFERENTIATION REGULATOR 1
NA	2.41**						2.60**		NA
319885				2.13*			1.82*	1.83*	ZINC FINGER, CCHC DOMAIN CONTAINING 7
67039	2.17*	2.60*					2.0*	1.66*	RIKEN CDNA 2600011C06 GENE
52680	1.88*					1.73*		1.53**	DNA SEGMENT, CHR 13, ERATO DOI 787, EXPRESSED
27981			1.95**				2.01**		DNA SEGMENT, CHR 4, WAYNE STATE UNIVERSITY 53, EXPRESSED
	Fold change Q_{10}								
	Liver		Heart		Brain		Kidney		
Gene ID	6M	14M	6M	14M	6M	14M	6M	14M	Gene name
15511		3.67*				2.17*		4.14**	HEAT SHOCK PROTEIN 1B
94089		2.17**					3.63**		TRIPARTITE MOTIF PROTEIN 7
76044	1.69*				1.69*		1.66*		LEUCINE ZIPPER PROTEIN 5

$*p \leq 0.05$, $**p \leq 0.01$

$Q_{10}H_2$ as well as Q_{10} treatment (Tables 2 and 3). These transcripts were designated as "CoQ_{10}-sensitive." As shown in Tables 2 and 3, 14 CoQ_{10}-sensitive transcripts were differentially expressed in the kidney at 6 months but not at 14 months after supplementation with Q_{10} and $Q_{10}H_2$. In the liver, 16 CoQ_{10}-sensitive transcripts were affected by Q_{10} at 14 months after supplementation. In contrast, $Q_{10}H_2$ affected most of these genes at 6 months after supplementation. Taken together, our data in SAMP1 mice suggested that liver and kidney are the main target tissues of $Q_{10}H_2$ or Q_{10} intervention regarding gene expression. Furthermore, $Q_{10}H_2$ may have stronger impact on gene expression. In accordance to this hypothesis, $Q_{10}H_2$-sensitive transcripts (liver, 14 M supplemented) which suggest stronger regulatory effects ("Top 10," $p<0.05$) are characterized by higher expression fold-change values in comparison to Q_{10}-sensitive transcripts (Tables 4 and 5).

3.3 Detailed analysis of gene expression data obtained from liver samples of $Q_{10}H_2$-supplemented SAMP1 mice

With regard to the effects of $Q_{10}H_2$ and Q_{10} on gene expression in different tissues of SAMP1 mice, liver was shown to be strongly affected. Regarding the senescence deceleration process in SAMP1 mice, $Q_{10}H_2$ was more effective than Q_{10} (Fig. 1). This was also shown by a previous study [14]. To study long-term effects of $Q_{10}H_2$-supplementation on gene expression in more detail, liver samples of $Q_{10}H_2$-supplemented animals (14 M) were used. Initially, up and downregulated $Q_{10}H_2$-sensitive transcripts displaying the highest fold-change values ("Top 50," $p<0.05$) were selected. To unravel the functional connections of these genes we performed a text mining approach using the Genomatix BibliospherePathwayEdition Software

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Table 2. Expression profiles of transcripts both sensitive for $Q_{10}H_2$ and Q_{10} in different tissues: fold change $Q_{10}H_2$

	Fold change $Q_{10}H_2$								
	Liver		Heart		Brain		Kidney		
Gene ID	6M	14M	6M	14M	6M	14M	6M	14M	Gene name
63830#	3.34**#	3.87*					2.31**#		KCNQ1 OVERLAPPING TRANSCRIPT 1
552902				1.67*			1.92**		HYPOTHETICAL LOC552902
68186	2.87*						2.03*		RIKEN CDNA 4632427E13 GENE
233489							2.43*		PHOSPHATIDYLINOSITOL BINDING CLATHRIN ASSEMBLY PROTEIN
78265	2.92**						2.57**		RIKEN CDNA 4632418H02 GENE
27981			2.12*				2.61**		DNA SEGMENT, CHR 4, WAYNE STATE UNIVERSITY 53, EXPRESSED
68371	3.41**						1.91*		RIKEN CDNA 0610038K03 GENE
96982	3.48*							1.85**	EXPRESSED SEQUENCE C79248
76719	2.51**						1.92**		RIKEN CDNA 1700081L11 GENE
319263	2.61**			1.59*		1.52*		1.66*	PROTEIN-L-ISOASPARTATE (D-ASPARTATE) O-METHYLTRANSFERASE DOMAIN CONTAINING 1
20239		2.00**					1.60*		ATAXIN 2
208618	4.26*						1.87*		CDNA SEQUENCE BC026657
108829	2.40*							1.66*	JUMONJI DOMAIN CONTAINING 1C
622943						1.67*	2.24**		DNA SEGMENT, CHR 5, ERATO DOI 579, EXPRESSED
320861	4.15*						1.60*		RIKEN CDNA C130047D21 GENE
13196		2.21*					2.01*		DEVELOPMENT AND DIFFERENTIATION ENHANCING
72739	2.41**						2.43**		ZINC FINGER PROTEIN 306

*$p \leq 0.05$, **$p \leq 0.01$, #presented by ≥ 2 probe set IDs.

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Table 3. Expression profiles of transcripts both sensitive for $Q_{10}H_2$ and Q_{10} in different tissues: fold change Q_{10}

	Fold change Q_{10}								
	Liver		Heart		Brain		Kidney		
Gene ID	6M	14M	6M	14M	6M	14M	6M	14M	Gene name
63830#		1.64*					3.07*		KCNQ1 OVERLAPPING TRANSCRIPT 1
552902		1.89*					1.71**		HYPOTHETICAL LOC552902
68186		1.51*					2.04*		RIKEN CDNA 4632427E13 GENE
233489		2.59*					2.79*		PHOSPHATIDYLINOSITOL BINDING CLATHRIN ASSEMBLY PROTEIN
78265		1.86**					3.34*		RIKEN CDNA 4632418H02 GENE
27981			2.31**				2.23*		DNA SEGMENT, CHR 4, WAYNE STATE UNIVERSITY 53, EXPRESSED
68371		1.90*					1.54*		RIKEN CDNA 0610038K03 GENE
96982		1.73*					2.18**		EXPRESSED SEQUENCE C79248
76719		1.51*					1.95**		RIKEN CDNA 1700081L11 GENE
319263		2.03*					1.53*		PROTEIN-L-ISOASPARTATE (D-ASPARTATE) O-METHYLTRANSFERASE DOMAIN CONTAINING 1
20239		2.53**					1.92**		ATAXIN 2
208618		2.46*					1.71*		CDNA SEQUENCE BC026657
108829		1.77*					2.08*		JUMONJI DOMAIN CONTAINING 1C
622943		1.94*					2.51**		DNA SEGMENT, CHR 5, ERATO DOI 579, EXPRESSED
320861		2.25*					1.54*		RIKEN CDNA C130047D21 GENE
13196		1.66**					1.84*		DEVELOPMENT AND DIFFERENTIATION ENHANCING
72739		1.77*					2.57**		ZINC FINGER PROTEIN 306

*$p \leq 0.05$, **$p \leq 0.01$, #presented by ≥ 2 probe set IDs.

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Table 4. ''Top 10'' of up- and down-regulated $Q_{10}H_2$-regulated transcripts in the liver of SAMP1 mice (14M)

Gene ID	FC	Gene symbol	Gene name
Upregulated			
21822	21.24*	TGTP	T-CELL SPECIFIC GTPASE
27007	14.18*	KLRK1	KILLER CELL LECTIN-LIKE RECEPTOR SUBFAMILY K, MEMBER 1
18439	12.52*	P2RX7	PURINERGIC RECEPTOR P2X, LIGAND-GATED ION CHANNEL, 7
19363	12.07*	RAD51L1	RAD51-LIKE 1 (S. CEREVISIAE)
328563	10.68**	APOL11B	RIKEN CDNA A330102K04 GENE
22368	9.68*	TRPV2	TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL, SUBFAMILY V, MEMBER 2
328563	9.55*	APOL11B	RIKEN CDNA A330102K04 GENE
20846	9.21*	STAT1	SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 1
11801	9.11*	CD5L	CD5 ANTIGEN-LIKE
14131	8.87*	FCGR3	FC RECEPTOR, IGG, LOW AFFINITY III
Downregulated			
17840#	−6.12**	MUP1	MAJOR URINARY PROTEIN 1
56631	−4.60**	TRIM17	TRIPARTITE MOTIF PROTEIN 17
18113	−4.16*	NNMT	NICOTINAMIDE N-METHYLTRANSFERASE
23985	−4.16**	SLC26A4	SOLUTE CARRIER FAMILY 26, MEMBER 4
13089	−3.47**	CYP2B13	CYTOCHROME P450, FAMILY 2, SUBFAMILY B, POLYPEPTIDE 13
78894	−3.40*	AACS	ACETOACETYL-COA SYNTHETASE
76574	−3.38*	MFSD2	MAJOR FACILITATOR SUPERFAMILY DOMAIN CONTAINING 2
17844	−3.30*	MUP5	MAJOR URINARY PROTEIN 5
53901	−3.27**	RCAN2	DOWN SYNDROME CRITICAL REGION GENE 1-LIKE 1
13897	−3.26*	ES22	ESTERASE 22

$*p \leq 0.05$, $**p \leq 0.01$, #presented by ≥ 2 probe set IDs.

Table 5. ''Top 10'' of Q_{10} up and downregulated transcripts in the liver of SAMP1 mice (14M)

Gene ID	FC	Gene symbol	Gene name
Upregulated			
70945	7.15**	MMRN1	MULTIMERIN 1
NA	5.37**	NA	NA
207921	4.48*	A830093I24RIK	RIKEN CDNA A830093I24 GENE
381280	4.19*	6430706D22RIK	RIKEN CDNA 6430706D22 GENE
217166	4.00*	NR1D1	NUCLEAR RECEPTOR SUBFAMILY 1, GROUP D, MEMBER 1
15511	3.90*	HSPA1B	HEAT SHOCK PROTEIN 1B
52822	3.86**	RUFY3	RUN AND FYVE DOMAIN CONTAINING 3
71972	3.85*	DNMBP	RIKEN CDNA 2410003L07 GENE
67039	3.84*	RBM25	RIKEN CDNA 2600011C06 GENE
11430	3.78**	ACOX1	ACYL-COENZYME A OXIDASE 1, PALMITOYL
Downregulated			
12592	−5.13**	CDX4	CAUDAL TYPE HOMEO BOX 4
16625	−3.90*	SERPINA3C	SERINE (OR CYSTEINE) PEPTIDASE INHIBITOR, CLADE A, MEMBER 3C
14803	−3.09*	GRID1	GLUTAMATE RECEPTOR, IONOTROPIC, DELTA 1
233987	−2.38*	BC003267	CDNA SEQUENCE BC003267
22648	−2.33*	ZFP11	ZINC FINGER PROTEIN 11
75458	−2.30*	CMTM2A	RIKEN CDNA 1700001K04 GENE
66658	−2.21**	CCDC51	COILED-COIL DOMAIN CONTAINING 51
216805	−2.18*	FLCN	FOLLICULIN
81011	−2.15*	V1RD14	VOMERONASAL 1 RECEPTOR, D14
75424	−2.04*	ZFP820	HYPOTHETICAL GENE MGC29393

$*p \leq 0.05$, $**p \leq 0.01$.

(GFG level B3). Co-cited transcripts having been represented by multiple probe sets for one gene and/or were shown by high intensity levels were selected. Based on these criteria, we identified 11 $Q_{10}H_2$-sensitive transcripts which seem to be primarily involved in cholesterol and lipid metabolism as well as in inflammatory processes and cell differentiation (Table 6). Moreover, a part of the identified $Q_{10}H_2$-sensitive genes is functionally connected by the

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1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55

Table 6. Identification of $Q_{10}H_2$-sensitive genes and their functional connections

Gene ID	FC $Q_{10}H_2$	FC Q_{10}	Gene symbol	Gene name
Fatty acid and cholesterol synthesis				
208715#	−1.68*	n.s.	HmgCs1	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1
15356	−1.40*	n.s.	HmgCl	3-Hydroxy-3-methylglutaryl-coenzyme A lyase
15357	−2.44*	n.s.	HmgCr	3-Hydroxy-3-methylglutaryl-coenzyme A reductase
78894	−3.40*	n.s.	Aacs	Acetoacetyl-CoA Synthetase
20787	−1.97*	−1.52*	Srebf1	Sterol regulatory element-binding factor-1
Lipid mobilization				
16592#	3.63**	n.s.	Fabp5	Fatty acid binding protein 5, epidermal
Lipoprotein metabolism				
18830#	8.30*	n.s.	Pltp	Phospholipid transfer protein
Inflammation				
20846	9.21*	n.s.	Stat1	Signal transducer and activator of transcription 1
Cell differentiation and activation				
75104#	−3.17*	n.s.	Mmd2	Monocyte to macrophage differentiation-associated 2
17476	7.20*	n.s.	Mpeg1	Macrophage-expressed gene 1
100702	5.74**	n.s.	Mpa2l	Macrophage activation-2 like

$*p \leq 0.05$, $**p \leq 0.01$, #presented by ≥ 2 probe set IDs.

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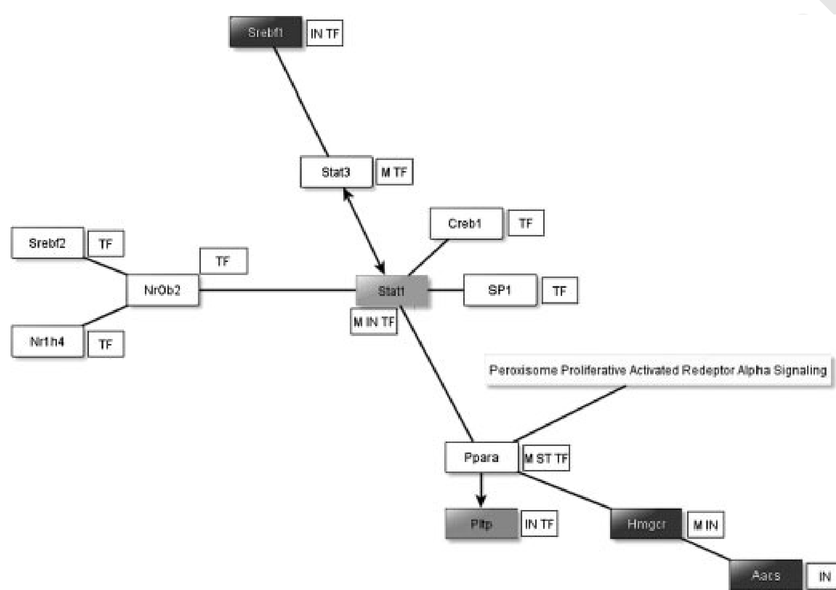

Figure 2. Bibliosphere network of $Q_{10}H_2$-sensitive genes regulated in the liver of SAMP1 mice. Based on co-citations with transcription factors and functional co-citations with other genes in the network (GFG level B3), 5 $Q_{10}H_2$-inducible genes were connected with each other by BibliospherePathwayEdition Software. According to this, the uploaded genes seem to play a key role in PPAR-α signaling. IN, input gene; TF, transcription factor; M, gene product is part of a metabolic pathway; ST, gene product is part of a Genomatix signal transduction pathway.

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peroxisome proliferator-activated receptor-α (PPAR-α) signaling pathway (Fig. 2).

3.4 Accumulation of CoQ_{10} in liver samples of $Q_{10}H_2$- and Q_{10}-supplemented SAMP1 mice

Next, we tested whether CoQ_{10} accumulates in the liver of SAMP1 mice supplemented for 14 months with $Q_{10}H_2$ or Q_{10}. Total CoQ_{10} as well as its redox state was determined in liver homogenates of SAMP1 mice which were used for microarray experiments ($n = 3$ *per* each group). In comparison to control animals, total CoQ_{10} levels increased about 54.92-fold ($p = 0.0027$) and 30.36-fold ($p = 0.0006$) in the liver of $Q_{10}H_2$- and Q_{10}-supplemented mice, respectively (Fig. 3A). As shown in Figs. 3B and C, 77.53% ($Q_{10}H_2$-supplemented) and 80.72% ($Q_{10}H_2$-supplemented) of CoQ_{10} was present in its reduced form. Because CoQ_9 is the predominant CoQ form in rodents [15], CoQ_9 levels were additionally determined in liver samples. As shown in Fig. 3D, the CoQ_9 concentration did not significantly change between treatment and control groups. In conclusion,

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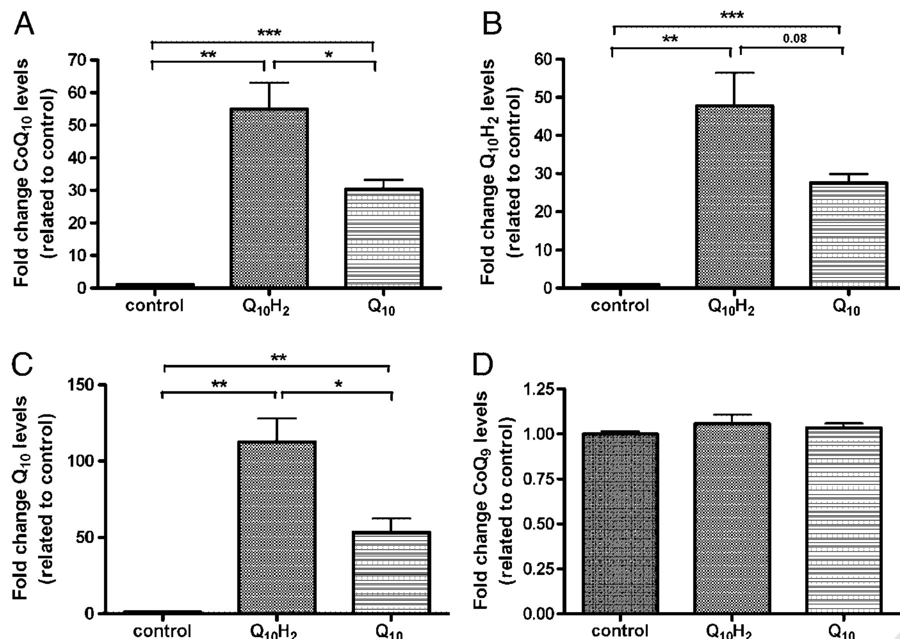

Figure 3. Effect of Q_{10} and $Q_{10}H_2$ supplementation on levels of total CoQ_{10} (A), redox status (B, C) and CoQ_9 (D) in liver tissues of SAMP1 mice. SAMP1-mice were supplemented with either $Q_{10}H_2$ or Q_{10} (500 mg/kg BW/d), or a respective control diet for 14 months. Thereafter, liver samples were collected, homogenized and used for HPLC analysis. Total CoQ_{10} levels increased about 54.92-fold ($p = 0.0027$) and 30.36-fold ($p = 0.0006$) in liver tissues of $Q_{10}H_2$- and Q_{10}-supplemented mice (A). Supplementation with $Q_{10}H_2$ and Q_{10} increased $Q_{10}H_2$ levels significantly about 47.86-fold ($p = 0.0054$) and 27.54-fold ($p = 0.0003$), respectively (B). $Q_{10}H_2$ supplementation induces also the strongest increase of Q_{10} levels in liver when related to controls (112.10-fold, $p = 0.0019$) (C). The CoQ_9 level did not significantly change between treatment and control groups (D). All data are means $\pm$ SEM of three animals *per* each group ($Q_{10}H_2$, Q_{10}, control), respectively.

$Q_{10}H_2$ supplementation was more effective than Q_{10} to increase the absolute levels of $Q_{10}H_2$ and Q_{10} in the liver of SAMP1 mice. Thereby, $Q_{10}H_2$ was the predominant form of CoQ_{10} in liver tissues.

4 Discussion

Data from previous [14] and present experiments reveal significant effects on decelerated senescence processes in SAMP1 mice supplemented with $Q_{10}H_2$ (Fig. 1). Additionally, distinct differences in gene expression profiles of $Q_{10}H_2$- and Q_{10}-supplemented SAMP1 mice in liver, heart, brain and kidney were identified. Because the oxidized form of CoQ_{10} can be reduced to $Q_{10}H_2$ by the plasma membrane redox system [16, 17], different effects of $Q_{10}H_2$ and Q_{10} on aging processes and gene expression are not obvious. However, the conversion of Q_{10} to $Q_{10}H_2$ is accompanied by the generation of reactive oxygen species [18],] which affects cellular redox-dependent gene regulation cascades [19]. A study in platelets also indicated a less effectiveness of the oxidized form of CoQ_{10} on oxidative stress parameters, despite the presence of quinone reductase activities [20]. Indeed, the activity of the plasma membrane redox system is modulated by different conditions including oxidative stress and aging [21, 22]. However, studies in perfused rat liver and isolated rat hepatocytes clearly indicated an antioxidant effect of exogenous $Q_{10}H_2$ [23, 24]. Some studies also reported about age-related decreases of CoQ_{10} levels in organs of both rates and humans [25]. In general, aging is considered as a process that seems to require an increase of antioxidant defenses to cope enhanced oxidative stress conditions [26, 27]. Additionally, differences in the tissue-dependent bioavailability of $Q_{10}H_2$ and Q_{10} may also have an impact on gene expression. Indeed, we found that $Q_{10}H_2$ supplementation was more effective than Q_{10} to increase levels of CoQ_{10} in the liver of SAMP1 mice. This might be in agreement with our previous results in monocytic cell lines, where incubation with raising doses of $Q_{10}H_2$ also induced a dose-dependent increase of the intracellular $Q_{10}H_2/Q_{10}$ ratio [5] when compared with Q_{10} incubation [1]. This $Q_{10}H_2$-specific effect additionally reveals the effectiveness of the exerted $Q_{10}H_2$ storage conditions for *in vitro* and *in vivo* studies. With regard to our present study, liver seems to be the main target tissue of CoQ_{10} intervention regarding gene expression, followed by kidney, heart and brain. This might be due to the fact that CoQ_{10} is mainly incorporated in LDL [28],] which are taken up by the liver. In this context, it was also shown that CoQ_{10} is capable to prevent LDL particles from oxidation *in vitro* and *in vivo* [29–32]. This effect is considered to be mediated through the radical scavenging activity of the reduced form of CoQ_{10}, at least in part by the regeneration of tocopheryl radicals [31, 33–35]. In this context our preliminary data (unpublished results) from

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another study in mice (C57BL6J) revealed a significant reduction (about $45 \pm 11.9\%$, $p = 0.0140$) of the pro-inflammatory chemokine MCP-1 in $Q_{10}H_2$ supplemented animals.

The observed consistence of CoQ_9 levels in tissue samples of CoQ_{10} supplemented mice were already described before [33].

With regard to gene expression data, a detailed analysis of whole genome expression profiles was performed for liver samples of mice supplemented with $Q_{10}H_2$ for 14 months. The results of the applied text-mining tool indicate an involvement of $Q_{10}H_2$-sensitive genes in the PPAR-α signaling pathway (Fig. 2). PPARs belong to the group of nuclear receptors and are negative regulators of numerous genes involved in lipid metabolism and cholesterol synthesis [36–40]. Moreover, it was shown that PPAR-α is predominantly expressed in tissues with high lipid catabolic activity [37]. In this context we identified a number of genes (Table 6) strongly downregulated by $Q_{10}H_2$-supplementation in the liver of SAMP1 mice. These genes were primarily involved in fatty acid and cholesterol synthesis (*e.g.* HMGCS1, HMGCL and HMGCR), lipid metabolism (FABP5) as well as lipoprotein metabolism (PLTP). Moreover, a regulatory role of PPAR-α in lipid metabolism and inflammatory processes is indicated in the literature [41–45]. The activation of PPAR-α occurs through, *e.g.* fatty acids and fibrates, a known class of hypolipidemic drugs. PPAR-α forms a heterodimer with retinoid X receptor (RXR) enhancing its binding to DNA sequence elements termed peroxisome proliferator response elements (PPRE) [46]. Liver X receptor (LXR) was found to inhibit the binding of the PPAR-α-9-cis retinoic acid receptor (PPAR-α/RXR) complex to PPRE [37]. Thus, ligand binding of LXR inhibits PPAR-α signaling and the activation of its downstream-target genes. Because LXR is activated by oxidized sterol and cholesterol metabolites [47, 48], antioxidant compounds might effectively inhibit activated LXR/RXR heterodimerization. Based on this data, we propose three putative functions for $Q_{10}H_2$ in PPAR-α-mediated signaling processes (Fig. 4): (i) as an antioxidant, leading to decreased levels of the LXR agonist oxidized LDL; (ii) as an antagonist of LXR, leading to PPAR-α/RXR heterodimers and PPRE activation; and (iii) as an agonist of PPAR-α, leading to PPAR-α/RXR heterodimers and PPRE activation. Moreover, the protein product of the Sterol regulatory element binding transcription factor-1 gene (SREBF-1, SREBP-1), described as a primary target gene of the RXR/LXR heterodimer [48, 49], was downregulated in liver samples of $Q_{10}H_2$-supplemented animals (Table 6). This might be a first indication of a $Q_{10}H_2$-mediated effect on PPAR-α signaling that leads to a reduced activation of the SREBF-1 promoter, a transcription factor that is known to activate fatty acid synthesis by increasing transcription of lipogenic genes [50–52]. Data of a recent study also demonstrate effects of CoQ_{10} treatment on lipid metabolism in obese ob/ob mice [53]. These effects were supposed to be mediated by PPAR-mediated activity. Moreover, a connection between PPAR-α signaling, inflammatory processes and neurodegenerative diseases in aging rats has been previously described [54]. These metabolic effects may explain, at least in part, the observed diminished effects on senescence characteristics in $Q_{10}H_2$-supplemented SAMP1 mice. Noteworhty is that the identified regulation of PPAR-α related genes observed in $Q_{10}H_2$-supplemented SAMP1 mice was not found in liver samples of Q_{10}-supplemented mice, indicating a $Q_{10}H_2$-

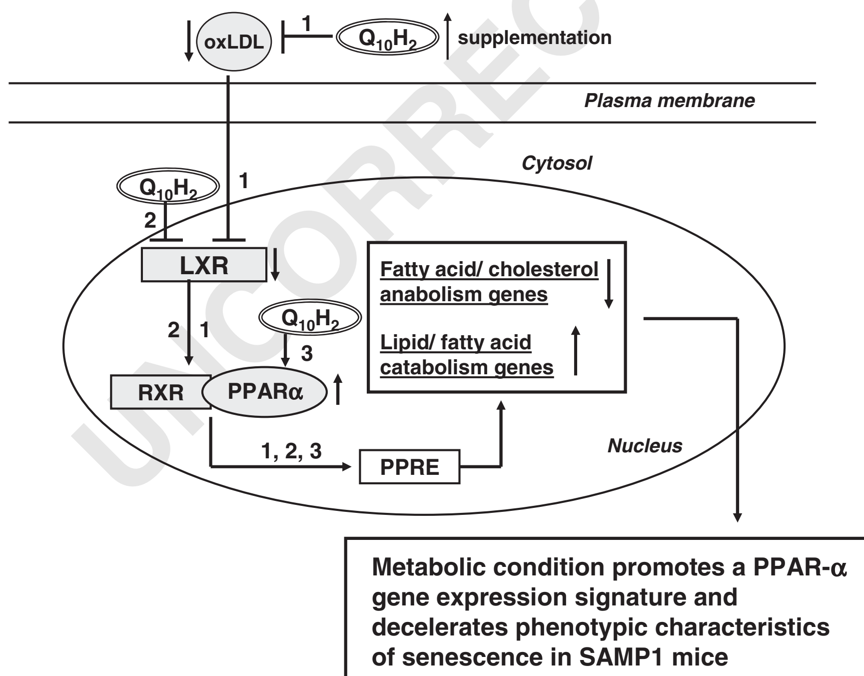

Figure 4. Putative mechanisms of $Q_{10}H_2$ action on PPAR-α signaling in liver tissues of SAMP1 mice. Three putative functions are proposed for $Q_{10}H_2$ in PPAR-α signaling: 1. As an antioxidant, leading to decreased levels of the LXR agonist oxidized LDL; 2. As an antagonist of LXR, leading to PPAR-α/RXR heterodimers and PPRE activation; and 3. As an agonist of PPAR-α, leading to PPAR-α/RXR heterodimers and PPRE activation.

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specific effect. Hence, between $Q_{10}H_2$ and control animals, no differences in expression levels of PPAR-α have been identified (fold-change: 1.06, $p = 0.827$). This might be a further hint for a $Q_{10}H_2$-modulatory effect on PPAR-α at the protein level, the most important mechanism of transcription factor regulation.

Even if the exact mechanism of $Q_{10}H_2$-PPAR-α interaction cascades is not definitely clear, these results support our conclusions regarding a regulatory role of $Q_{10}H_2$ in PPAR-α signaling processes.

The authors have declared no conflict of interest.

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Effects of the Reduced Form of Coenzyme Q_{10} on Gene Expression, Inflammation and Cell Differentiation in Humans

Running title: $Q_{10}H_2$ and gene expression in humans

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Abstract

Previous *in vitro* studies indicate a role of Coenzyme Q_{10} (CoQ_{10}) in gene expression, inflammation and apoptosis. To determine these effects in humans, a 2-week supplementation study with the reduced form of CoQ_{10} ($Q_{10}H_2$, 150 mg/d) was performed in 53 healthy male volunteers. Mean CoQ_{10} plasma levels increased significantly (4.8-fold) after supplementation. Gene expression studies in isolated monocytes of study subjects revealed 7 $Q_{10}H_2$-sensitive genes (TNFα, CXCL2, CCL3, GIMAP7, NR4A2, BRE and PMAIP1) that are related to inflammation and apoptosis. These genes are functionally connected by NFκB and PPAR signalling pathways, which are involved in the regulation of lipid metabolism and cell differentiation. Biochemical as well as NMR-based analyses showed a significant reduction of LDL cholesterol plasma levels after $Q_{10}H_2$ supplementation. At the cellular level, we obtained alterations of haematological parameters including a reduced count of erythrocytes but an increased number of reticulocytes. In conclusion, $Q_{10}H_2$-supplementation reduces LDL cholesterol and affects cell differentiation processes in humans. These effects are hypothesized to be mediated by the antioxidant property of $Q_{10}H_2$ through NFκB and PPARα dependent gene expression cascades.

Introduction

Coenzyme Q_{10} (CoQ_{10}) acts as a cofactor in the electron transport in the respiratory chain [1,2] and is required for the biosynthesis of pyrimidine nucleotides [3] and the function of uncoupling proteins (UCPs) [4]. More recently, CoQ_{10} has been identified as a modulator of gene expression *in vitro* [5] and in mice [6-8]. Moreover, our group and others observed anti-inflammatory [9-11] and anti-apoptotic [12-16] effects of CoQ_{10} *in vitro*. These effects are hypothesized to be mediated by the antioxidant properties of the reduced form of CoQ_{10} ($Q_{10}H_2$) [2]. To reveal putative gene-regulatory effects of $Q_{10}H_2$ on inflammatory processes and apoptosis *in vivo*, 53 healthy male volunteers were supplemented with $Q_{10}H_2$ (150 mg/d) for 2 weeks. So far, CoQ_{10} supplementation studies in a healthy study population are deficient when compared to those performed in diseased study groups, e.g. those with cardiovascular disorders [17]. Moreover, most published studies used oxidized CoQ_{10} (Q_{10}) [18-21]. Although Q_{10} can be enzymatically converted into its reduced form ($Q_{10}H_2$) [22], our previous results in the monocytic cell line THP-1 indicated stronger anti-inflammatory effects of $Q_{10}H_2$ when compared to Q_{10} [9,10]. Additionally, the conversion of Q_{10} to $Q_{10}H_2$ is accompanied by the generation of reactive oxygen species (ROS) [23], with an additional impact on gene expression. In fact, it has been shown that reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) are effective modulators of apoptotic processes by acting through gene expression [24,25]. Because monocytic cells have been described as key players in a variety of inflammatory and apoptotic processes [26-29], isolated monocytes were used for our gene expression studies.

To our knowledge, this is the first study investigating gene-regulatory effects of the reduced form of CoQ_{10} ($Q_{10}H_2$) on inflammatory and apoptotic processes in a healthy study population.

Subjects and Methods

$Q_{10}H_2$ formulation

$Q_{10}H_2$ capsules ($Q_{10}H_2$, rapeseed oil, diglyceryl monooleate, bee wax, lecithin) were obtained from KANEKA Corporation, Japan. Capsule stabilization was ensured by a method disclosed in patent applications (WO 03/06408, WO 03/06409, WO 03/06410, WO 03/06411, WO 03/06412, WO 03/08363 and WO 03/32967).

Subjects and study design

53 healthy male volunteers received 150 mg $Q_{10}H_2$ daily in form of three softgel capsules with each principal meal (a´ 50 mg) for 14 days. Fasting blood samples (54 ml each) were taken before (T_0) and after (T_{14}) supplementation with $Q_{10}H_2$ from all study participants as well as 4 weeks after study completion (T_{42}, washout period) from 9 subjects, respectively. The study design is a simple one-group pre-post analysis. Based on clinical laboratory tests, all study participants fulfilled the following inclusion criteria: 1) no history of gastrointestinal, hepatic, cardiovascular or renal disease; 2) no supplemental vitamin use for $\geq$ 2 weeks before the start of the study; 3) non- or occasional smoking ($\leq$ 3 cigarettes/d); and 4) perpetuation of usual nutrition habits. Three males were dropped out for their elevated TNFα or CRP levels, three dropped out from the study for personal reasons and one person had invalid $Q_{10}H_2$ plasma values after supplementation. The study was approved by the ethics committee of the Medical Faculty of Kiel University, Germany, and was conformed to Helsinki Declaration. All volunteers gave written informed consent.

Plasma CoQ_{10} content

HPLC-analysis

Analysis was based on the method of high-pressure liquid chromatography (HPLC) with electrochemical detection and internal standardisation using ubihydroquinone-9 and ubiquinone-9 as standards and is described elsewhere [30]. In brief, as internal standard 15 pmol of ubihydroquinone-9 in 50 µl ethanol were added to 100 µl plasma sample. Subsequently, the sample was mixed for 1 minute, and the suspension was immediately extracted with 500 µl hexane after mixing for further 2

minutes. After centrifugation (1000 x g, 5 minutes, 4° C), 300 µl of the supernatant was transferred to a separate tube and dried under a stream of argon. Finally, the dried residue was redissolved in 40 µ ethanol and injected into the HPLC system.

Gene expression

Monocyte isolation and RNA extraction

Monocytes were isolated from EDTA-blood samples (36 ml) of volunteers at each indicated time point (T_0, T_{14}, T_{42}). Monovettes were stored on ice for a maximum of 1.5 h until isolation of monocytes. CD14-positive cells were isolated by density centrifugation with LymphoPrep™ (Fresenius Kabi Norge, Oslo, Norway) and successive magnetic antibody cell sorting (MACS) using micro bead-conjugated anti-CD14 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously [31]. Monocytes were counted and stored at -80°C until all samples of the study had been completely collected. Total RNA was extracted with the miRNeasy Isolation Kit, including on-column DNA digestion, according to the manufacturer´s instructions (Qiagen, Hilden, Germany). RNA concentration was determined by using an ultraviolet spectrometer; RNA integrity number was measured with a 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, USA).

Microarray analysis

Microarray experiments were performed from monocytes of three volunteers at the indicated time points (T_0, T_{14}), including a total of six human genome U133 Plus 2.0 GeneChip® microarrays. Sample selection from volunteers for microarray analysis was based on quality and quantity of isolated RNA. RNA integrity, indicated as RNA integrity number (RIN), was in a range between 7.5 and 9.2. For generation of biotin-labeled cRNA (MessageAmp™ II-Biotin *Enhanced* Kit, Ambion/Applied Biosystems, Darmstadt, Germany), 2 µg total RNA were used for each array. Poly-A RNA controls were applied according to the manufacturer´s instructions (GeneChip® Eukaryotic Poly A-RNA Control Kit). Using a hybridization oven 640, each GeneChip® array was hybridized for 16 h at 45°C with 15 µg fragmented, biotin-labeled cRNA, including hybridization controls (GeneChip® Eukaryotic Hybridization Control Kit). Subsequently thereafter, arrays were washed and stained using Fluidics Station 450. Hybridization, washing and staining

solutions were obtained from analogous GeneChip® kits. After hybridization and washing procedures, microarrays were scanned with the GeneChip® scanner 3000, using GCOS software. If not stated otherwise, all kits and equipment were purchased from Affymetrix (High Wycombe, United Kingdom). Fluorescence data were obtained in CEL file format. Quality control and normalization procedure of the files was performed with R software 2.7.1 and BioConductor 2.0.1 provided by the MADMAX database (https://madmax.bioinformatics.nl). Data were normalized with the GC-RMA algorithm.
Initially, only probe sets were selected having the same direction of expression changes in all three subjects at each time point, respectively. Secondly, only probe sets displaying the highest fold-change values at T_{14} when compared to T_0 ("Top 50" for up- and down-regulated, respectively, $p \leq 0.05$) were chosen. Based on gene ontology (GO) terms (http://amigo.geneontology.org), transcripts related to inflammation/immune response and/or apoptosis were selected. The complete microarray data sets and information about study design and methodology will be subsequently submitted to Minimal Information about Microarray Experiments (MIAME) at NCBI Gene expression omnibus (GEO) in an applicable format [32].

Quantitative real-time PCR

SYBR® Green

Primer sequences for real-time quantitative RT-PCR (qRT-PCR) experiments were designed with Primer Express® Software 3.0 (Applied Biosystems, Darmstadt, Germany). Primer pairs (Table 1) were obtained from MWG Biotech AG (Ebersberg, Germany). cDNA synthesis was initially carried out with the reverse transcriptase core kit (Eurogentec, Köln, Germany) on a thermocycler (Biometra, Göttingen, Germany). qRT-PCR amplification was performed with the Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) on an Applied Biosystem 7300 qRT-PCR system. Ct-values of target genes were related to those of the corresponding housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

TaqMan®

The TaqMan® MicroRNA Assay (hsa-mir-21) and endogenous control (RNU48) were obtained from Applied Biosystems (Darmstadt, Germany). miRNA quantification was carried out as two-step RT-PCR. Reverse transcription was performed on a thermocycler (Biometra, Goettingen, Germany) with specific miRNA primers supplied by the TaqMan® MicroRNA and endogenous control assays as well as reagents from the TaqMan® MicroRNA Reverse Transcription Kit. During the PCR step, PCR products were amplified from cDNA samples using the TaqMan® MicroRNA Assay together with the TaqMan® Universal PCR Master Mix (Applied Biosystems). qRT-PCR reactions were performed on an Applied Biosystems 7300 Real-Time PCR System. Target sequences were as follows: hsa-miR-21, UAGCUUAUCAGACUGAUGUUGA; RNU48, GAUGACCCCAGGUAACUCUGAGUGUGUCGCUGAUGCCAUCACCGC AGCGCUCUGACC.

In silico **analysis**

For analysis of common pathways between regulated genes, Genomatix Software 2009 (www.genomatix.de) was used. Probe set IDs of the selected genes were uploaded to BibliospherePathwayEdition (BSPE) software. This text mining tool identifies putative functional connections of genes based on co-citations with transcription factors and other genes in the network from NCBI pubmed [33]. The co-citation filter “signal transduction associations” (level ST) was applied.

Secretion levels of pro-inflammatory molecules in native serum and *ex vivo* stimulated whole blood samples

Ex vivo whole blood stimulation

Venous blood from study participants was diluted 1:5 with cell culture medium (RPMI 1640, Invitrogen, Karlsruhe, Germany) and plated in 24-well format. Subsequently after one hour, blood samples were stimulated with LPS (*Salmonella enteriditis*, 20 ng/ml). Unstimulated (-LPS) samples served as controls. Plates were further incubated for 6 h at 37 °C in humidified atmosphere. Subsequently thereafter, plates were centrifuged and supernatants were removed. Samples were stored at -80°C until further analysis.

ELISA

Supernatants of native and/or *ex vivo* stimulated whole blood samples were measured with commercially available ELISA kits for TNFα, MCP-1 (R&D Systems, Minneapolis, MN), oxLDL (KAMIYA Biomedical Company, Seattle, USA), CXCL2 (Promocell, Heidelberg, Germany) and ADMA (DLD Diagnostika, Hamburg, Germany). Optical density was read on a microplate reader (Spectramax® 190, Molecular Devices).

Comparison of individual mRNA and protein levels

Relative mRNA levels (qRT-PCR data) for TNFα and CXCL2 from T_{14} monocyte samples were correlated to the respective native serum protein levels of study subjects (n = 9). Methods of mRNA (qRT-PCR) and protein (ELISA) quantification were already described before.

Determination of LDL cholesterol

^{1}H NMR analysis

For analysis of LDL cholesterol levels in serum samples of study subjects, ^{1}H NMR spectroscopy was carried out on a Bruker AVANCE II spectrometer operating at 600 MHz ^{1}H resonance frequency. As NMR-probe a TXI-probe was used and the samples were run in 5 mm NMR-tubes with a total of 400 µl. For serum samples, 190 µl aliquots of neat serum and 190 µl of PBS were transferred into the NMR tube and 20 µl standard solution (deuterium oxide with Sodium 4,4-dimethyl-4-silapentane-1-sulfonate) were added. ^{1}H NMR spectra were acquired immediately after preparation of each individual sample. All spectra were recorded at 37 °C. ^{1}H NMR spectra were measured over a spectral width of 12019 Hz which resulted in an acquisition time of 2 s. Plasma samples were analyzed using the one-dimensional nuclear Overhauser and Exchange Spectroscopy (1D NOESY) and one-dimensional Carr-Purcell-Meiboom-Gill (1D CPMG) sequence. Data analysis was performed using principal component analysis (PCA).

Determination of blood parameters

Immediately after blood withdrawal, values of haematological variables (leukocytes, erythrocytes, reticulocytes, haemoglobin, haematocrit, mean corpuscular volume,

mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration) were determined by using a haematology autoanalyzer (Sysmex K-1000).

Statistical analysis

To determine anti-inflammatory and anti-apoptotic effects of $Q_{10}H_2$ supplementation both on gene expression and protein levels, power calculation was involved. Based on power analysis including F-value [95 % power, $p \leq 0.05$] of 12.99, a standard deviation of TNFα levels of $\sigma = 0.7$ and literature-based differences in TNFα levels between controls and treatment groups of $d = 0.5$, the minimum number of study subjects was determined as 51. Thus, 53 study subjects were finally included in the study. Statistics were calculated with SPSS 11.5 software (SPSS GmbH Software, München, Germany), Microsoft Excel 2003 and GraphPad Prism 4.0. Before statistical analysis, normal distribution of the parameters was tested. Results were analyzed by a two-sided, paired Students *t*-test if not stated otherwise. Levels of statistical significance were set at $p \leq 0.05$.

Results

$Q_{10}H_2$ supplementation induces significant increases of total CoQ_{10} plasma levels and its antioxidant redox state

CoQ_{10} mediates anti-inflammatory and anti-apoptotic effects *in vitro* [9-16]. These effects were thought to be mediated by the reduced form of CoQ_{10} ($Q_{10}H_2$). To study these effects in humans, 53 healthy male volunteers were supplemented with 150 mg/d $Q_{10}H_2$ for 2 weeks. Fasting blood samples were taken before (T_0) and after (T_{14}) supplementation with $Q_{10}H_2$. As shown in table 2, basic characteristics (e.g. BMI, fasting glucose, creatinine, blood pressure) of the study subjects were in accordance with the inclusion criteria of the study and show values within the physiological range for healthy men. Because previous studies with CoQ_{10} indicated effects on markers of endothelial dysfunction [34,35], blood pressure and asymmetric dimethylarginine (ADMA) was additionally determined. We found no effect of CoQ_{10} on these parameters (Table 2).

$Q_{10}H_2$ plasma concentrations above baseline levels were an essential precondition for the study. According to this, cholesterol-related plasma CoQ_{10} levels (µmol/mol) increased significantly from 229.24 ± 61.34 to 1109.75 ± 343.76 ($p < 0.0001$) for T_0 and T_{14}, respectively (Figure 1A). Four weeks after study completion (T_{42}, washout period, n = 9), plasma levels returned to baseline levels of 236.33 ± 77.01 µmol/mol. Additionally, $Q_{10}H_2$ supplementation reduced plasma levels of the oxidized form of CoQ_{10} (Q_{10}) significantly from 7.47 ± 0.97 % at T_0 to 5.95 ± 0.91 % at T_{14} ($p < 0.0001$) (Figure 1B). However, 4 weeks after washout period, the redox state returned nearly to pre-treatment levels with 6.97 ± 0.82 % in the oxidized form ($p = 0.024$). Thus, we were able to increase CoQ_{10} levels about 4.8-fold in $Q_{10}H_2$-supplemented persons, with significant lower amounts of the oxidized form. These effects were abolished after 4-week washout period.

Identification of 7 $Q_{10}H_2$-sensitive genes involved in inflammatory and apoptotic processes

For the identification of $Q_{10}H_2$-sensitive genes, mRNA steady state levels of 25.044 probe sets in monocyte samples of 3 study subjects before (T_0) and after (T_{14}) $Q_{10}H_2$ supplementation were determined. Based on selection criteria (see Materials and Methods) for the microarray experiment, 272 unique probe sets were

significantly regulated (fold change $\geq$ 1.5, $p \leq 0.05$) through $Q_{10}H_2$ treatment. Out of these, 56 probe sets were up-regulated and 216 probe sets were down-regulated with a fold change ranging from 1.52 to 2.85 and -1.51 to -27.30, respectively (Tables 3 and 4). Based on gene ontology (GO) terms, nine transcripts (TNFα, CXCL2, IL8, CCL3, GIMAP7, NR4A2, BRE, PMAIP1 and miR21) related to inflammatory/immune response and/or apoptotic processes were selected for further analysis. In order to identify common pathways of these genes, we performed a text mining approach. As shown in figure 3, the $Q_{10}H_2$-sensitive genes seem to play a role in peroxisome proliferator-activated receptor (PPAR)-signalling and cell proliferation (TP53) pathways, finally connected by NFκB.

Control and extended analysis confirms microarray data

As shown in table 5, technical verification supported the results of the microarray data (Table 5). Biological verification and an extended analysis of these data was performed in six additional samples from volunteers as well as in monocyte samples from 4 weeks after discontinuation of $Q_{10}H_2$-supplementation ("washout"). With exception for the CXCL2 gene, expression levels of TNFα, CCL3, GIMAP7, NR4A2, BRE and PMAIP1 returned nearly to pre-treatment levels after 4 weeks of study completion. In more detail, the pro-inflammatory Tumour necrosis factor alpha (TNFα) gene shows 61.1 ± 5.3 % reduced expression levels at T_{14} ($p = 0.0021$) (Figure 2A). This effect was nearly declined after an one-month washout period (T_{42}), even though to a reduced extent (73 ± 14.1 % of T_0, $p = 0.025$). The chemokine (C-X-C motif) ligand 2 gene (CXCL2, macrophage inflammatory protein-2-alpha, MIP-2α) and chemokine (C-C motif) ligand 3 (CCL3, MIP-1α) were also shown to be down-regulated about 72 ± 9.8 % ($p < 0.0001$) and 41.7 ± 16.1 % ($p = 0.0171$) at T_{14}, respectively (Figure 2B and 2C). In comparison to the other $Q_{10}H_2$-sensitive genes, the $Q_{10}H_2$-mediated effects on CXCL2 and CCL3 expression seem to be more persistent. At washout period, only 44.8 ± 11.8 % and 66.4 ±14.5 % of the baseline levels were achieved (Figure 2B and 2C). Two further genes that were significantly down-regulated at T_{14} are the nuclear receptor subfamily 4, group A, member 2 (NR4A2 or NURR1) gene and the brain and reproductive organ expressed (BRE) gene, namely to 37.3 ± 9.9 % ($p = 0.0285$) and 64.9 ± 9.7 % ($p = 0.0009$), respectively (Figure 2D and 2F). After washout period, the effects on NR4A2 and BRE gene expression returned to 67.1 ± 10 %

and 83.5 ± 8.6 % of the baseline (T_0) levels (Figure 2D and 2F). Another gene that was significantly down-regulated to 52.6 ± 9.7 % ($p = 0.0019$) in monocytes of $Q_{10}H_2$-supplemented persons was phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1) (Figure 2G). After washout period, the effects returned to 97.7 ± 23.6 % of the baseline (T_0) level. Only one gene, the GTPase, IMAP family member 7 (GIMAP7), was significantly up-regulated about 44.7 % at T_{14} ($p = 0.032$), but returned also nearly to baseline levels (about 86.7 ± 10.3 % of T_0) after washout period (Figure 2E).

$Q_{10}H_2$ supplementation shows significant anti-inflammatory effects on the transcriptional but not on the protein level

To confirm the observed anti-inflammatory effects of CoQ_{10} on gene expression at the protein level, relevant inflammatory markers were examined in serum samples. However, no significant effects of $Q_{10}H_2$ supplementation were observed for Tumour necrosis factor alpha (TNFα), Monocyte chemotactic protein-1 (MCP-1), chemokine (C-X-C motif) ligand 2 (CXCL2) and C-reactive protein (CRP) (Table 7). Of note, comparison between mRNA and protein levels of the inflammatory mediators TNFα and CXCL2 revealed no correlation (Figure 4). Additionally, we analysed anti-inflammatory effects of $Q_{10}H_2$ at the protein level with an *ex vivo* approach. Before and after CoQ_{10} supplementation whole blood samples were stimulated with LPS and the resulting secretion levels of TNFα and MCP-1 were determined. As shown in table 6, mean levels of TNFα and MCP-1 increased significantly in LPS stimulated whole blood samples when compared to controls (-LPS). TNFα responses increased 26.42- and 29.67-fold ($p < 0.0001$) at T_0 and T_{14}, respectively. MCP-1 levels increased 1.76- and 1.70-fold after LPS stimulation ($p < 0.0001$). However, the stimulation values showed no differences between T_0 and T_{14}. Taken together, $Q_{10}H_2$ supplementation mediates anti-inflammatory effects on the transcriptional but not on the protein level.

$Q_{10}H_2$ supplementation induces a significant reduction of serum LDL cholesterol levels

With respect to the results from our *in silico* analysis (Figure 3), $Q_{10}H_2$-mediated effects on peroxisome proliferator-activated receptors (PPAR) signalling pathways were hypothesized. Because PPARs are known key players in lipid and cholesterol

metabolism [36], effects on serum lipid parameters were additionally analyzed. As determined by routine laboratory tests, a significant reduction of low-density lipoprotein (LDL) cholesterol levels has been found in serum samples of study subjects (from 95.51 ± 28.89 mg/dl to 90.60 ± 27.21 mg/dl, p = 0.022) (Table 8). No significant effects were found for other lipid parameters including total cholesterol, high-density lipoprotein (HDL) cholesterol, oxidized LDL (oxLDL) and triglycerides (TG) (Table 8). The reducing effect on LDL cholesterol levels was additionally shown by ^{1}H NMR analysis (from 75.0 to 65.5 mg/dl) with no effects on LDL particle size. The observed effects on total LDL cholesterol levels are mediated through significant reductions of three LDL subfractions including B, C and E with relative reductions of 33.02 % (p = 0.00002), 14.62 % (p = 0.0098) and 16.52 (p = 0.008), respectively (Figure 5). In contrast, no $Q_{10}H_2$-mediated alterations were found for the LDL subfractions A and D. Taken together, $Q_{10}H_2$ supplementation mediates distinct reducing effects on LDL cholesterol levels.

$Q_{10}H_2$ supplementation alters haematological parameters in the blood

Caspases are involved in various cell differentiation processes [37-39]. The production and differentiation of red blood cells (erythropoiesis) is related to caspase 8 activation [40]. Because we identified the monocyte to macrophage differentiation gene (MMD, Table 4) and the caspase 8 and FADD-like apoptosis regulator gene (CFLAR, Table 3) as $Q_{10}H_2$-senstive genes in isolated human monocytes and liver tissues of SAMP1-mice as well (*unpublished results*), haematological parameters were determined in study subjects before and after $Q_{10}H_2$ supplementation. As shown in table 9, parameters of erythropoiesis [41] were significantly changed in the blood. The number of erythrocyte and haematocrit values decreased significantly from 4.95 ± 0.29 to 4.89 ± 0.31 (p = 0.02) and 44.48 ± 2.36 to 43.78 ± 2.40 (p = 0.01), respectively, for T_0 and T_{14}. Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and reticulocyte count increased significantly from 30.62 ± 1.12 to 30.82 ± 1.17 (p = 0.02), 34.09 ± 0.53 to 34.33 ± 0.53 (p = 0.02) and 10.94 ± 3.03 to 12.09 ± 2.46 (p = 0.0006), respectively, at T_0 and T_{14}. No significant effects were found for leukocyte count and haemoglobin concentration (Table 9). In conclusion, $Q_{10}H_2$-supplementation affects parameters related to erythroid differentiation processes.

Discussion

Assumption 1

$Q_{10}H_2$ modulates the expression of inflammatory genes at mRNA level but not at protein level.

In the present study, 53 male volunteers were supplemented with the reduced form of CoQ_{10} ($Q_{10}H_2$) for 2 weeks. To identify $Q_{10}H_2$-sensitive genes related to inflammatory and apoptotic processes, gene expression profiles in monocytic cells of study subjects were analysed. Monocytes have been described as a reliable model to study anti-inflammatory and anti-apoptotic effects *in vivo* [26,27,42]. TNFα, CXCL2, CCL3, GIMAP7, NR4A2, BRE and PMAIP1 were assigned as $Q_{10}H_2$-sensitive genes involved in inflammatory and apoptotic processes. GIMAP7 belongs to the group of GTPases which are exclusively expressed in cells of the immune system including macrophages and peripheral blood leukocytes [43,44]. GTPases are thought to regulate a variety of different processes in cells including their action as tumor suppressors [43]. TNFα, CXCL2 and CCL3 are described as key players in the maintenance of inflammatory responses in monocytes and macrophages [45-50]. NR4A2 has been shown to modulate apoptotic processes [51,52] but to be also involved in inflammatory cascades [53,54]. BRE was reported to play a significant key role in proliferative responses [55], endocrine and immune functions [56] as well as apoptosis [57]. PMAIP1 has been shown to be involved in apoptotic processes [58,59].

For verification of $Q_{10}H_2$-mediated transcriptional effects on the protein level, concentrations of relevant inflammatory mediators (TNFα, CXCL2, MCP-1, CRP) were additionally determined in serum samples of study subjects. However, no significant differences were observed. Comparisons between individual mRNA and protein levels of the pro-inflammatory mediators TNFα and CXCL2 – each playing a distinct role in monocyte/macrophage activation processes [60-63] – revealed no significant correlation as well. Because triggering of monocytes with LPS leads to an activation of the transcription factor NFκB and, hence, the release of pro-inflammatory mediators [64,65], this *ex vivo* approach was additionally applied to determine putative anti-inflammatory effects of $Q_{10}H_2$ on the protein level. $Q_{10}H_2$-mediated effects on the reduction of several pro-inflammatory molecules have

been already described *in vitro* [9-11]. With regard to our *ex vivo* approach, no $Q_{10}H_2$-mediated effect on LPS-induced secretion levels of TNFα and MCP-1 was found. One explanation for the observed discrepancies regarding the $Q_{10}H_2$ effects on inflammation at the protein level in *in vitro* and *in vivo* studies could be the heterogeneity of blood plasma and serum matrices when compared to isolated cell culture systems. $Q_{10}H_2$-independent posttranscriptional (e.g. micro RNAs) [66] and posttranslational mechanisms [67] leading to a reduced activity or stability of the synthesized mRNAs and proteins may account for different effects of $Q_{10}H_2$ on mRNA and protein level. In view of the *in vivo* and *ex vivo* data, the $Q_{10}H_2$-mediated effects on inflammation are considered to occur mainly at the transcriptional level.

Assumption 2

$Q_{10}H_2$ affects cell differentiation through PPARα signalling cascade.

Putative common pathways between the $Q_{10}H_2$-regulated genes were identified by text mining analysis. Based on co-citations with transcription factors and other genes in the network, 6 genes (TNFα, CCL3, CXCL2, NR4A2, BRE and PMAIP1) were functionally connected with each other. The associated genes were identified to play a role in PPAR-signalling and cell proliferation (TP53) pathways, centrally connected by NFκB. Effects of $Q_{10}H_2$ supplementation on PPARα signalling pathways were already found in liver tissues of SAMP1 mice [6]. In this context, the identified $Q_{10}H_2$-sensitive genes showed a partial involvement in cell differentiation processes. As cell differentiation plays a crucial role in human monocytes [68,69], putative effects of $Q_{10}H_2$ supplementation on genes involved in monocyte/macrophage differentiation processes were additionally determined. In agreement to the consistent results of microarray [6] and qRT-PCR results (*unpublished data*) from liver tissue of 14-months supplemented (14 M) SAMP1 mice, the human monocyte to macrophage differentiation gene (MMD) was also significantly down-regulated (-1.73, $p = 0.04$) in monocytes of $Q_{10}H_2$-supplemented subjects (Table 4). A modulatory role of $Q_{10}H_2$ in PPARα mediated differentiation processes is additionally indicated by the down-regulation of the PMAIP1 (Noxa) gene, which is also involved in cell differentiation cascades by induction of apoptosis signalling pathways [59].

With regard to previous studies [12-16] and our present data on gene expression (Table 5), anti-apoptotic effects of $Q_{10}H_2$ are suggested. Because the monocyte to macrophage differentiation process is linked to apoptosis [68], caspases are thought to be putative upstream regulators in the modulation of the monocyte to macrophage differentiation gene (MMD). Moreover, although in most cases caspase activation is linked to apoptosis, recent evidence indicates nonapoptotic functions of caspase 8 [70-72]. As caspase 8 activation has been shown to down-regulate the transcription factor NFκB, which in turn is hypothesized to favour the macrophage differentiation process [73], caspase 8 and FADD-like apoptosis regulator gene (CFLAR) was ascribed as further putative $Q_{10}H_2$ target gene. In fact, microarray analysis of monoycte samples revealed a 2.13-fold increase of CFLAR gene expression (p = 0.02) in monocytes of $Q_{10}H_2$ supplemented volunteers (Table 3). Moreover, a significant induction of CFLAR gene expression (1.77-fold, p = 0.02) was also found in liver tissues of SAMP1 mice supplemented with $Q_{10}H_2$ for 14 months (*unpublished results*) [6]. Taken together, $Q_{10}H_2$ may inhibit the monocyte to macrophage differentiation process via modulation of MMD and CFLAR gene expression.

Because processes such as liver hematopoietic stem cell proliferation and red blood cell differentiation (erythropoiesis) are also related to caspase 8-dependent mechanisms [40,72], effects on haematological parameters were determined. Significant effects of $Q_{10}H_2$ supplementation have been found for parameters related to erythrocyte differentiation and proliferation processes (erythropoiesis) [41]. Thus, the number of mature erythrocyte and haematocrit values decreased significantly in blood samples of study subjects after $Q_{10}H_2$ supplementation. In contrast, values of mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and reticulocyte count were significantly increased. Reticulocytes are described as immature erythrocytic cells in the peripheral blood that are in a penultimate phase of maturation [74]. Because the number of reticulocytes was significantly increased after $Q_{10}H_2$-supplementation, as well as the number of mature erythrocytes reduced, $Q_{10}H_2$-mediated effects on differentiation and/or proliferation processes related to erythropoiesis were hypothesized. Taken together, gene expression data from mice and humans as

well as cellular parameters from the human study indicate an involvement of $Q_{10}H_2$ in cell differentiation processes through PPARα signalling cascade.

Assumption 3

$Q_{10}H_2$ reduces LDL cholesterol.

Because PPARs are known key players in lipid and cholesterol metabolism [36,75,76], effects on serum lipid parameters were additionally determined. Significant reducing effects were found for LDL cholesterol in serum samples of study subjects after $Q_{10}H_2$ supplementation. More recently, $Q_{10}H_2$-mediated effects on PPARα gene expression patterns were discussed in liver tissues of SAMP1 mice [6]. In this context, several genes related to lipid and cholesterol metabolism as well as cell differentiation processes were identified to be regulated in liver tissues of mice by $Q_{10}H_2$-treatment. Because $Q_{10}H_2$ is primarily incorporated in LDL particles during its transport in the blood [77], it is able to prevent LDL oxidation processes *in vivo* and *in vitro* [78-80]. This in turn may inhibit the oxLDL-mediated liver X receptor (LXR) activation [81,82] and subsequent up-regulation of ABC transporter genes, finally leading to a decreased cellular cholesterol efflux as well [83-85]. In general, the physiological relevance of the observed lowering effect of $Q_{10}H_2$ supplementation on LDL cholesterol levels in our human study (12.7 % from NMR-based data) is comparable to these described for plant sterols in different cohorts. Plant sterols, which are structurally related to cholesterol, mediate distinct lowering effects on LDL plasma levels [83]. It has been shown that an usual daily intake of 2 – 2.5 g plant sterols or stanols results in an average reduction of LDL cholesterol levels of up to 14 % [86,87].

Assumption 4

$Q_{10}H_2$ affects NFκB/PPAR-mediated signalling pathways which connect cell differentiation and inflammatory processes.

Although in most cases caspase activation is linked to apoptosis, recent evidence indicates nonapoptotic functions of caspase 8 and/or FADD-like apoptosis regulator gene [59,70-72,88]. This might also explain the results from previous *in vitro* experiments [12-16] as well as from our present gene expression data in human monocytes where anti-apoptotic effects of CoQ_{10} are evident. Furthermore, caspase 8 has been shown to prevent the activation of the nuclear transcription

factor NFκB in monocytes [73] and thus, an undergoing differentiation process into macrophages. In addition, PPARs have been described to interact with the nuclear transcription factor NFκB [89]. PPARα activators can inhibit the translocation process of NFκB into the nucleus [90,91], thereby preventing the expression of pro-inflammatory genes. Moreover, mechanisms of transrepression could also account for the $Q_{10}H_2$-mediated effects on gene expression, which are due to protein-protein interactions between PPARα and the promoter-bound transcription factor NFκB [89]. However, independently of the molecular mechanisms underlying NFκB inhibition, PPARs are known key regulators of inflammatory processes and lipid homeostasis [92]. PPARα has been shown to be already expressed in undifferentiated monocytes, whereas PPARγ expression is initially induced upon differentiation into macrophages [76]. Additionally, PPARα activators have been described to prevent differentiation processes in human monocytes [76], whereas PPARγ seems to be absent in these cells [93]. In general, a distinct role of $Q_{10}H_2$ in PPARα mediated cell differentiation processes is suggested by the down-regulation of the PMAIP1 and MMD gene in human monocytes, probably mediated by caspase 8 and/or PPARα mediated inhibition of NFκB.

Conclusion

In conclusion, $Q_{10}H_2$-supplementation in humans modulates the expression of genes involved in inflammation at mRNA level but not at protein level, reduces LDL cholesterol and affects cell differentiation processes. These effects are hypothesized to be mediated by the antioxidant properties of $Q_{10}H_2$ through NFκB and PPARα dependent gene expression.

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Figure legends

Figure 1

Analysis of total CoQ_{10} levels and its redox state in plasma samples of study subjects before (T_0) and after supplementation with $Q_{10}H_2$ (T_{14}) as well as after 4-wk washout period (T_{42})

Data present effects on total CoQ_{10} levels (A), and its redox state (B) before (T_0) and after $Q_{10}H_2$ supplementation (T_{14}) as well as after 4-week washout period (T_{42}). Data are calculated from means (± SEM) of 53 measurements for T_0 and T_{14}, as well as 9 measurements for T_{42}, respectively.

Figure 2

Expression levels of $Q_{10}H_2$-sensitive genes involved in inflammatory and apoptotic processes in human monocytes

Technical as well as biological verification experiments (qRT-PCR) of monocyte samples revealed the expression of 7 $Q_{10}H_2$-sensitive genes related to inflammatory and apoptotic processes: TNFα (A), CXCL2 (B), CCL3 (C), NR4A2 (D), GIMAP7 (E), BRE (F) and PMAIP1 (G). Data are means (± SEM) of samples from 9 volunteers, respectively. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$

Figure 3

Bibliosphere network of genes regulated in monocytes of $Q_{10}H_2$ supplemented volunteers

Based on co-citations with transcription factors and other genes in the network (ST level), the identified $Q_{10}H_2$-sensitive genes were connected with each other by BiblioSpherePathwayEdition Software. According to this, these genes seem to play a role in PPAR-signalling and cell proliferation (TP53) pathways, finally connected by NFκB.

Figure 4

Correlation between individual mRNA and protein levels of TNFα and CXCL2 after $Q_{10}H_2$ supplementation

Relative mRNA levels for TNFα and CXCL2 were correlated to respective serum protein levels of study subjects (n = 9) after $Q_{10}H_2$ supplementation (T_{14}).
No correlation was found between these parameters.

Figure 5

Effects of $Q_{10}H_2$ supplementation on LDL subfractions in serum samples of study subjects

Based on NMR analysis, effects of $Q_{10}H_2$ supplementation on concentrations (nmol/l) of LDL subfractions (A-E) were analysed in serum samples of study subjects (n = 53). Significant effects have been found for the LDL subfractions B, C and E with relative reductions of 33.02, 14.62 and 16.52 %, respectively.

$^{**}p \leq 0.01$; $^{***}p \leq 0.001$

Tables

Table 1 Nucleotide sequences of primer pairs used for the real-time qRT-PCR experiments

Gene	Primer sequence *forward* (5´-3´)	Primer sequence *reverse* (5´-3´)
NR4A2	CCG CCA GCA ATA ATT GAC AA	TGC TTG GGA GGA GGT CTT AGA A
GIMAP7	GCT CCC TGA GGA TCG TTC TG	GGT GTT CGC TGT TGC ACT TTT
IL8	CAC CGG AAG GAA CCA TCT CA	AGA GCC ACG GCC AGC TT
KLF6	TCC TGT AAG AAG CGG CAT AGC	GAG TCC AGG GTC ACC CAC AT
PMAIP1	AGC TGG AAG TCG AGT GTG CTA CT	CTG CCG GAA GTT CAG TTT GTC
TAGAP	GAG TTA TGC TGT TTC TCC CAT TCT TTA	TGG AGG GTC TCT AGC CAG AGT T
BRE	GGA GGC AGC ATT TGC CAA T	GGC TGG CCA CCT CTC AAG A
CXCL2	GAT GCT GAA AAA TGG CAA ATC C	CAG GAA CAG CCA CCA ATA AGC
CCL3	CCG TCA CCT GCT CAG AAT CAT	GGT GCA GAG GAG GAC AGC AA
TNFα	GCA GGT CTA CTT TGG GAT CAT TG	GCG TTT GGG AAG GTT GGA
GAPDH	ATG GAA ATC CCA TCA TCT T	CGC CCC ACT TGA TTT TGG

Table 2 Characteristics of study subjects before (T_0) and after 2-wk supplementation period (T_{14}) with $Q_{10}H_2$ (n = 53)

Parameters	T_0	T_{14}
Age (years)	30.13 ± 6.71	30.13 ± 6.71
Weight (kg)	79.11 ± 10.17	79.18 ± 10.01
Height (m)	1.81 ± 0.06	1.81 ± 0.06
BMI (kg/m^2)	24.12 ± 2.50	24.14 ± 2.44
Glucose	86.47 ± 10.68	84.26 ± 10.10
Creatinine	1.05 ± 0.10	1.07 ± 0.12
GOT[1]	30.09 ± 8.67	33.06 ± 35.95
GPT[2]	37.79 ± 14.73	37.26 ± 16.52
γ-GT[3]	20.49 ± 10.36	17.79 ± 7.68***
Blood pressure (mm Hg)		
systolic	126.79 ± 12.00	126.11 ± 12.29
diastolic	82.74 ± 9.31	81.79 ± 8.46
ADMA (µmol/l)	0.98 ± 0.27	1.11 ± 0.50

Data are described as means $\pm$ SD
*** T_0 vs. T_{14}, $p \leq 0.001$
[1]Glutamate-Oxalacetate-Transferase (ASAT, Aspartat-Aminotransferase)
[2]Glutamat-Pyruvat-Transaminase (ALAT, Alanin-Aminotransferase)
[3]Gamma-Glutamyl-Transferase

Table 3 $Q_{10}H_2$ up-regulated genes (FC ≥ 1.5, p < 0.05)

Affymetrix Probe Set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
206765_at	KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2	36.10	21.24	102.99	19.49	2.85
228071_at	GIMAP7	GTPase, IMAP family member 7	1468.59	1013.58	3738.86	176.80	2.55
224917_at	MIRN21	microRNA 21	630.29	229.14	1540.31	285.74	2.44
229543_at	---	---	180.90	83.70	439.44	24.44	2.43
228362_s_at	RP1-93H18.5	Hypothetical protein LOC441168	101.95	63.32	241.44	17.00	2.37
235837_at	---	Transcribed locus, weakly similar to NP_775735.1 l(3)mbt-like 4 [Homo sapiens]	37.23	17.81	82.71	4.73	2.22
239629_at	CFLAR	CASP8 and FADD-like apoptosis regulator	210.89	77.04	448.26	82.51	2.13
206584_at	LY96	lymphocyte antigen 96	778.64	445.09	1633.35	38.54	2.10
1563357_at	---	MRNA; cDNA DKFZp564C203 (from clone DKFZp564C203)	123.81	57.72	251.93	50.51	2.03
214149_s_at	ATP6V0E	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e	178.41	69.51	357.57	77.63	2.00
236492_at	PPP2R2A	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform	106.20	36.21	210.57	49.63	1.98
229391_s_at	RP1-93H18.5	hypothetical protein LOC441168	933.38	506.28	1849.49	158.35	1.98
230466_s_at	RASSF3	Ras association (RalGDS/AF-6) domain family 3	592.99	311.33	1152.57	157.69	1.94
1569129_s_at	---	Homo sapiens, clone IMAGE:4695648, mRNA	129.13	56.43	248.60	28.00	1.93
225580_at	MRPL50	mitochondrial ribosomal protein L50	60.68	23.54	115.14	12.34	1.90
229970_at	---	---	11.38	1.20	21.53	2.73	1.89
231697_s_at	TMEM49	Transmembrane protein 49	98.53	36.39	186.01	14.77	1.89
230559_x_at	FGD4	FYVE, RhoGEF and PH domain containing 4	118.96	16.08	221.95	19.05	1.87
241617_x_at	---	---	33.26	5.03	61.93	10.23	1.86
227284_at	ZNF766	zinc finger protein 766	61.83	15.84	113.61	10.72	1.84

Affymetrix Probe Set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
232034_at	LOC203274	hypothetical protein LOC203274	99.20	38.23	180.30	21.02	1.82
218732_at	PTRH2	peptidyl-tRNA hydrolase 2	169.90	78.36	308.20	23.61	1.81
217144_at	UBB /// LOC648390	ubiquitin B /// similar to ubiquitin B precursor	38.91	9.12	69.98	4.76	1.80
236165_at	---	---	39.95	6.13	71.45	8.15	1.79
220992_s_at	C1orf25	chromosome 1 open reading frame 25 /// chromosome 1 open reading frame 25	67.36	8.50	119.34	14.04	1.77
204531_s_at	BRCA1	breast cancer 1, early onset	24.59	6.70	43.20	5.21	1.76
1560327_at	---	CDNA clone IMAGE:5273088	104.58	30.60	182.30	12.64	1.74
228964_at	PRDM1	PR domain containing 1, with ZNF domain	18.94	5.22	32.72	4.88	1.73
209007_s_at	C1orf63	chromosome 1 open reading frame 63	2693.11	1150.59	4625.48	258.16	1.72
219146_at	C17orf42	chromosome 17 open reading frame 42	61.16	18.97	104.66	0.72	1.71
240452_at	GSPT1	G1 to S phase transition 1	22.12	5.23	37.76	8.03	1.71
229934_at	---	Transcribed locus	263.06	62.99	448.45	66.40	1.70
229804_x_at	CBWD1 /// CBWD2 /// CBWD3 /// CBWD6	COBW domain containing 1 /// COBW domain containing 2 /// COBW domain containing 3 /// COBW domain containing 6	305.28	41.21	507.59	93.68	1.66
210528_at	MR1	major histocompatibility complex, class I-related	32.26	7.54	53.55	6.34	1.66
209850_s_at	CDC42EP2	CDC42 effector protein (Rho GTPase binding) 2	70.68	17.39	116.76	14.78	1.65
203739_at	ZNF217	zinc finger protein 217	764.24	233.92	1256.33	79.59	1.64
224321_at	TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2 /// transmembrane protein with EGF-like and two follistatin-like domains 2	203.39	37.27	334.07	38.49	1.64
231252_at	FLJ23861	hypothetical protein FLJ23861	14.27	2.31	23.41	4.39	1.64

Affymetrix Probe Set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
235306_at	GIMAP8	GTPase, IMAP family member 8	1826.70	455.55	2986.27	363.18	1.63
222701_s_at	CHCHD7	coiled-coil-helix-coiled-coil-helix domain containing 7	297.13	95.72	485.68	35.06	1.63
227636_at	THAP5	THAP domain containing 5	151.52	49.58	245.00	25.51	1.62
1561179_s_at	AMZ1	archaemetzincin-1	23.00	5.02	36.52	5.36	1.59
226077_at	FLJ31951	hypothetical protein FLJ31951	569.70	180.89	903.10	18.93	1.59
221634_at	RPL23AP7	ribosomal protein L23a pseudogene 7	42.56	6.66	67.33	11.51	1.58
230259_at	C10orf125	chromosome 10 open reading frame 125	108.71	26.40	171.74	28.56	1.58
206141_at	MOCS3	molybdenum cofactor synthesis 3	19.63	5.72	30.92	1.92	1.57
213233_s_at	KLHL9	kelch-like 9 (Drosophila)	116.55	15.77	182.48	20.04	1.57
223477_s_at	FLJ38663	hypothetical protein FLJ38663	22.22	7.29	34.67	1.24	1.56
228714_at	---	CDNA FLJ46701 fis, clone TRACH3014063	14.30	0.77	22.10	1.55	1.55
1564820_at	RAB6A	RAB6A, member RAS oncogene family	21.79	4.07	33.53	2.94	1.54
1564207_at	FLJ35390	hypothetical protein FLJ35390	138.82	24.87	213.15	21.19	1.54
213891_s_at	---	CDNA FLJ37747 fis, clone BRHIP2022986	172.43	28.72	264.00	36.52	1.53
236703_at	---	Transcribed locus, strongly similar to NP_036361.1 5'-nucleotidase, cytosolic II; purine 5' nucleotidase; 5'-nucleotidase (purine), cytosolic type B; IMP-specific 5'-NT [Homo sapiens]	107.42	11.89	164.45	22.30	1.53
230852_at	STAC3	SH3 and cysteine rich domain 3	54.10	6.09	82.79	8.40	1.53
1554948_at	---	---	122.16	11.34	186.59	23.81	1.53
224346_at	RNPS1	RNA binding protein S1, serine-rich domain /// RNA binding protein S1, serine-rich domain	73.66	16.02	111.67	6.00	1.52

Table 4 $Q_{10}H_2$ down-regulated genes (FC ≤ -1.5, p < 0.05)

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
211506_s_at	IL8	interleukin 8	1525.09	1372.25	55.87	12.92	-27.30
202859_x_at	IL8	interleukin 8	5053.70	4423.82	328.78	176.29	-15.37
209774_x_at	CXCL2	chemokine (C-X-C motif) ligand 2	1149.75	865.42	93.01	29.81	-12.36
205114_s_at	CCL3 /// CCL3L1 /// CCL3L3 /// LOC643930	chemokine (C-C motif) ligand 3 /// chemokine (C-C motif) ligand 3-like 1 /// chemokine (C-C motif) ligand 3-like 3	4591.46	2758.68	658.29	390.95	-6.97
204621_s_at	NR4A2	nuclear receptor subfamily 4, group A, member 2	664.26	412.31	100.46	37.53	-6.61
1568768_s_at	BRE	brain and reproductive organ-expressed (TNFRSF1A modulator)	94.61	8.87	14.32	5.31	-6.61
1569203_at	CXCL2	chemokine (C-X-C motif) ligand 2	95.67	70.08	18.62	5.84	-5.14
204622_x_at	NR4A2	nuclear receptor subfamily 4, group A, member 2	225.64	124.59	47.81	23.25	-4.72
207113_s_at	TNF	tumor necrosis factor (TNF superfamily, member 2)	158.26	92.90	36.66	11.52	-4.32
216248_s_at	NR4A2	nuclear receptor subfamily 4, group A, member 2	339.55	175.82	81.18	31.04	-4.18
239845_at	---	Transcribed locus	204.27	36.38	51.10	5.82	-4.00
215322_at	LONRF1	LON peptidase N-terminal domain and ring finger 1	228.80	85.12	67.15	15.69	-3.41
AFFX-r2-Bs-phe-5_at	---	---	519.41	182.15	156.45	25.67	-3.32
AFFX-PheX-5_at	---	---	205.75	56.79	62.29	6.86	-3.30
234050_at	TAGAP	T-cell activation GTPase activating protein	589.38	87.94	180.95	76.67	-3.26
AFFX-r2-Bs-thr-3_s_at	---	---	846.31	326.33	267.05	13.67	-3.17
AFFX-ThrX-3_at	---	---	643.53	255.90	204.79	34.05	-3.14

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
239405_at	RAB7	RAB7, member RAS oncogene family	34.02	5.70	10.95	1.58	-3.11
217591_at	SKIL	SKI-like	406.23	127.63	131.90	3.45	-3.08
204470_at	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	85.90	24.87	28.07	8.40	-3.06
AFFX-PheX-M_at	---	---	281.60	50.70	98.82	19.88	-2.85
202014_at	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	756.92	300.49	268.40	46.19	-2.82
1566901_at	TGIF	TGFB-induced factor (TALE family homeobox)	56.39	3.69	20.25	4.07	-2.78
242587_at	SLC9A9	solute carrier family 9 (sodium/hydrogen exchanger), member 9	31.40	11.26	11.66	1.13	-2.69
231904_at	U2AF1	U2 small nuclear RNA auxiliary factor 1	550.70	188.82	211.10	13.47	-2.61
1556072_at	FLJ40542	hypothetical protein FLJ40542	249.57	43.03	95.92	16.41	-2.60
AFFX-r2-Bs-lys-5_at	---	---	359.81	9.17	138.98	30.87	-2.59
1556602_at	SLC19A2	Solute carrier family 19 (thiamine transporter), member 2	40.29	3.21	15.57	3.80	-2.59
AFFX-r2-Bs-phe-M_at	---	---	596.38	129.10	233.22	70.92	-2.56
AFFX-LysX-M_at	---	---	203.52	10.10	79.88	18.84	-2.55
1552542_s_at	TAGAP	T-cell activation GTPase activating protein	300.79	52.43	122.45	55.35	-2.46
240094_at	TXNDC13	Thioredoxin domain containing 13	427.84	138.66	177.58	51.24	-2.41
221765_at	---	---	43.71	7.44	18.50	1.77	-2.36
AFFX-LysX-5_at	---	---	244.06	2.50	105.50	29.04	-2.31
AFFX-r2-Bs-phe-3_at	---	---	486.84	44.95	213.37	35.26	-2.28
1557459_at	SNF1LK2	SNF1-like kinase 2	64.20	16.64	28.32	12.16	-2.27
239494_at	LOC646725	hypothetical protein LOC646725 /// hypothetical protein	102.67	30.34	45.30	10.86	-2.27

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
	/// LOC649431	LOC649431					
242210_at	ZNF24	Zinc finger protein 24	17.48	3.75	7.78	0.22	-2.25
232392_at	SFRS3	Splicing factor, arginine/serine-rich 3	60.67	13.34	27.22	7.54	-2.23
204285_s_at	PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	1270.73	35.11	577.55	106.44	-2.20
AFFX-PheX-3_at	---	---	308.89	33.47	140.89	20.77	-2.19
224854_s_at	KIAA1458	KIAA1458	31.03	4.26	14.20	3.23	-2.18
AFFX-r2-Bs-lys-3_at	---	---	393.18	21.67	182.30	42.96	-2.16
215415_s_at	LYST	lysosomal trafficking regulator	44.71	10.62	21.49	5.26	-2.08
217649_at	---	Transcribed locus, strongly similar to XP_371170.1 PREDICTED: similar to Zinc finger protein 216 [Homo sapiens]	200.13	12.39	97.50	14.50	-2.05
AFFX-r2-Bs-lys-M_at	---	---	399.84	50.81	195.60	49.93	-2.04
232141_at	U2AF1	U2 small nuclear RNA auxiliary factor 1	518.65	64.09	254.12	43.49	-2.04
210204_s_at	CNOT4	CCR4-NOT transcription complex, subunit 4	17.78	4.89	8.75	1.22	-2.03
210976_s_at	PFKM	phosphofructokinase, muscle	81.96	15.32	40.35	5.54	-2.03
235716_at	---	Transcribed locus	332.97	65.32	166.37	17.67	-2.00
204293_at	SGSH	N-sulfoglucosamine sulfohydrolase (sulfamidase)	194.17	53.70	97.58	21.56	-1.99
216985_s_at	STX3	syntaxin 3	51.45	13.02	25.95	3.97	-1.98
219349_s_at	EXOC2	exocyst complex component 2	101.68	23.11	51.42	9.67	-1.98
1555106_a_at	CTDSPL2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase like 2	26.86	7.11	13.73	1.94	-1.96
228955_at	---	Transcribed locus, weakly similar to NP_990560.1 very low density lipoprotein (VLDL)/vitellogenin receptor [Gallus	40.02	9.18	20.53	7.21	-1.95

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
		gallus]					
233303_at	UBE2D3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	273.45	19.66	140.82	65.67	-1.94
1560274_at	WTAP /// LOC653150	Wilms tumor 1 associated protein /// similar to Wilms tumor 1 associated protein	52.62	10.38	27.12	3.12	-1.94
231989_s_at	LOC23117 /// LOC440345	KIAA0220-like protein /// hypothetical protein LOC440345	292.42	76.06	150.81	37.69	-1.94
205997_at	ADAM28	ADAM metallopeptidase domain 28	126.85	34.75	65.78	9.20	-1.93
209383_at	DDIT3	DNA-damage-inducible transcript 3	174.47	44.71	90.56	24.06	-1.93
1560058_at	LOC399900	hypothetical gene supported by AK093779	339.34	20.10	176.52	41.87	-1.92
204286_s_at	PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	253.10	50.92	132.15	15.69	-1.92
201502_s_at	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	7748.34	1315.85	4050.19	295.88	-1.91
AFFX-LysX-3_at	---	---	539.89	71.81	282.41	34.38	-1.91
201531_at	ZFP36	zinc finger protein 36, C3H type, homolog (mouse)	9825.24	2601.42	5168.46	1084.77	-1.90
224572_s_at	IRF2BP2	interferon regulatory factor 2 binding protein 2	647.26	90.70	342.18	87.24	-1.89
230733_at	MRCL3	Myosin regulatory light chain MRCL3	66.81	16.82	35.60	9.51	-1.88
236213_at	HNRPA3	Heterogeneous nuclear ribonucleoprotein A3	27.55	0.90	14.74	6.13	-1.87
235369_at	C14orf28 /// SYPL2	chromosome 14 open reading frame 28 /// synaptophysin-like 2	30.51	5.59	16.38	3.36	-1.86
239045_at	ERN1	Endoplasmic reticulum to nucleus signalling 1	71.30	11.69	38.40	6.37	-1.86
235088_at	LOC201725	hypothetical protein LOC201725	41.74	7.39	22.48	0.88	-1.86
1560486_at	STXBP3	syntaxin binding protein 3	45.12	7.16	24.35	10.62	-1.85
1556322_a_at	TJAP1	Tight junction associated protein 1 (peripheral)	29.26	6.80	15.85	2.37	-1.85

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
231165_at	DDHD1	DDHD domain containing 1	173.88	36.18	94.29	14.80	-1.84
219717_at	C4orf30	chromosome 4 open reading frame 30	60.13	10.29	32.62	11.44	-1.84
1554522_at	CNNM2	cyclin M2	93.82	24.03	51.11	5.64	-1.84
236752_at	PKP4	Plakophilin 4	19.99	0.56	10.91	1.96	-1.83
1558783_at	WTAP	Wilms tumor 1 associated protein	284.92	63.12	155.62	20.75	-1.83
212225_at	EIF1	eukaryotic translation initiation factor 1	1560.64	332.57	856.65	193.25	-1.82
225484_at	TSGA14	testis specific, 14	39.22	1.70	21.59	3.56	-1.82
201918_at	SLC25A36	Solute carrier family 25, member 36	271.19	52.37	149.28	49.07	-1.82
232463_at	CXYorf10	chromosome X and Y open reading frame 10	12.63	1.71	6.96	0.47	-1.81
229676_at	PAPD1	PAP associated domain containing 1	129.44	30.66	71.39	10.71	-1.81
212655_at	ZCCHC14	zinc finger, CCHC domain containing 14	86.83	22.21	47.91	7.45	-1.81
201466_s_at	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	965.50	111.03	533.41	151.13	-1.81
215850_s_at	NDUFA5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5, 13kDa	58.99	8.88	32.60	9.02	-1.81
205045_at	AKAP10	A kinase (PRKA) anchor protein 10	54.08	13.55	29.92	2.68	-1.81
211272_s_at	DGKA	diacylglycerol kinase, alpha 80kDa	122.76	31.89	68.17	5.75	-1.80
226663_at	ANKRD10	ankyrin repeat domain 10	395.88	29.23	219.94	31.42	-1.80
218401_s_at	ZNF281	zinc finger protein 281	640.56	115.35	357.28	23.43	-1.79
221986_s_at	KLHL24	kelch-like 24 (Drosophila)	199.67	31.82	111.78	17.09	-1.79
212434_at	GRPEL1	GrpE-like 1, mitochondrial (E. coli)	383.96	93.78	215.12	18.12	-1.78
227501_at	WSB1	WD repeat and SOCS box-containing 1	193.85	50.27	109.25	12.25	-1.77
215300_s_at	FMO5	flavin containing monooxygenase 5	52.37	4.76	29.70	0.81	-1.76
202972_s_at	FAM13A1	family with sequence similarity 13, member A1	66.24	13.43	37.62	1.80	-1.76

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
201727_s_at	ELAVL1	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)	254.36	59.47	144.65	16.08	-1.76
243134_at	LOC440309	Hypothetical LOC440309	511.53	28.94	290.98	10.40	-1.76
244778_x_at	GPIAP1	GPI-anchored membrane protein 1	138.32	15.01	78.69	21.77	-1.76
222669_s_at	SBDS	Shwachman-Bodian-Diamond syndrome	436.75	109.04	248.67	26.22	-1.76
219961_s_at	C20orf19	chromosome 20 open reading frame 19	57.71	4.93	32.90	4.64	-1.75
227290_at	---	CDNA FLJ13598 fis, clone PLACE1009921	161.52	29.01	92.42	28.72	-1.75
202723_s_at	FOXO1A	forkhead box O1A (rhabdomyosarcoma)	170.03	34.37	97.68	11.02	-1.74
225408_at	MBP	myelin basic protein	126.71	33.02	72.83	3.98	-1.74
227762_at	---	Transcribed locus	63.03	16.30	36.35	2.43	-1.73
226735_at	FLJ90013	hypothetical protein FLJ90013	96.61	20.84	55.91	10.34	-1.73
243857_at	MORF4L2	Mortality factor 4 like 2	99.99	9.79	57.90	7.80	-1.73
203414_at	MMD	monocyte to macrophage differentiation-associated	146.68	30.03	85.02	19.30	-1.73
214805_at	EIF4A1	Eukaryotic translation initiation factor 4A, isoform 1	1041.38	164.23	604.32	41.72	-1.72
206877_at	MXD1	MAX dimerization protein 1	93.68	17.53	54.37	13.46	-1.72
226644_at	MIB2	mindbomb homolog 2 (Drosophila)	99.74	4.25	57.94	4.61	-1.72
219634_at	CHST11	carbohydrate (chondroitin 4) sulfotransferase 11	479.71	88.50	279.64	25.39	-1.72
1555334_s_at	SLC30A5	solute carrier family 30 (zinc transporter), member 5	47.87	7.61	28.00	1.96	-1.71
218374_s_at	C12orf4	chromosome 12 open reading frame 4	61.63	14.74	36.12	2.96	-1.71
219599_at	PRO1843	hypothetical protein PRO1843	210.26	27.93	124.02	18.83	-1.70
206059_at	ZNF91	zinc finger protein 91	283.17	52.13	167.71	6.43	-1.69
223430_at	SNF1LK2	SNF1-like kinase 2	118.15	13.48	70.05	10.79	-1.69
227620_at	---	---	111.10	18.45	66.04	16.28	-1.68

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
238156_at	RPS6	Ribosomal protein S6	453.67	70.80	269.78	8.25	-1.68
202773_s_at	SFRS8	splicing factor, arginine/serine-rich 8 (suppressor-of-white-apricot homolog, Drosophila)	100.34	20.94	59.75	6.26	-1.68
212912_at	RPS6KA2	ribosomal protein S6 kinase, 90kDa, polypeptide 2	9.19	0.94	5.48	0.60	-1.68
209287_s_at	CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3	437.88	95.03	261.12	53.24	-1.68
214318_s_at	FRY	furry homolog (Drosophila)	42.76	9.07	25.54	5.10	-1.67
201464_x_at	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	2478.03	219.98	1482.79	264.52	-1.67
1554015_a_at	CHD2	chromodomain helicase DNA binding protein 2	62.09	7.08	37.21	5.46	-1.67
236836_at	TBCA	Tubulin-specific chaperone a	158.44	13.33	95.18	23.82	-1.66
225963_at	KLHDC5	kelch domain containing 5	78.15	8.06	46.99	4.38	-1.66
210512_s_at	VEGF	vascular endothelial growth factor	396.72	69.92	238.80	9.45	-1.66
209053_s_at	WHSC1	Wolf-Hirschhorn syndrome candidate 1	49.68	9.19	29.97	4.91	-1.66
201580_s_at	TXNDC13	thioredoxin domain containing 13	298.28	68.07	179.97	0.95	-1.66
1557263_s_at	---	CDNA FLJ35536 fis, clone SPLEN2002451	236.85	50.22	143.12	25.77	-1.65
209349_at	RAD50	RAD50 homolog (S. cerevisiae)	59.71	14.05	36.19	3.93	-1.65
218051_s_at	NT5DC2	5'-nucleotidase domain containing 2	21.30	3.95	12.92	1.19	-1.65
217246_s_at	DIAPH2	diaphanous homolog 2 (Drosophila)	24.47	4.56	14.89	1.26	-1.64
214960_at	API5	apoptosis inhibitor 5	91.81	16.89	55.91	5.59	-1.64
202726_at	LIG1	ligase I, DNA, ATP-dependent	14.38	0.46	8.76	2.25	-1.64
230270_at	PRPF38B	PRP38 pre-mRNA processing factor 38 (yeast) domain containing B	502.76	64.95	306.53	77.77	-1.64
1555905_a_at	C3orf23	chromosome 3 open reading frame 23	66.50	7.10	40.58	4.04	-1.64
1556007_s_at	CSNK1A1	Casein kinase 1, alpha 1	240.85	31.93	146.99	13.76	-1.64

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	**± SD**	**Mean**	**± SD**	
228702_at	FLJ43663 /// LOC641825 /// LOC647017	hypothetical protein FLJ43663 /// hypothetical protein LOC641825 /// hypothetical protein LOC647017	76.10	12.06	46.46	8.11	-1.64
205704_s_at	ATP6V0A2	ATPase, H+ transporting, lysosomal V0 subunit a2	32.87	5.97	20.08	0.23	-1.64
226679_at	SLC26A11	solute carrier family 26, member 11	307.71	6.39	188.49	50.78	-1.63
212677_s_at	CEP68	centrosomal protein 68kDa	69.71	10.76	42.74	9.80	-1.63
235745_at	ERN1	endoplasmic reticulum to nucleus signalling 1	84.00	10.72	51.54	3.95	-1.63
239963_at	---	Transcribed locus	36.93	6.37	22.67	5.91	-1.63
231357_at	CLEC12B	C-type lectin domain family 12 member B	74.77	10.88	46.18	13.92	-1.62
213459_at	RPL37A	ribosomal protein L37a	109.69	14.35	67.78	4.13	-1.62
212960_at	TBC1D9	TBC1 domain family, member 9 (with GRAM domain)	173.99	27.30	107.59	19.40	-1.62
232280_at	SLC25A29	Solute carrier family 25, member 29	29.16	4.72	18.05	0.42	-1.62
214931_s_at	SRPK2	SFRS protein kinase 2	84.91	17.46	52.59	6.38	-1.61
227113_at	ADHFE1	alcohol dehydrogenase, iron containing, 1	98.89	14.62	61.40	6.40	-1.61
204233_s_at	CHKA	choline kinase alpha	79.48	14.32	49.38	2.08	-1.61
239734_at	LOC401320	Hypothetical LOC401320	57.51	2.39	35.82	8.59	-1.61
211578_s_at	RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	82.34	9.04	51.30	3.68	-1.61
209336_at	PWP2H	PWP2 periodic tryptophan protein homolog (yeast)	115.66	18.47	72.06	13.92	-1.61
203803_at	PCYOX1	prenylcysteine oxidase 1	48.92	4.38	30.50	8.39	-1.60
218918_at	MAN1C1	mannosidase, alpha, class 1C, member 1	121.81	11.07	76.06	12.00	-1.60
33494_at	ETFDH	electron-transferring-flavoprotein dehydrogenase	111.81	14.68	70.11	6.44	-1.59
1564274_at	C9orf47	chromosome 9 open reading frame 47	16.94	3.50	10.65	0.83	-1.59

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
225896_at	M-RIP	Myosin phosphatase-Rho interacting protein	49.05	5.98	30.89	6.65	-1.59
220408_x_at	FAM48A	family with sequence similarity 48, member A	232.33	38.93	146.40	13.54	-1.59
223171_at	DYM	dymeclin	259.99	58.73	163.90	4.07	-1.59
209876_at	GIT2	G protein-coupled receptor kinase interactor 2	307.62	53.10	194.13	36.21	-1.58
209294_x_at	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	48.56	2.71	30.71	4.57	-1.58
220702_at	---	---	62.30	12.63	39.51	2.07	-1.58
231630_at	FLJ16341	Hypothetical gene supported by AK122786	13.35	2.47	8.47	0.58	-1.58
230099_at	---	Transcribed locus	381.13	80.53	241.98	9.74	-1.58
219437_s_at	ANKRD11	ankyrin repeat domain 11	284.34	25.71	180.54	28.45	-1.57
226533_at	---	---	72.93	2.31	46.37	10.76	-1.57
213211_s_at	TAF6L	TAF6-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor, 65kDa	29.60	6.25	18.82	2.33	-1.57
211938_at	EIF4B	eukaryotic translation initiation factor 4B	2103.11	275.84	1339.42	362.38	-1.57
201810_s_at	SH3BP5	SH3-domain binding protein 5 (BTK-associated)	367.07	59.30	233.88	32.97	-1.57
241425_at	NUPL1	Nucleoporin like 1	160.72	17.53	102.50	20.63	-1.57
35626_at	SGSH	N-sulfoglucosamine sulfohydrolase (sulfamidase)	776.86	127.80	495.97	93.33	-1.57
222147_s_at	ACTR5	ARP5 actin-related protein 5 homolog (yeast)	44.11	7.11	28.17	5.87	-1.57
226688_at	C3orf23	chromosome 3 open reading frame 23	51.98	3.47	33.26	3.48	-1.56
222427_s_at	LARS	leucyl-tRNA synthetase	550.35	115.62	352.48	19.25	-1.56
229246_at	FLJ44342	hypothetical protein LOC645460	85.85	16.30	55.00	4.91	-1.56
230050_at	BTBD14A	BTB (POZ) domain containing 14A	78.08	1.34	50.12	12.73	-1.56
212227_x_at	EIF1	eukaryotic translation initiation factor 1	11007.74	1811.55	7077.43	1189.29	-1.56
233563_s_at	CPSF3L	cleavage and polyadenylation specific factor 3-like	234.47	22.74	150.76	19.07	-1.56

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
238736_at	REV3L	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	589.15	108.94	379.71	51.07	-1.55
203198_at	CDK9	cyclin-dependent kinase 9 (CDC2-related kinase)	227.36	29.32	146.54	10.90	-1.55
202973_x_at	FAM13A1	family with sequence similarity 13, member A1	845.30	133.28	545.05	120.03	-1.55
202469_s_at	CPSF6	cleavage and polyadenylation specific factor 6, 68kDa	632.31	112.45	407.73	38.69	-1.55
242255_at	WDR37	WD repeat domain 37	52.68	8.62	34.02	3.07	-1.55
201433_s_at	PTDSS1	phosphatidylserine synthase 1	1358.81	213.82	877.91	135.36	-1.55
217100_s_at	UBXD7	UBX domain containing 7	140.86	11.65	91.05	16.88	-1.55
227056_at	KIAA0141	KIAA0141	337.61	53.05	218.36	19.58	-1.55
227946_at	OSBPL7	oxysterol binding protein-like 7	74.51	12.62	48.19	1.14	-1.55
238987_at	B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	446.95	31.51	289.48	65.69	-1.54
218177_at	CHMP1B	chromatin modifying protein 1B	317.34	62.80	205.60	24.68	-1.54
211769_x_at	SERINC3	serine incorporator 3 /// serine incorporator 3	194.84	37.57	126.34	9.21	-1.54
202021_x_at	EIF1	eukaryotic translation initiation factor 1	11117.73	1379.83	7216.36	999.49	-1.54
203245_s_at	FLJ35348	FLJ35348	32.51	4.24	21.12	3.77	-1.54
222824_at	SEC61A2	Sec61 alpha 2 subunit (S. cerevisiae)	121.71	14.20	79.09	12.20	-1.54
238455_at	---	CDNA FLJ45742 fis, clone KIDNE2016327	248.53	22.51	161.95	47.80	-1.53
223129_x_at	MYLIP	myosin regulatory light chain interacting protein	231.14	17.84	150.63	41.90	-1.53
209191_at	TUBB6	tubulin, beta 6	238.01	14.13	155.25	36.17	-1.53
200779_at	ATF4	activating transcription factor 4 (tax-responsive enhancer element B67)	2806.53	281.64	1830.90	174.43	-1.53
233647_s_at	CDADC1	cytidine and dCMP deaminase domain containing 1	99.43	14.92	64.96	7.77	-1.53
212130_x_at	EIF1	eukaryotic translation initiation factor 1	10499.00	1324.06	6870.47	1152.71	-1.53

Affymetrix Probe set ID	Gene symbol	Gene name	Mean fluorescence data ± SD (n = 3)				FC
			T_0		T_{14}		
			Mean	± SD	Mean	± SD	
1554155_at	MCPH1	microcephaly, primary autosomal recessive 1	41.97	7.11	27.51	2.29	-1.53
225588_s_at	TMEM129	transmembrane protein 129	154.93	11.70	101.55	26.50	-1.53
222303_at	---	---	684.77	31.29	448.95	65.91	-1.53
231431_s_at	LOC388114 /// LOC649557	hypothetical LOC388114 /// hypothetical protein LOC649557	272.64	47.72	178.89	19.53	-1.52
214482_at	ZBTB25	zinc finger and BTB domain containing 25	73.63	4.74	48.33	4.76	-1.52
228174_at	GOLGA1	Golgi autoantigen, golgin subfamily a, 1	35.72	4.75	23.50	5.80	-1.52
212508_at	MOAP1	modulator of apoptosis 1	1096.57	175.42	723.02	85.48	-1.52
226030_at	ACADSB	acyl-Coenzyme A dehydrogenase, short/branched chain	86.02	11.61	56.75	7.99	-1.52
240106_at	GNPTAB	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	41.49	6.74	27.40	4.31	-1.51
225993_at	EARS2	glutamyl-tRNA synthetase 2 (mitochondrial)(putative)	16.29	2.73	10.76	0.93	-1.51
235463_s_at	LASS6	LAG1 longevity assurance homolog 6 (S. cerevisiae)	84.62	4.48	55.94	14.01	-1.51
204313_s_at	CREB1	cAMP responsive element binding protein 1	932.93	94.18	618.42	71.64	-1.51
223266_at	ALS2CR2	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 2	144.29	15.46	95.66	3.03	-1.51
201473_at	JUNB	jun B proto-oncogene	4874.58	515.01	3234.97	781.83	-1.51
1567080_s_at	CLN6	ceroid-lipofuscinosis, neuronal 6, late infantile, variant	90.44	8.33	60.05	2.42	-1.51

Table 5 Mean fluorescence data and fold change values of target genes from microarray and qRT-PCR experiments in monocytes of $Q_{10}H_2$-supplemented subjects

Gene	Gene name	Mean fluorescence data ± SD (n = 3)		FC Microarray T_0 vs. T_{14}	qRT-PCR (verifications)	
		T_0	T_{14}		Technical (n = 3)	Biological (n = 9)
Inflammation/ Immune response						
TNFα	tumor necrosis factor alpha	158.26 ± 92.90	36.66 ± 11.52	-4.3*	-4.0 ± 0.2**	-2.6 ± 0.1**
CXCL2	chemokine (C-X-C motif) ligand 2	1149.75 ± 865.42	93.01 ± 29.81	-8.8*#	-20.9 ± 0.2**	-3.6 ± 0.1***
IL8	interleukin 8	1525.09 ± 1372.25	55.87 ± 12.92	-21.3*#	-6.3 ± 0.7*	-1.3 ± 0.5
CCL3	chemokine (C-C motif) ligand 3	4591.46 ± 2758.68	658.29 ± 390.95	-7.0*	-2.5 ± 0.2**	-1.7 ± 0.1*
GIMAP7	GTPase, IMAP family member 7	1468.59 ± 1013.58	3738.86 ± 176.80	2.6*	1.8 ± 0.1*	1.4 ± 0.1*
Apoptosis						
NR4A2 (NURR1)	nuclear receptor subfamily 4, group A, member 2	664.26 ± 412.31	100.46 ± 37.53	-5.2*#	-6.4 ± 1.1*	-2.7 ± 0.5*
BRE	brain and reproductive organ-expressed	94.61 ± 8.87	14.32 ± 5.31	-6.6***	-1.8 ± 0.1*	-1.5 ± 0.1***
miR21	microRNA 21	630.29 ± 229.14	1540.31 ± 285.74	2.4*	1.6 ± 0.1***	1.1 ± 0.1
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	1270.73 ± 35.11	577.55 ± 106.44	-2.2*	-1.5 ± 0.1*	-1.9 ± 0.2**

Mean of $\geq$ 2 probe set IDs, $p \leq 0.05$; * T_0 vs. T_{14}, $p \leq 0.05$; ** T_0 vs. T_{14}, $p \leq 0.01$; *** T_0 vs. T_{14}, $p \leq 0.001$

Table 6 Release of pro-inflammatory markers in serum samples of study subjects before (T_0) and after (T_{14}) $Q_{10}H_2$ supplementation

Parameters	T_0	T_{14}
TNFα (pg/ml)	139.37 ± 122.24	144.60 ± 140.00
CXCL2 (pg/ml)	251.97 ± 68.78	252.02 ± 69.47
MCP-1 (ng/ml)	184.88 ± 108.55	183.23 ± 107.59
CRP (mg/dl)	0.30 ± 0.01	0.31 ± 0.05

Data are described as means ± SD

Table 7 Release of TNFα and MCP-1 in *ex vivo* LPS stimulated whole blood samples of study subjects before (T_0) and after (T_{14}) $Q_{10}H_2$ supplementation

	T_0			T_{14}		
	-LPS	**+LPS**	**+LPS/ -LPS**	**-LPS**	**+LPS**	**+LPS/ -LPS**
TNFα (pg/ml)	79.17 ± 30.53	2091.28 ± 648.81	30.43 ± 19.13	86.65 ± 31.65	2571.12 ± 908.45	31.86 ± 12.44
MCP-1 (ng/ml)	90.84 ± 41.36	159.66 ± 57.49	1.92 ± 0.72	94.62 ± 41.39	160.66 ± 59.64	1.88 ± 0.86

Data are described as means ± SD

Table 8 Serum levels of lipid parameters before (T_0) and after (T_{14}) $Q_{10}H_2$ supplementation

Parameters	T_0	T_{14}
Cholesterol (mg/dl)	166.09 ± 29.79	163.85 ± 27.85
HDL cholesterol (mg/dl)	51.13 ± 12.93	51.3 ± 11.9
LDL cholesterol (mg/dl)	95.51 ± 28.89	90.60 ± 27.21*
oxLDL (U/ml)	20.62 ± 14.67	20.43 ± 14.46
Triglycerides (mg/dl)	97.40 ± 49.04	112.32 ± 73.13

* T_0 vs. T_{14}, $p \leq 0.05$; Data are described as means ± SD

Table 9 Blood parameters of study subjects before (T_0) and after 2-week supplementation period (T_{14}) with $Q_{10}H_2$ (n = 53)

	T_0	T_{14}
Leukocytes	5.65 ± 1.43	5.74 ± 1.28
Erythrocytes	4.95 ± 0.29	4.89 ± 0.31*
Hemoglobin	15.16 ± 0.82	15.04 ± 0.85
Hematocrit	44.48 ± 2.36	43.78 ± 2.40**
MCV[1]	89.82 ± 2.87	89.76 ± 2.78
MCH[2]	30.62 ± 1.12	30.82 ± 1.17*
MCHC[3]	34.09 ± 0.53	34.33 ± 0.53*
Reticulocytes	10.94 ± 3.03	12.09 ± 2.46***

Data are described as means ± SD

* T_0 vs. T_{14}, $p \leq 0.05$

** T_0 vs. T_{14}, $p \leq 0.01$

*** T_0 vs. T_{14}, $p \leq 0.001$

[1]Mean corpuscular volume

[2]Mean corpuscular haemoglobin

[3]Mean corpuscular haemoglobin concentration

Figures

Figure 1

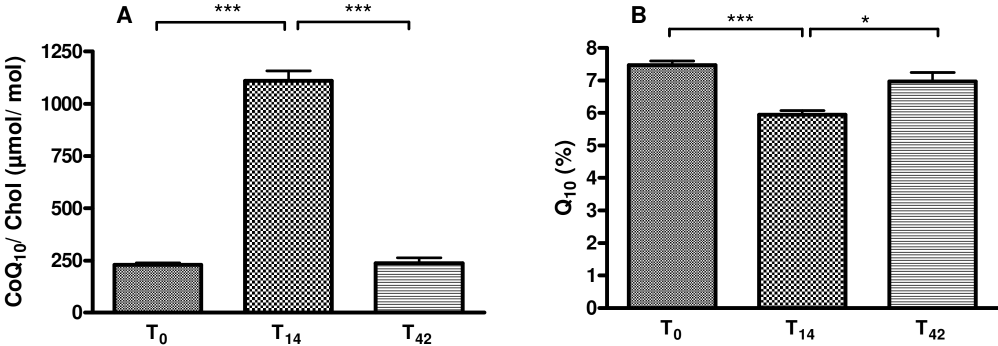


Figure 2

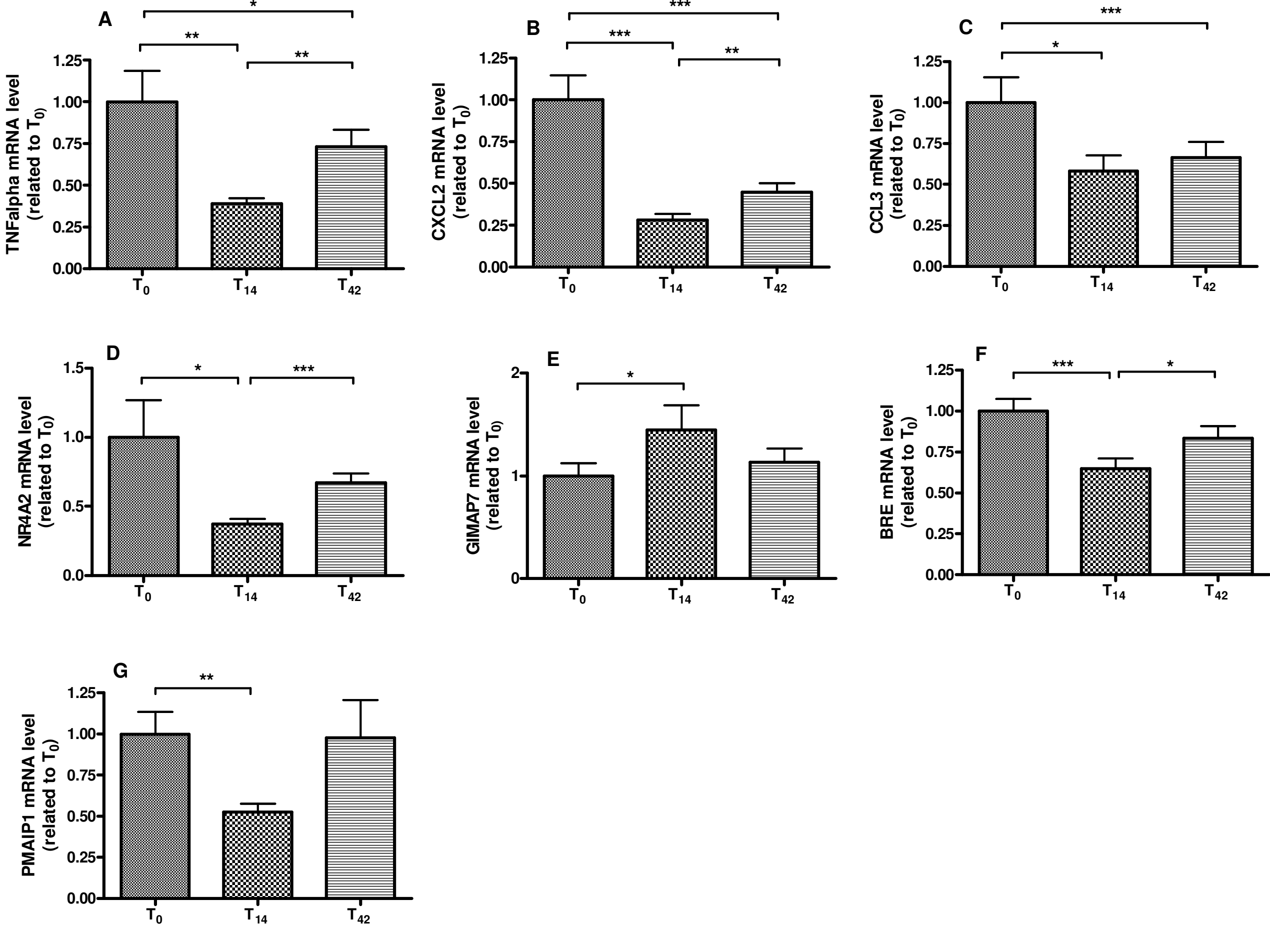


Figure 3

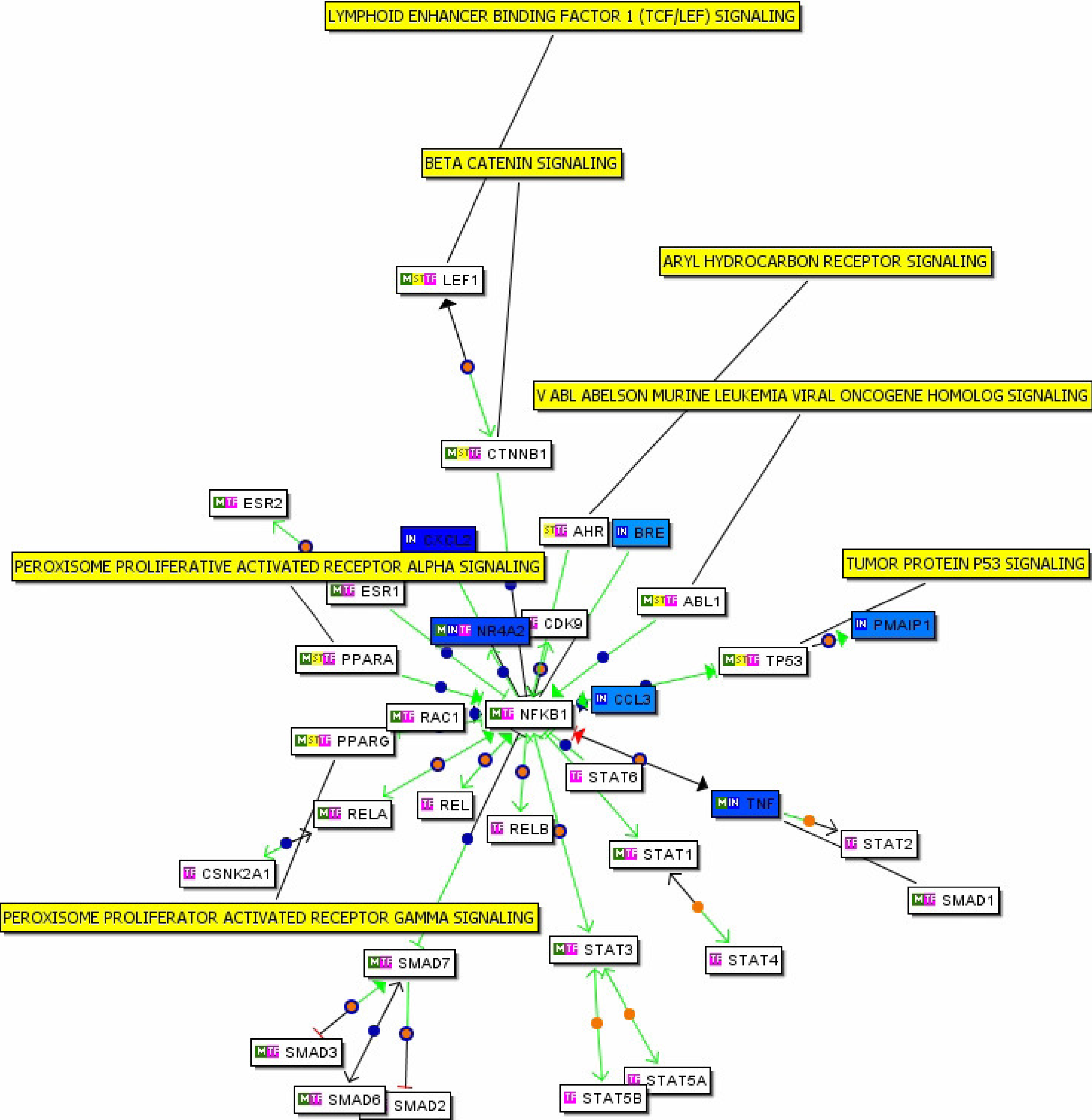
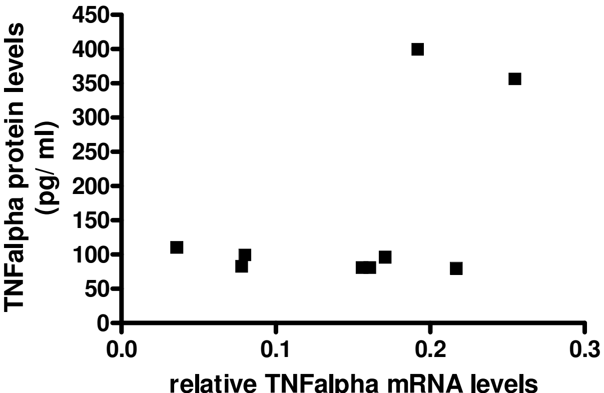


Figure 4



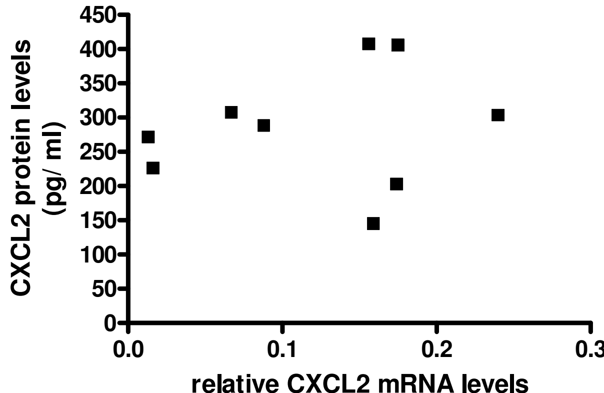
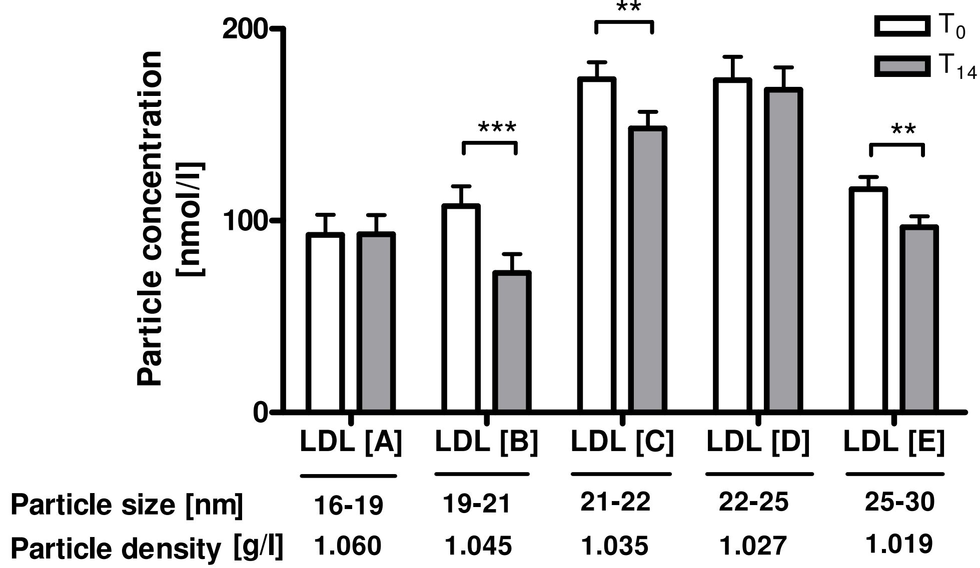


Figure 5



Danksagung

Ein großer Dank gilt Prof. Dr. Frank Döring, der mir trotz meiner anfänglichen minimalistischen Laborkenntnisse die Bearbeitung dieses interessanten Themas überließ. Mit dieser Chance bestärkte er mich auch in meiner grundsätzlichen Auffassung, dass nicht allein das gegenwärtige Wissen die Fähigkeiten eines Menschen ausmachen, sondern die Einstellung sich notwendiges Wissen aneignen zu können und zu wollen.

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Lebenslauf

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Staatsangehörigkeit: deutsch

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04/2004	Bachelor of Science
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08/1998	Freiwilliges soziales Jahr
06/1998	Abitur

Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorgelegte Dissertation mit dem Titel „Effects of Coenzyme Q_{10} on Gene Expression and Inflammation: Results from *In silico*, *In vitro* and *In vivo* Studies" selbständig und ohne unerlaubte Hilfe angefertigt habe und dass ich die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorgelegt habe.

Hiermit erkläre ich, dass gegen mich kein strafrechtliches Ermittlungsverfahren schwebt.

Constance Schmelzer