Effect of Foods on Basal and LPS induced NF-κB Activity

Master Thesis

by

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Siril Garmannvik Johansen

Summary

The content of phytochemicals has been proposed to contribute to the beneficial effects of a diet rich in fruits and vegetables. One proposed mechanism of action of phytochemicals is down regulation of NF-κB activity. This transcription factor is important in inflammatory and immune responses, however aberrant activity of NF-κB is associated with some chronic diseases and cancers.

This thesis is part of a screening project that investigates the ability of foods to modulate NF-κB activity by the use of a monocytic cell line stably transfected with a luciferase reporter gene coupled to a promoter of three NF-κB binding sites. Foods from five different food groups were tested, and both modulation of basal and LPS induced NF-κB activity was screened. Furthermore, potent inhibitors of LPS induced NF-κB activity *in vitro* were tested *in vivo*.

Of the 27 extracts tested, 12 had the ability to increase basal NF-κB activity, and all food groups were represented with at least one item. 12 extracts inhibited LPS induced NF-κB activity, with red wine, green- and black tea and dark chocolate as the most potent inhibitors, all these reduced LPS induced NF-κB activity to less than 20 % of control. Only two extracts, unhydrosed barley “Olve” and unhydrolysed oat “Hurdal”, further increased LPS induced NF-κB activity. No correlation was found between the antioxidant capacity and the ability to modulate basal NF-κB activity, however there was a correlation between FRAP and ability to modulate LPS induced NF-κB activity.

The most potent inhibitors of LPS induced NF-κB activity, were combined and tested in transgenic mice. No significant reduction in NF-κB activity occurred, however there was a trend towards inhibition of NF-κB activity in whole mice, intestine and ovaries that had been treated with the mixed extract.

Based on the results in this thesis, further screening should be performed with new foods, and more investigation regarding pre-conditioning effects of foods and *in vivo* effects should be done.

Contents

1. List of Abbrevations

AGE	Advanced Glycosylated End products
AMP	Adeninemonophosphate
ATP	Adeninetriphosphate
AUC	Area Under Curve
CAT	Catalase
CBP	Cyclic AMP response element Binding Protein
CD14	Cluster of Differentiation 14
CHD	Coronary Heart Disease
DHA	Docosahexaenoic Acid
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
EGCG	Epigallocatechin gallate
EMSA	Electrophoretic Mobility Shift Assay
FBS	Fetal Bovine Serum
FRAP	Ferric Reducing Ability of Plasma
GSH	Glutathione
GSSH	Oxidized form of glutathione
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HPLC	High performance liquid chromatography
IκB	Inhibitory kappa B
IKK	IκB kinase

IL	Interleukin
LBP	LPS Binding Protein
LDH	Lactate Dehydrogenase
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
LUC	Luciferase
MAPK	Mitogen Activated Protein Kinase
MeOH	Methanol
MSK1	Mitogen- and Stress Activated Kinase-1
NAC	N-acetylcysteine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor kappa B
NLS	Nuclear Localization Sequence
NNR	Nordic Nutrition Recommendations
NO	Nitric Oxide
O_2-	Superoxide Anion
$OH^{\cdot}$	Hydroxyl Radical
$ONOO^-$	Peroxynitrite
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate Buffered Saline
PKA	Protein Kinase A
POTC	Pyrrolidine dithiocarbamate

RHD	Rel Homology Domain
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TAD	Transactivation Domain
TBP	TATA Binding Protein
TEAC	Trolox Equivalent Antioxidant Capacity
TFIIB	Transcription factor II B
TFIID	Transcription factor II D
TLR4	Toll-like Receptor 4
TNF	Tumor Necrosis Factor

2. Introduction

2.1 Nutrition Recommendations

The relationship between diet and health has long been investigated, and guidelines for dietary intakes are published[1]. These guidelines are based on the knowledge of diseases of deficiencies and toxicity related to vitamins and micronutrients, as well as diet-related diseases associated with a high energy and/or fat intake, such as cardiovascular disease, obesity, diabetes[1,2] and some cancers[3].

The Nordic Nutrition Recommendations (NNR) was first published in 1954. These recommendations are based on the regular diet in the Nordic countries, and aims to give guidelines for a nutritional composition that satisfies the need of micro- (vitamins, minerals and trace elements) and macronutrients (energy containing compounds; fat, carbohydrates, proteins and alcohol), and gives the basis for good health[1,4]. The recommendations are continuously updated based on scientific evidence, and are not only focused on single compounds in the diet, such as fat, carbohydrates and vitamins. Also food-based guidelines are included, with a focus on an increased intake of fruits and vegetables, potatoes, low-fat milk, lean meat and cereal products, in addition to a reduced intake of simple sugars[1].

Despite recommendations for all nutrients, the NNR focuses mainly on the energy containing compounds, the macronutrients. Guidelines for the distribution between fats, carbohydrates and proteins are given, and if followed, other nutrients like vitamins and minerals, are likely to be consumed as recommended. The recommended distribution between the macronutrients is given in **Figure 2.1**.

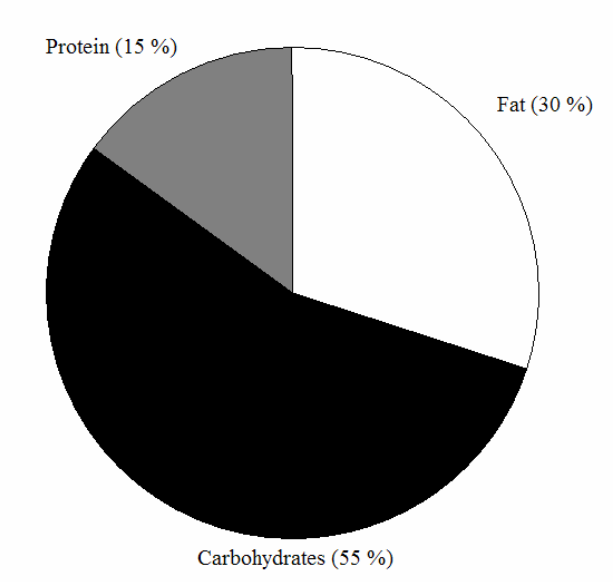


Figure 2.1 Recommended distribution between the energy containing nutrients; fats, carbohydrates and proteins.

In general, the intake of fat is recommended to contribute maximum 30 % of daily energy intake (E %), however the composition of fat is of great importance. The intake of saturated fat should not exceed 10 E %, and the main contributors to saturated fats in the Nordic diets are milk and dairy products, meat and meat products, and margarines and butters[5]. Furthermore mono-unsaturated and poly-unsaturated fatty acids are recommended to constitute to 10-15 and 5-10 E % respectively, for which the main contributors are olive oil and poultry, and soft margarines, oils and fat fish respectively[5]. To secure a varied diet, protein recommendations are 10-20 E %, despite the fact that an intake of 10 E % from proteins would be adequate to fulfill the protein needs[2]. Good sources of protein in traditional Nordic diets are meat, cereals and grains, and dairy products[5]. Concerning carbohydrates, an intake of 55 E % is recommended. This should not, however, be covered by refined sugars, which are contributors to diet related diseases[6]. Grains, fruits and vegetables are large contributors to carbohydrates in the diet[5].

2.2 Prevention of Disease by Diets Rich in Plant Based Foods

In addition to the general nutrition recommendations, many countries have special recommendations and campaigns to encourage to an increased intake of fruits and vegetables. A vast number of epidemiological studies suggest a strong, inverse association between fruit and vegetable intake and the risk of developing several chronic and degenerative diseases, such as cancers, cardiovascular diseases and Alzheimer's disease[7,8]. A recent meta-analysis including more than 270.000 individuals in independent cohort studies, showed that an increased consumption of fruit and vegetables from less than three servings per day to more than five servings per day is related to a 17 % reduction in the risk of coronary heart disease (CHD)[9].

Despite the general consensus pointing toward a reduction in the risk of chronic and degenerative diseases related to an increased intake of fruit and vegetables, the mechanisms and the compounds involved in the protective effects have not been fully established. Plant foods contain a diverse range of compounds, such as carotenoids, vitamin C, vitamin E, folic acid and fiber, in addition to polyphenols[10,11], that may be involved in the protective effects. Effects of single compounds however are not convincing[12], as risk reductions of disease generally are found only with intake of whole fruits and vegetables and not supplements. This suggest that several components of fruits and vegetables form an intricate network and together reduce the risk of developing chronic and degenerative diseases[13].

Oxidative stress reduction by dietary antioxidants is one commonly suggested mechanism for the observed protective effect of fruits and vegetables[7,8]. Fruits and vegetables are rich in phytochemicals[14], many of these with antioxidant properties. It has been suggested that phytochemicals influence the generation or eradication of reactive oxygen and nitrogen species (ROS/RNS)[15], and that this mechanisms may contribute to the beneficial effects of fruit and vegetable intake. Still, mechanisms other than direct antioxidant effects are increasingly introduced when studying phytochemicals and disease. Thus, whether the protective effect of fruits and

vegetables are firstly, -due to the content of phytochemicals,- and secondly the antioxidant properties of the phytochemicals, remains to be fully established[15].

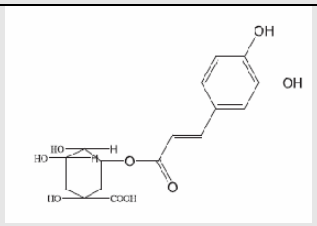
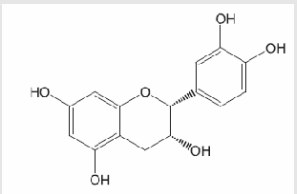
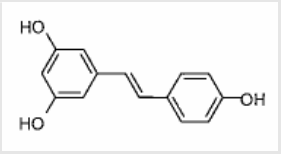
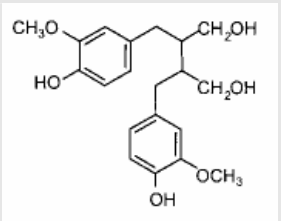
2.3 Phytochemicals

Phytochemicals are non-nutrient compounds in plants and serves various functions. These compounds are important in growth, flavour and coloration of plants, and contribute antimicrobial properties[16]. Phytochemicals are also important in the protection against UV-radiation, with the ability to heal damaged areas of the plant[17], furthermore they have the ability to reduce oxidative damage as they serve as antioxidants[18]. So far thousands of phytochemicals have been characterized, and more than hundred of these can be found in one serving of vegetables[17]. The phytochemicals can be divided into several groups of which the polyphenols is the largest, and hence is the only group that will be further discussed.

2.3.1 Polyphenols

The polyphenols are abundant in plant based foods, and are recognized by at least one aromatic ring with minimum one hydroxyl group. Based on additional structural groups, the polyphenols are further divided into the phenolic acids, flavonoids, and the less common stilbenes and lignans[16,19] (**Table 2.1**). The flavonoids consist of several subgroups, such as the flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols. It is estimated that the intake of polyphenols is about 1g/d, where fruits and beverages are the main contributors[19], and in the Norwegian diet coffee is a major source[20]. It should be noted, however, that the polyphenol content of fruits and vegetables varies with ripeness, processing and storage, and a complete polyphenol profile in specific foods is thus hard to establish[16].

Table 2.1 The subgroups of polyphenols, their chemical structure and sources. Adapted from Lotito and Frei[21] and Manach *et al.*[16].

Polyphenol group	Example of chemical structure	Sources
Phenolic acids	 Chlorogenic acid	Tea, coffee, apples, cereals
Flavonoids	 Epicatechin	Apple, green tea, black tea, red wine, cereals, broccoli, chocolate
Stilbenes	 Resveratrol	Grapes, red wine, blueberries
Lignans	 Secoisolariciresinol	Seeds, grains

2.3.2 Bioavailability and Metabolism

To achieve a biological effect, the phytochemicals must be absorbed and transported to the appropriate sites in the body. In plants most of the polyphenols are usually glycosylated to sugars such as glucose and rhamnose, and removal of these sugars, by

enzymes in the small intestine, is needed before absorption[16]. An exception is anthocyanin glycosides, which are absorbed in their conjugated forms[22].

The aglycones that are formed and absorbed will be conjugated after absorption. This involves glucuronidation, methylation and sulfation, and will make the polyphenols more hydrophilic. This xenobiotic metabolism may be done by intestinal enzymes, however phase 1 and 2 enzymes in the liver are the major modulators. Conjugation will increase the excretion of polyphenols in the urine and the bile, however polyphenols have been detected in tissues such as brain, spleen, kidney, liver and skin in animal models[16]. Polyphenols excreted in the bile can be the target of bacterial enzymes, i.e. β-glucuronidase, leading to reabsorption from the intestine. Furthermore, polyphenols that are not absorbed in the small intestine, may still exert an biological effect in the colon[15] (**Figure 2.2**).

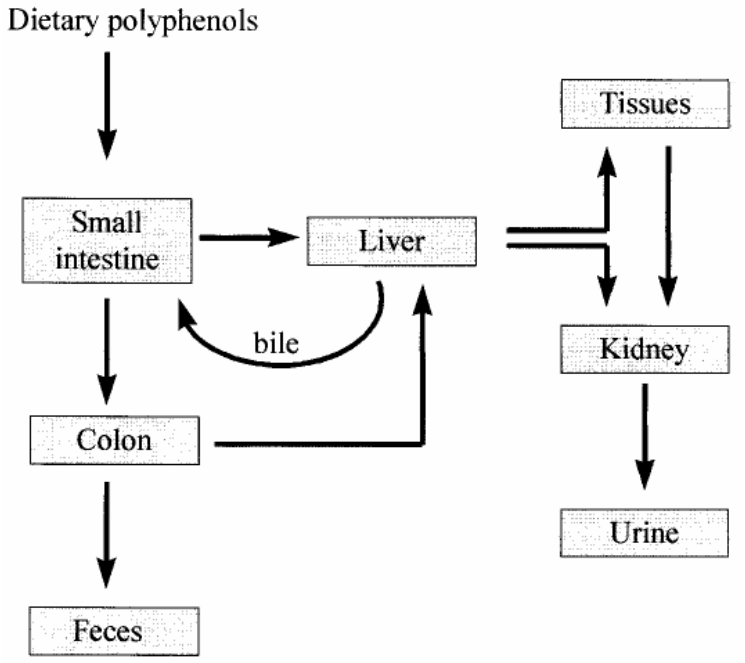


Figure 2.2 Polyphenol uptake and excretion. Polyphenols can be absorbed in the intestine and transported to the liver, where conjugation occurs. The conjugated polyphenols will often be excreted in the urine, however some might be distributed in tissues. Polyphenols can also be excreted in the bile, this may lead to reabsorption. Polyphenols not absorbed in the small intestine may possibly have beneficial effects in the colon. From Scalbert and Williamson[19].

2.3.3 Phytochemicals and Human Health

The consumption of foods known to be rich in phytochemicals has been associated with health benefits, and several mechanisms behind this have been proposed. The French Paradox is only one example of this, where in spite of a high intake of saturated fat the French population has a low incidence of coronary heart disease[23]. This is thought to be due to a high intake of red wine, fruits and vegetables, hence a diet rich in phytochemicals, leading to a reduced cholesterol uptake and reduced oxidation of low density lipoproteins (LDL)[21,24]. The antioxidant capacity of phytochemicals can be one of the underlying mechanisms, however phytochemicals may act independently of their antioxidant capacity[17,25,26].

Studies have also shown that phytochemicals can reverse features related to aging, such as decreased cognitive function [27]. Phytochemicals may promote induction of cytoprotective enzymes, such as phase 2 enzymes, which might promote the excretion of toxic and carcinogenic substances[28], thus phytochemicals may contribute to the modulation of cancer development[17]. These findings may be part of the beneficial health effects associated with a high intake of fruit and vegetables.

2.4 Oxidative Stress and Antioxidant Defence Mechanisms

Oxidative stress appears to be one of the underlying causes of several chronic and neurodegenerative diseases, such as cancers, cardiovascular, and Parkinson's and Alzheimer's disease[8,29,30]. A common feature of oxidative stress is the increased production of ROS/RNS [13,31]. The ROS/RNS are produced by all aerobic organisms in normal cellular metabolic reactions, however they can also be formed as a consequence of exogenous factors, such as smoking, drugs, environmental pollutions and radiation among others[13,32]. ROS/RNS consists of both radical and non-radical species, such as the superoxide anion (O_2-), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), nitric oxide and (NO) and peroxynitrite ($ONOO^-$)[29,33]. At normal conditions, the endogenous antioxidant defense and the production of ROS/RNS will be at balance, and imbalances towards increased ROS/RNS may be corrected by an ability to combat oxidative stress. However, if the amount of free radicals exceeds the ability of the endogenous antioxidant defense to eradicate these, the highly reactive ROS/RNS species will react with and may alter the structure and function of proteins, carbohydrates, lipids, RNA and DNA[30,34]. The resulting oxidative damage range from mutations of DNA to advanced glycated end products (AGEs) and oxidized low density lipoproteins (LDL) which again may lead to the formation of atherosclerotic plaques[34].

The endogenous antioxidant defense consists of several enzymes and non-enzymatic molecules, making an intricate defense network[35]. Among the antioxidant enzymes; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase and glutathione reductase are the most commonly known. SOD eliminates the superoxide radical (O_2-), creating hydrogen peroxide (H_2O_2), which itself is less reactive than O_2. However, hydrogen peroxide might take part in other reactions resulting in more hazardous ROS, such as the hydroxyl radical ($OH^{\cdot}$), a very reactive radical. The catalase catalyzes the conversion of H_2O_2 to water and oxygen, and hence might reduce the production of $OH^{\cdot}$[35].

The thiol-containing tripeptide glutathione is one of the main endogenous antioxidants. It is found in high concentrations (millimolar) in most cells, and can be found in both a reduced (GSH) and an oxidized form (GSSG)[31]. The glutathione peroxidase catalyzes the oxidation of GSH to GSSG, eliminating hydrogen peroxide or other reactive molecules. To maintain the amount of GSH in the cells, glutathione reductase will convert GSSG to GSH with the help of nicotinamide adenine dinucleotide phosphate (NADPH). Myhrstad *et al.*[36] found that the flavonoid quercetin stimulate the synthesis of glutathione by up-regulating γ-glutamylcysteine synthetase, the rate limiting step in the glutathione synthesis.

In addition to glutathione, other non-enzymatic compounds, such as vitamin E and C, are important in the endogenous antioxidant defence. Vitamin E and C are antioxidants derived from the diet, however increased intakes above required levels of these nutrients have not shown decreased oxidative damage in an already well-nourished population[32]. Whether antioxidants in foods, other than vitamin E and C, can directly eliminate ROS/RNS, is a highly discussed topic.

In cases of oxidative alterations to DNA, several DNA repair systems normally eliminate such damage. The repair systems include strand break repair, base excision repair, nucleotide excision repair, mismatch repair and lesion bypass. If no such repair occurs before the cell replicates, oxidative damage can cause permanent mutations associated with disease[37].

2.5 Nutrients and Gene Regulations

2.5.1 Regulation of Gene Expression

In general, every cell in a multi-cellular organism contains every gene in that specific organism's genome[38]. For each cell to acquire and maintain its proper function, the spatial and temporal regulation of gene expression is important. Cells respond to physiological and environmental signals by regulating expression of genes. DNA-binding transcription factors, non-DNA-binding co-regulators and the RNA polymerase II machinery are important for mediating specific patterns of gene expression[39].

DNA contains genes described as nucleotide sequences that code for proteins, catalytic RNAs or non-coding RNA molecules. The DNA is packaged into chromatin which again is further packed into chromosomes, and each chromosome consists of a double-stranded molecule of DNA in a complex with proteins. The genetic information lies in the DNA, whilst the proteins regulate the physical properties of the DNA[38]. The proteins are called histones and due to their positive charge they attract DNA and make the complex tightly packed when transcription does not occur. The interaction between the histones and DNA forms beadlike units called nucleosomes, the basic unit of the chromatin. Each nucleosome consists of 147 base pairs of DNA wrapped around a core of 8 histones, two each of the histone molecules, H2A, H2B, H3 and H4. Another histone, named H1, stabilize the chromatin complex further. Between each nucleosome are small segments of DNA, linking the nucleosomes[40].

Eukaryotic cells have both coding and non-coding DNA segments. The human genome has approximately 21000 protein-coding genes[41], however the DNA - segments of coding genes only represent a small percentage of the total genome[42]. Some of the non-protein-coding segments flank the protein-coding parts of the DNA

and regulate their transcription, to assure that certain genes are expressed and proteins synthesized in the right cells at the appropriate time.

The transcription of a gene into a RNA-molecule consists of three parts – initiation, elongation and termination. The transcription starts at the promoter, which contains a sequence rich in adenine-thymine (AT) located 25 base pairs away from the initiation site, and is referred to as the TATA box[38]. RNA polymerase II is responsible for all synthesis of RNA in eukaryotes, however it is not capable of recognizing the promoter and initiate transcription without various regulatory proteins called transcription factors[43]. Transcription factors are activated by extracellular signals which through signal transduction pathways promote the binding of the transcription factors to specific binding sites of the promoter. Transcription factor IID (TFIID) is the first transcription factor to bind to DNA as TFIID binds to the TATA box and changes the shape of the DNA, attracting other transcription factors. Both gene-specific and unspecific transcription factors, in addition to proteins binding to enhancer regions and silencers, are necessary for transcription to occur[38].

As the transcription factors have bound to the DNA, RNA polymerase II initiates transcription and unwinds the DNA strand about 20 base pairs at a time. The elongation process, where RNA polymerase adds new nucleotides to the strand with the help of elongation factors, proceeds until the termination site of the DNA is reached, and the RNA transcript is released from the RNA polymerase II. An overview of the transcription of DNA to RNA and the further translation to protein can be seen in **Figure 2.3 A**.

Interspersed in the coding parts of the DNA are non-coding base sequences called introns, which are also transcribed during the transcription process. The pre-mRNA must thus be further processed with a removal of the introns from the expressed parts (exons) (**Figure 2.3 B**). When the 3’ exon has been spliced together to the 5’ exon, the mature mRNA can be transported out of the nucleus to the ribosomes, where translation to protein occurs.

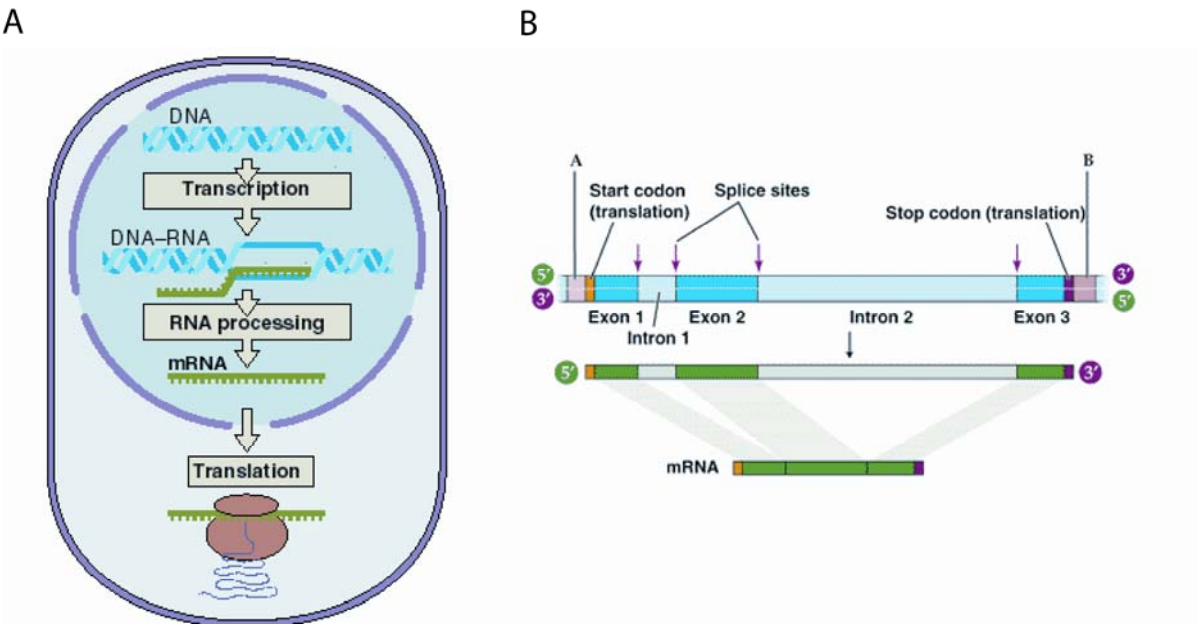


Figure 2.3 Transcription and translation of genes to RNA and ultimately proteins. A) An overview of the route from gene to protein via transcription and translation. B) Introns in the transcribed RNA must be spliced out to make the mature RNA. Mature RNA leaves the nucleus and is translated into proteins in the cytoplasma[44].

The production of proteins is controlled at all stages of transcription and translation, and even differential splicing can alter the gene product [38]. Exogenous and endogenous factors, such as oxidative stress, can alter gene regulation. Oxidative stress can cause strand breaks not only in the DNA and cause mutations, but also in RNA, and thereby control the expression of the genetic information[30].

2.5.2 Modulation by Nutrition

It is known that food components may act as signals that can influence gene regulation and thereby play an important role in the regulation of homeostasis. This field of research is referred to as nutrigenomics and tries to identify nutrients that modulate gene and/or protein expression[45]. Several technologies are being used in the nutrigenomics, including microarrays and transgenic- and knockout mice[45,46].

Dietary regulation of gene expression is complex, as each nutrient can affect several signalling pathways, resulting in cross-talk between several gene regulators. The main goal of the nutrigenomics is to increase the knowledge of how food components affect genes and thereby their products, hence learning more about the prevention of diet-related diseases.

Micro- and macronutrients have shown to be important modulators of gene expression, in addition to their functions as energy and cofactors for enzymes such as superoxide dismutase[35,45]. Especially well studied are nutrients such as retinoic acid, fatty acids and vitamin D, which modulate the activity of specific nuclear receptors. Also, glucose, amino acids and several other micronutrients have shown to have transcription factors as targets[45]. Some of these dietary components and their target transcription factors are listed in **Table 2.2**.

Table 2.2 Transcription factor pathways mediating nutrient-gene interactions. From Müller and Kersten[45].

Nutrient	Compound	Transcription Factor
Macronutrients		
Fats	Fatty acids	PPARs, SREBPs, LXR, HNF4,
	Cholesterol	SREBPs, LXRs, FXR
Carbohydrates	Glucose	USFs, SREBPs, ChREBP
Proteins	Amino acids	C/EBPs
Micronutrients		
Vitamins	Vitamin A	RAR, RXR
	Vitamin D	VDR
	Vitamin E	PXR
Minerals	Calcium	Calcineurin/NF-ATs
	Iron	IRP1, IRP2
	Zinc	MTF1
Other food		
	Flavonoids	ER, NF-κB, AP1
	Xenobiotics	CAR, PXR

AP1, activating protein 1; CAR, constitutively active receptor; C/EBP, CAAT/enhancer binding protein; ChREBP, carbohydrate responsive element binding protein; ER, oestrogen receptor; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; IRP, iron regulatory protein; LXR, liver X receptor; MTF1, metalresponsive transcription factors; NF-κB, nuclear factor kappa B; NF-AT, nuclear factor of activated T cells; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SREBP, sterol-responsive-element binding protein; USF, upstream stimulatory factor; VDR, vitamin D receptor.

Other non-nutrient components of foods may also influence gene expression. The transcription factor family of Nuclear Factor kappa B (NF-κB), involved in immune and inflammatory responses, has been observed to be modulated by phytochemicals such as flavonoids[45,47].

2.6 Nuclear Factor-κB

2.6.1 Regulation of Gene Expression by NF-κB

The nuclear factor kappa B (NF-κB) proteins are a group of transcription factors found in all mammalian cells. The family controls the transcription of diverse genes related to immune and acute phase inflammatory responses, oxidative stress responses, cell adhesion, differentiation and apoptosis[47,48], and is involved in pathological processes such as inflammatory diseases, cancers and atherosclerosis[49-51]. The NF-κB regulated genes can be divided into four major functional classes; negative feedback of the NF-κB activation, immunity, anti-apoptosis and proliferation (**Figure 2.4**)[52]. So far more than 150 activators of the NF-κB signalling pathway have been identified[48].

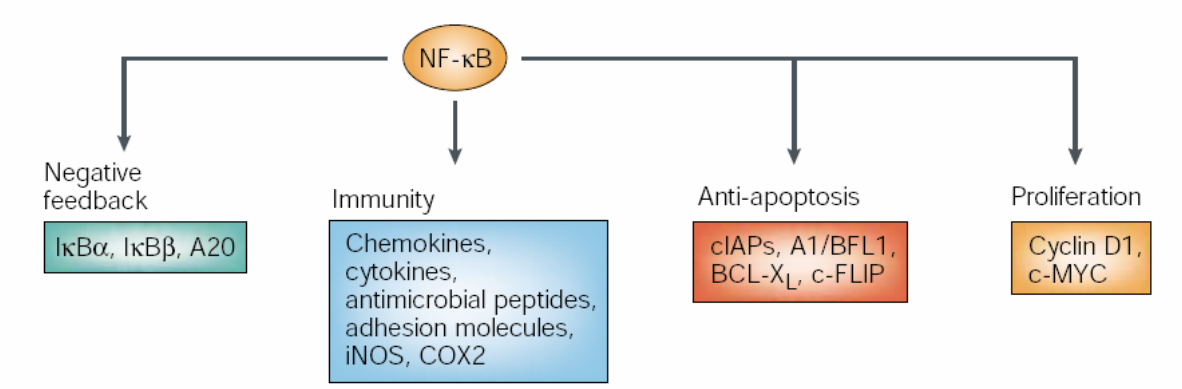


Figure 2.4 Genes regulated by NF-κB can be divided into four categories based on the function of their end-products; negative feedback, immunity, anti-apoptosis and proliferation. From Karin et al.[52].

The mammalian NF-κB family is comprised of several structurally related proteins, p50, p52, RelA/p65, RelB and c-Rel (**Figure 2.5**). These proteins form homo- and heterodimers, the most common one p50-RelA[48]. All the proteins share a rel-homology domain (RHD) which consists of 300 amino acids in the C-terminal end, [53]. The RHD mediates dimerization, interaction with inhibitory factors, the IκBs, and is responsible for binding the dimers to DNA regulatory sites of 10 bp, referred to as κB sites [48]. RelA, RelB and c-Rel each contain a transactivation domain (TAD) in the

carboxy terminal end, which interacts with members of the basal transcription machinery, such as transcription factor IIB (TFIIB), TATA-binding protein (TBP) and other co-activators[53]. Unlike the Rel-proteins, p50 and p52 do not contain the TAD, and will not promote transcription when present as homodimers. p50 and p52 are synthesized as large precursors, p105 and p100 respectively, and proteolytical cleavage is needed to form the active subunits[54].

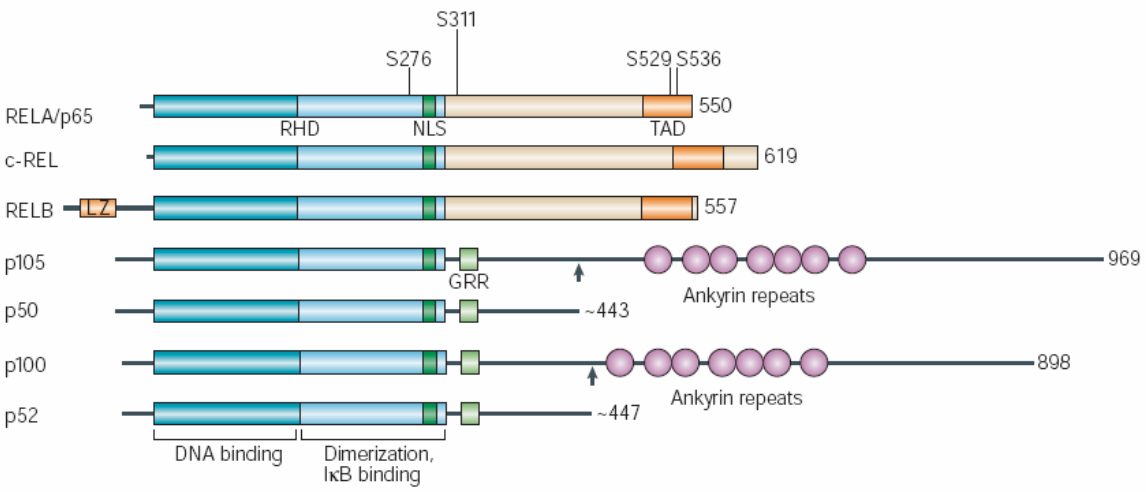


Figure 2.5 The five proteins in the NF-κB family and the two precursors of p50 and p52, p105 and p100 respectively. The rel-homology domain (RHD) mediates DNA binding, dimerization and binding with inhibitory proteins (IkBs), and contains the nuclear-localization sequence (NLS). The Rel proteins contain a transactivation domain (TAD). Serine residues, where phosphorylation occurs upon activation, are shown. Adapted from Chen and Greene[53].

In most cells NF-κB dimers are present in an inactive state in the cytoplasm through association with inhibitors, IκBs (IκBα, IκBβ, IκBε, IκBγ and BCL3). IκB will bind to the RHD, block the nuclear-localization sequence (NLS) in the dimers[55], thereby preventing translocation of the dimer to the nucleus[47]. However, after stimulation of cells through membrane bound receptors, IκB is rapidly phosphorylated, ubiquitinated and degraded by proteasomes. The phosphorylation of IκB is mediated by IκB kinases (IKK), which are composed of three subunits, the catalytic IKK-α and IKK-β and the associated regulatory subunit NF-κB Essential Modulator (NEMO)/IKK-γ. The IKK subunits will be phosphorylated upon signal mediated stimulation, this leads to phosphorylation of IκB at two serine residues (S), S32 and S36. When poly-ubiquitinated, the IκB is rapidly degraded through the 26S

proteasome, and NF-κB with its revealed NLS is free to enter the nucleus[47,55]. In the nucleus NF-κB will bind to its response elements, the κB sites, with the consensus sequence 5'-GGGRNYYYCC-3', where R is a purine, Y is a pyrimidine and N is any nucleic acid, thereby promote gene expression[53].

The induction of transcription by the NF-κB dimer may vary between the different target genes. Some dimers bind to their respective κB sites almost immediately after translocation, whilst others needs hours of stimulation to bind[56]. Despite these differences, common to all transcriptions is the need of post-translational modification of NF-κB, such as phosphorylation of RelA and histone acetylation, to promote maximal transcriptional response. Kinases, such as protein kinase A (PKA) and mitogen- and stress-activated kinase-1 (MSK1) in the cytoplasma and nucleus respectively, are activated upon stimulation, and will phosphorylate several serine residues in the RHD (S276 and S311) and the TAD (S529 and S536). This leads to the recruitment of co-activators essential for the transcriptional response, e.g. phosphorylation of S133 will facilitate the recruitment of p300 and cyclic-AMP-response element binding protein (CBP). To increase the transcriptional response further, acetylation of histones around the NF-κB responsive genes will occur. The acetylation finds place in the histone tails, which remodels the dense chromatin, and promotes access for the NF-κB dimer itself, general transcription factors and co-activators[53].

The NF-κB signalling pathway will be turned off by several mechanisms. IκBα is one of the target genes for NF-κB, and will be resynthesized when NF-κB binds to κB-sites. The increasing levels of IκB will lead to binding of NF-κB in the nucleus, which reduces transcription. Secondly, NF-κB can be removed by proteasomal degradation when bound to DNA[56].

The pathway of NF-κB stimulation explained above is referred to as the classical (or canonical) (**Figure 2.6 A**) pathway, however there is also one alternative (non-canonical) (**Figure 2.6 B**) pathway of NF-κB activation. p105 and p100, the

precursors of p50 and p52 respectively, contains ankyrin repeats and hence behave as IκBs[54]. p100 is usually coupled to RelB in the cytoplasma. Whilst the classical pathway mainly depends on activation by IKKβ, the processing and cleavage of p100 to form an active p52-RelB heterodimer is dependent on IKKα activation. The classical pathway is mainly activated by proinflammatory cytokines and is important for the expression of T and B cell receptors, and to inhibit programmed cell death. Regulation of premature B cells and the development of lymphoid organs are dependent on the alternative pathway, where members of the tumor necrosis factor (TNF) family activate IKKα[50].

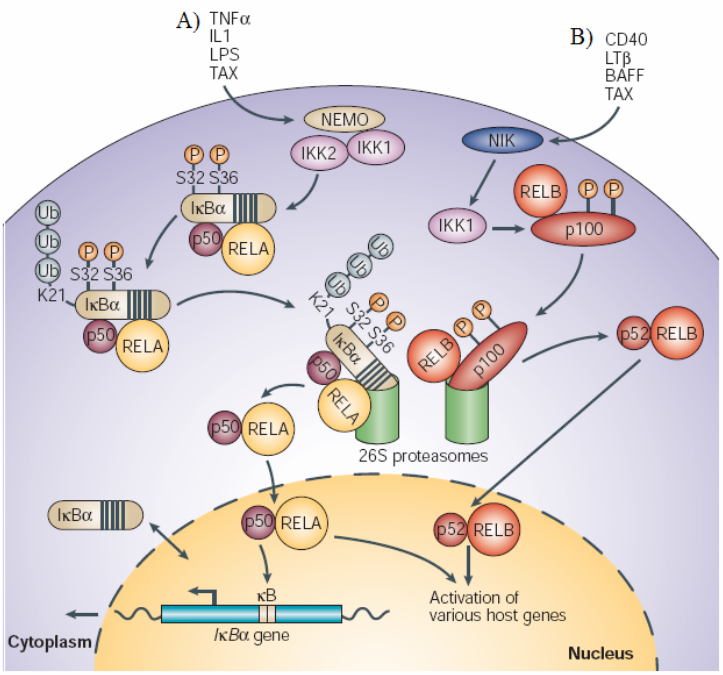


Figure 2.6 Illustration of the A) classical (canonical) and the B) alternative (non-canonical) pathway in the activation of NF-κB. From Chen and Greene[53].

2.6.2 Activators and Inhibitors

NF-κB regulates the transcription of many genes related to immune responses, hence several of the stimuli that activate NF-κB are induced by various stress conditions. Bacterial and viral agents are known to induce NF-κB activity in many cells, furthermore cytokines such as IL-1, IL2 and TNF-α are common activators. Physical stress, i.e. different kinds of radiation, and the exposure to environmental hazards, chemicals and many drugs also activate the NF-κB signalling pathway, thus leading to an immunological response[48]. Whether reactive oxygen species have the ability to increase NF-kB activity or not, has been discussed. Some has proposed that ROS increase NF-κB activity by stimulating the release of the IkB-complex from NF-κB, thereby causing translocation of NF-κB to the nucleus[57]. However, it has been shown that ROS only activate NF-κB in certain cells, and that ROS in general not mediate NF-κB signaling[58,59]. Still, ROS can lead to the formation of modified molecules, such as advanced glycated end products (AGE) and oxidized LDL, which do activate NF-κB[48,57]. **Table 2.3** shows some of the NF-κB inducers.

Table 2.3 Activators of NF-κB. Modified from Pahl[48].

Condition	Example
Bacterias/Bacterial products	Lipopolysaccharide
	Helicobacter pylori
	E. coli
Viruses	Adenovirus
	Cytomegalovirus
	Influenza Virus
Cytokines	IL-1
	IL-2
	TNF-α
Physical stress	Ultraviolet irradiation
	γ radiation
Oxidative stress	Hydrogen peroxide (H_2O_2)
Environmental hazards	Smoking
	Ccocidolite asbestos fibres
	Herbicides
Drugs	Methamphetamine
	Anthralin
Modified proteins	Advanced glycated end products
	Oxidized LDL
Endoplasmatic reticulum	MHC Class I
Apoptotic mediators	Anti-Fas/Apo-1
Growth factors and hormones	Insulin
	TGF-α
Physiological mediators	Angiotensin II
Chemical agents	Linoleic acid

A wide range of inhibitors of the NF-κB pathway have been described[47]. Many of these suppressors are general inhibitors by acting at an early step of induction. Others, however, inhibit specific steps of the NF-κB signalling pathway [26,47]. In general, NF-κB activity can be inhibited at three different steps: 1) Avoiding stimulators to bind to their cell surface receptors, thereby blocking activation at an

early stage; 2) Blocking any step in the cascade that occurs in the cytoplasm; or 3) Interfering with the nuclear activity, i.e. inhibiting translocation of NF-κB to the nucleus, its binding to DNA, or recruitment of other transcription factors[26,47].

More than 785 inhibitors of NF-κB activity have so far been found[47] (**Table 2.4**). These include antioxidants, peptides, proteasome inhibitors, dominant-negative or constitutively active polypeptides and small RNA/DNA[26,47]. Antioxidants may have both a direct and an indirect effect on NF-κB activity. They might interfere with several steps in the NF-κB signalling pathway, furthermore a decrease in the magnitude of reactive oxygen species may occur[26]. Phytochemicals, such as curcumin, gingerol, capsaicin, epigallocatechin gallate (EGCG), genistein and resveratrol, also suppress NF-κB activation[17], however these abilities are not known to be due to their antioxidant capacity[60].

Table 2.4 Inhibitors of NF-κB activity. Modified from Gilmore & Herscovitch [47] .

Category	Example
Antioxidants	α-tocopherol
	Epigallocatechin-3-gallate (EGCG)
	Glutathione
	Manganese Superoxide Dismutase (Mn-
	Quercetin
	Vitamin C
	Vitamin E derivatives
Proteasome inhibitors	Peptide aldehydes
	Ubiquitin ligase inhibitors
Inhibitors of IκBα phosphorylation and	Aspirin
	Ibuprofen
	Nitric oxide
	Capsaicin
	Estrogen
	Genistein
Others	Glucorticoids

2.6.3 NF-κB in Normal Physiology

Some of the proteins coded for by NF-κB target genes are acute phase proteins, immunoglobulins and stress response molecules [48]. To combat various forms of stress, such as inflammation, radiation and environmental hazards, a proper response of NF-κB hence is absolutely necessary. Defects in the B-cell proliferation in response to lipopolysaccharide occurs in mice who are p50 deficient[61]. NF-κB is a major regulator of apoptosis, and research done by Beg *et al*.[62] have shown that the anti-apoptotic effects orchestrated by NF-κB are necessary for proper liver development in mice. RelA knock-out mice will die 15-16 days post conception due to massive apoptosis of the fetal liver, suggesting an important role of NF-κB activity for normal fetal development. It has also been hypothesized that the anti-apoptotic effects of NF-κB may provide an opportunity to repair DNA damage in cells, however this has not been proven[63].

2.6.4 NF-κB and Disease

Although the activation of NF-κB is important in normal physiological responses such as inflammation, dysregulation of NF-κB activity is associated with several chronic diseases, including inflammatory bowel diseases, multiple sclerosis, psoriasis, asthma, rheumatoid arthritis and several cancers [49,64-66]. The link between chronic inflammation and cancer has been intensively investigated, and epidemiological studies have shown that as many as 15 % of human deaths caused by cancer are related to chronic bacterial or viral infections[67]. Examples are the human papilloma viruses, Helicobacter pylori and hepatitis C virus, which are associated with cervical cancer, gastric cancer and hepatocellular carcinoma respectively. Other forms of cancers, such as breast, pancreatic and prostate carcinomas also have aberrant activity of NF-κB[65]. To understand the link between chronic inflammation and cancer, one should consider the features of cancers, such as unlimited cell growth, insensitivity to growth inhibitory signals, evading apoptosis, tissue invasion and metastasis, and angiogenesis. A hyperactivation of the NF-κB signaling

pathways or mutations leading to inactivation of the inhibitory proteins, might link these pathophysiological processes together, as a dysregulation of NF-κB may influence tumor promotion and development in several ways (**Figure 2.6**)[66,68]. Firstly, hyperactivation of NF-κB leads to increased anti-apoptotic and proliferative signals, such as TNF-α and IL-6, which stimulate tumor growth and angiogenesis[66-68]. Secondly, NF-κB activity increases resistance to death-cytokines, thereby preventing necrosis of the malignant cells[50,66,68]. These findings have made NF-κB an attractive target for cancer therapeutic approaches[66,68].

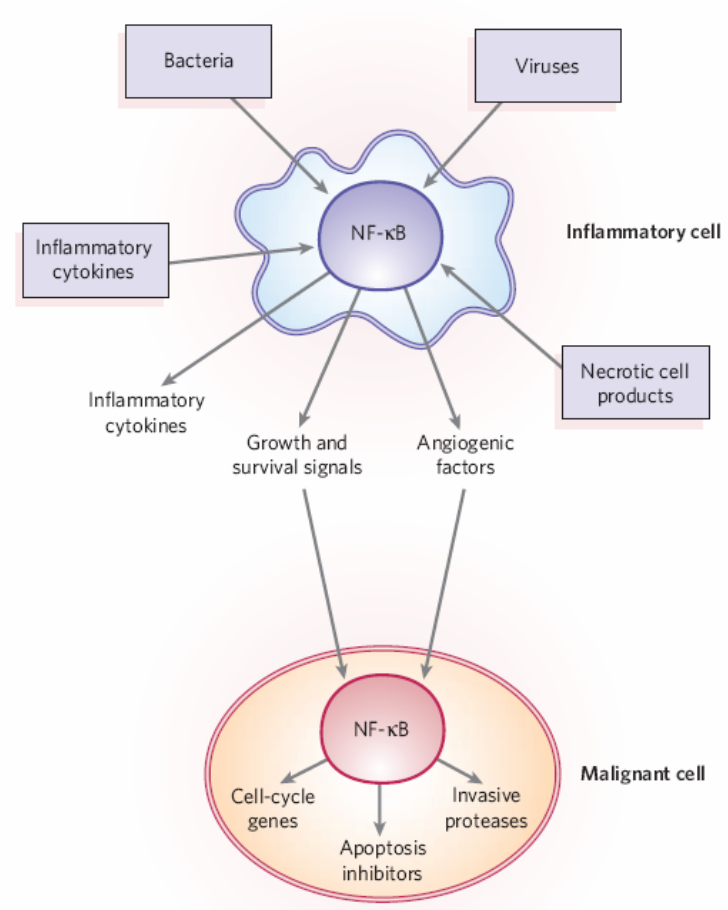


Figure 2.6 The link between the NF-κB signaling pathways and cancer. Dysregulation of NF-κB can lead to increased anti-apoptotic signals, cell-cycle genes and invasive proteases, thereby being a link between inflammation and tumor initiation and progression. From Karin[68].

Despite the association between persistent elevated NF-κB activity and several chronic diseases, small repetitive episodes of increased NF-κB activity have shown to protect against later, stronger insults. These findings, which include conditions such as coronary heart disease[69], sepsis[70] and cerebral diseases[71], suggest that substances with the ability to slightly increase basal NF-κB activity may be beneficial. This effect on NF-κB activity, referred to as pre-conditioning, has been seen with extracts of several dietary plants, and might explain some of the benefits associated with a high intake of fruits and vegetables[25].

The screening of foods in different food groups, and their substances, can identify promising candidates that either induce NF-κB activity, thereby causing a pre-conditioning effect, or inhibit or reduce continuously activation of NF-κB, hence minimizing the risk for chronic diseases. This screening can be of nutritional and medical interest, and might help explain some of the association between diet and health.

2.7 Foods and modulation of NF-κB activity

2.7.1 Modulation of NF-κB activity by foods

As previously mentioned, NF-κB activation can be inhibited by a vide range of substances[47] of which a substantial portion is derived form dietary plants. Most of the substances tested have been purified compounds from foods. However, from a nutritional point of view a screening based on individual foods would be of more interest. So far this has been done on many dietary plants[25], however the knowledge of modulation of NF-κB activity of foods in other food groups is scarce.

2.7.2 Food Groups

Foods in the diet can be divided into several groups based on their composition and use in daily life. One approach towards understanding the net effect of a complete diet in a nutrigenomic context, could be to study the effects of foods from different food groups on transcription factors such as NF-κB.

Fruits and vegetables

Vegetables are in reality all edible parts of plants, except the seed bearing compounds, - the fruits. Berries and nuts are also defined as fruits[72]. The fruits and vegetables generally contribute to only a small portion of the energy intake, however they contain many essential micronutrients, such as vitamin C, β-carotene, folate, iron and fibre[72]. A wide range of phytochemicals have already been identified as inhibitors of NF-κB, such as sulforaphane in broccoli and cabbage[73], and flavonoids including quercetin and epicatechin in apples[21]. Furthermore, whole fruits and vegetables, such as potato, carrots and tomatoes have shown to modulate NF-κB activity[25].

Beverages and liquids

Beverages include many forms of liquids, from teas with low energy content, to dairy products with a high percent of fat and protein, and alcoholic beverages such as wine. The milk and dairy products have been of great nutritional importance, and contain all the macronutrients, as well as vitamins and minerals[72]. Dietary products have not been tested on NF-κB activity, however it has been found that lactic acid bacteria and human breast milk decrease NF-κB activity in intestine[74,75]. In contrast to the milk and dairy products, teas and red wine have shown to have a high content of phytochemicals[16,28]. Resveratrol, one of the major phytochemicals in red wine, and epigallocatechin-3-gallate (EGCG) in green tea, reduces NF-κB activity[25,76], however there are few studies with pure beverages. Voluntarily subjects drinking red wine did however have a reduction in NF-κB activity in a study by Blanco-Colio *et al.*[77]

Fish and meat

Meat and fish are good sources of proteins, containing ~20 % and 16-21 % proteins respectively, providing all the essential amino acids. Clean-cut meat is usually low in fat content, however the fat is saturated. Meat is also one of the biggest contributors to cholesterol in the diet, where the greatest sources are the entrails. The fat content of fish varies from 0-7 %, these fatty acids are however unsaturated[72]. Little work has been done on possible effects of whole meats or fish on NF-κB, however the fatty acid docosahexoenoic acid (DHA 22:6n3), found in fish, has shown to have an anti-inflammatory effect that might be due to inhibition of NF-κB activation[78].

Chocolate and cocoa products

Chocolate and cocoa products are usually considered within the group of sweets, and hence should not comprise major contribution to the diet. The NNR recommends a maximum intake of refined sugar to 10 E % daily[2]. Chocolate bars generally have a high content of refined sugar and saturated fat. Still, cocoa have been found to contain high amounts of phytochemicals [79], and thus small amounts products with high cocoa content have been recommended by some[80]. Sies *et al.*[79] showed that

cocoa drinks increased flow-mediated dilatation in healthy subjects by enhancing nitric oxide (NO) bioactivity.

Grains

Grains are a large and ubiquitous group, including wheat, oat, barley and rice among others. The grains and their respective flours make up the basis of diets in many cultures. A number of nutrients are found in grains, including proteins, starch, and vitamins and minerals such as B-vitamins and iron[72], as well as phytochemicals[81]. Grains are also one of the major sources of fiber in the diet, however the fiber content relies on the degree of grinding of the flour[72]. The consumption of whole-grain is associated with reduced inflammatory diseases[82], which might be due to their antioxidant content. However, single compounds in grains, such as gliadin in wheat, has been found to increase NF-κB activity[83].

As shown above, few data are available when it comes to foods common in the Norwegian diet and their ability to modulate NF-κB activity. So far, mainly single compounds, and whole items of fruits and vegetables have been tested. Hence, the effect of whole foods is an unexplored area, which, when studied, might reveal interesting findings that can help to better understand the relationship between diet and health.

3. Aims of the Thesis

Aberrant activity of NF-κB, a transcription factor essential for immune- and inflammatory responses, has been related to several chronic diseases. Previously, a selection of dietary plants has been investigated for their effect on NF-κB, and many of these have shown to modulate both basal and LPS induced NF-κB activity in a monocytic cell line. This initiate screening is important for further testing of how dietary compounds can affect health. These dietary plants, however only contribute to a small portion of the foods commonly consumed in the Norwegian diet. Therefore this thesis focuses on the effect of food items from a wider range of food groups on NF-κB activity. Furthermore, potential modulators of NF-κB were tested in transgenic mice, to see whether the effects found *in vitro* can be reproduced into an *in vivo* situation.

The specific aims of this thesis were to:

- Identify foods common in the Norwegian diet that have the ability to modulate basal and/or LPS induced NF-κB activity in a monocytic cell line.
- Test the most potent inhibitors of LPS induced NF-κB activity *in vitro*, in an *in vivo* system, using transgenic NF-κB reporter mice.

4. Materials

4.1 Cell line

Cell line	Distributor	Location
U937 3xkB-LUC	The American Type Culture Collection	Manassas, VA

4.2 Cell culture equipment

Chemical/compound/ equipment	Manufacturer	Location
Cell culture flask, 25, 75, 225 cm^3	Corning Inc.	Corning, NY
Cell culture plates, 24 wells	Corning Inc.	Corning, NY
Cell culture plates, CulturPlate24	Perkin Elmer precisely, 6005168	Shelton, CT
RPMI-1640	Sigma, R-0883	St. Louis, MO
Fetal Bovine Serum	Sigma, F-7524	St. Louis, MO
Hygromycin B	Invitrogen, 10687-010	Carlsbad, CA
L-Glutamine	Sigma, G-7513	St. Louis, MO
Penicillin/Streptomycin Solution	Sigma, P-4458	St. Louis, MO

4.3 Transgenic mice

Mouse line	Distributor	Location
NF-κB-luciferase mice	Cgen	Oslo, Norway

4.4 Transgenic mice equipment

Equipment	Manufacturer	Location
Cameo 25 Gas Syringe Filter, 0.22 micron	Osmonics Inc, DGA02025SO	Minnetonka, MN
Centrifuge tubes, 15 and 50 ml	BD Biosciences	Franklin Lakes, NJ
Feeding tube for oral gavage feeding		
Micro tubes 1.5 ml	Sarstedt	Nümbrecht, Germany
Omnifix (syringes) 1 and 10 ml	Braun Melsungen AG	Melsungen, Germany
Sterican (needles) 0.15x16 mm	Braun Melsungen AG	Melsungen, Germany
96 well plates	Sigma, M2936	St.Louis, MO

4.5 Extracts of foods

Name	Latin name/Commercial name	Producer	Location
Apple	Malus pumila	Enza, Royal gala	New Zealand
Banana	Musa sp.		Ica, Grünerløkka, Oslo and pre-clinical cafeteria, UiO, Oslo
Broccoli	Brassica oleraceae var. italica	Thor Graff	Ica, Grünerløkka, Norway
Brussels sprouts	Brassica oleraceae var. gemmifera	Haluco bv	Bleiswijk, Netherlands
Sesame seed	Økologiske Sesamfrø	Møllerens	Bergen, Norway
Green tea	Sencha Japanese Green Tea	Clearspring Organic	London, England
Black tea	Yellow Label	Lipton Tea Company	Crawley, UK
Red wine	Da Luca, Primitivo Merlot Tarantino	MGM Mondo del Vino	Italia
Skim milk (0.01 % fat)	Tine Skummet Melk	Tine BA	Oslo, Norway

Whipping cream (38 % fat)	Q Kremfløte	Q-meieriene	Bergen, Norway
Chicken	Kyllingfilet	Prior	Oslo, Norway
Beef	Indrefilet		Ica, Ullevål, Norway
Cod	Torskefilet		ICA, Storo, Norway
Salmon	Laksefilet		ICA, Storo, Norway
Cocoa	Freia Regia Originalkakao	Freia Kraft Foods	Oslo, Norway
Cocoa 100 %	Mørk Bakekakao	Confecta AS	Oslo, Norway
Dark chocolate	Bocca Mørk Sjokolade Original, 70 % kakao	Nidar	Trondheim, Norway
Wheat "Standard"	Triticum aestivum		
Wheat "Mjølner"	Triticum aestivum		
Buckwheat "Lileja"	Fagopyrum esculentum		
Barley "Olve"	Hordum vulgare		
Oat "Hurdal"	Avena sativa		

4.6 Chemicals

Chemical/compound	**Manufacturer**	**Location**
2-Propanol (HPLC – grade)	Merck	Darmstadt, Germany
2,4,6-tripyridyl-s-triazine (TPTZ)	Fluka Chemie AG	Deisenhofen, Switzerland
Acetic acid	Merck	Damstadt, Germany
Albumin	Bio-Rad Laboratories Inc., 500-0007	Hercules, CA
Argon	AGA, 100325	Oslo, Norway
ATP (adenine triphosphate)	Roche Diagnostics	Ottweiler, Germany
Coenzyme A	Roche Diagnostics	Ottweiler, Germany
Corn oil Biochemika	Sigma, C-8267	St. Louis, MO
D-Luciferin	Biosynth AG	Staad, Switzerland
DMSO (dimethylsulphoxide)	Sigma, D-5879	St. Louis, MO
DTT (dithiothreitol)	Sigma	St. Louis, MO
EDTA (diethylenediamine	Merck	Darmstadt, Germany

tetra-acetic acid)		
Ethanol	Arcus AS	Oslo, Norway
FeCl3 • 6H20	BDH Laboratory Supplies	Dorset, England
FeSO4 • 7H2O	Riedel-deHaën AG	Seelze, Germany
Hydrochloric acid	Merck	Darmstadt, Germany
Isoflourane	Baxter AS	Oslo, Norway
Methanol (HPLC-grade)	Merck	Darmstadt, Germany
MgSO4 • 7H2O	Merck	Darmstadt, Germany
MilliQ water	Millipore	Bedford, MA
PBS (Phosphate buffered saline)	Bio Whittaker, BE17-512F	Verviers, Belgium
Recombinant luciferase (1 mg/ml)	Promega	Madison, WI
Reporter Lysis Buffer	Promega, E3971	Madison, WI
Sodiumacetate trihydrate	Riedel-deHaën AG	Seelze, Germany
Tricine	Sigma, T5816	St. Louis, MO
Trypan blue	Sigma, T-6146	St. Louis, MO

4.7 Kits

Kit Name	**Manufacturer**	**Location**
Bio-Rad Protein Assay	Bio-Rad Laboratories Inc., 500-0006	Hercules, CA

4.8 Instruments

Instrument	**Manufacturer**	**Location**
Biofuge Fresco	Heraeus Instruments	Osterode, Germay
IVIS Imaging System 100 Series	Xenogen Corporation	Alameda, CA
Labofuge 400e	Heraeus Instruments	Osterode, Germany
Luminometer TD 20/20	Turner Designs	Sunnydale, CA
Megafuge 1.0 R	Heraeus Sepatech GmbH	Harz, Germany
Sonicator, 2510 Branson	Branson Ultrasonics Corp.	Dansbury, CT
Synergy 2	Bio Tek® Instruments, Inc	Winooski, VT

Technicon RA 1000 system	Technicon Instruments Corporation	New York, NY
Titertek Multiskan Plus	ELFAB	Finland

4.9 Software

Software	**Manufacturer**	**Location**
Adobe Illustrator 10	Adobe Systems Incorporated	San Jose, CA
Gen 5™ PC	Bio Tec® Instruments, Inc	Winooski, VT
Living Image Software	Xenogen Corporation	Alameda, CA
Microsoft Office XP	Microsoft Corporation	Redmond, WA
Reference manager 11	ISI Research Soft	Carlsbad,CA
SPSS 15 for Windows	SPSS Inc.	Chicago,IL

5. Methods

5.1 Food Extracts

Foods and liquids were bought in grocery stores and local markets in Oslo. The flours were donated by the Norwegian Food Research Institute (Matforsk). Non-liquid foods were pulverized/homogenized in food-processor before extraction. To 10 g of the product, - liquids were added 10 ml methanol (MeOH), while dried foods were added 10 ml MeOH plus an additional 10 ml of MilliQ-water (MQ-water). In some cases a total amount of 20 ml MeOH and 20 ml MQ-water was used. Fruits and vegetables were added 10 ml MeOH plus MQ-water depending on the water-content of the actual food (i.e. apple contains 86 % water and thereby was added 10 ml MeOh and 10 ml – (10 ml*86 %) = 1.4 ml MQ-water). After vortexing, the samples were sonicated in a water bath at 0^{o}C for 30 minutes.

The extracts were transferred to 50 ml tubes, centrifuged at 10^{o}C at 4000 rpm for ten minutes, and the liquid phase transferred to Erlenmeyer flasks. This step was often performed twice to retrieve as much as possible of the supernatant. The supernatant was concentrated to a viscid fluid (≤4ml) under nitrogen gas. Concentrated extracts were diluted to a total amount of 5 ml in phosphate buffered saline (PBS) or dimethyl sulfoxide (DMSO)/PBS, depending on solubility, or corn oil for animal experiments. The extracts were then sterile filtered using a 0.2 µm filter and stored under argon gas in airtight tubes at -70^{o}C. The final concentration was 2 g original product per ml extract.

Hydrolysis of flour extracts

Phytochemicals in grains may be bioactive, however a substantial portion of these phytochemicals are bound and will not be extracted in a regular methanol:H_2O extraction. Hydrolysis will facilitate extraction of the bound phytochemicals, hence both hydrolysed and unhydrolysed extracts of grains were used in this study.

The hydrolysed flour extracts were donated by the Norwegian Food Research Institute (Matforsk), and all extraction procedures were performed by them.

Briefly, 0.2 g milled grain was extracted with 10 ml cold acetone/water (60 %) and shaken at 350 rpm for ten minutes. The solution was centrifuged at 2800 rpm and the acetone mixture was removed. Unbound phenolic compounds and FRAP was measured in this unhydrolysed solution.

The extract was hydrolysed with 10 ml 2 M sodium hydroxide (NaOH) over night, and the sample adjusted to a pH of 1.45-1.55 with 6 M hydrogen chloride (HCl) to make the phenols polar for ethylacetate extraction. The bound phenols were extracted with 10 ml ethylacetate, shaken for 10 minutes, and then centrifuged at 2800 rpm. The latter step was performed four times. If gelating occurred, which made the phenols less available, one drop 2 M HCl was added. The ethylacetate supernatants were collected and concentrated under vacuum. The resulting dried pellet was dissolved in 1 ml DMSO, and total phenols and FRAP content were measured (hydrolysed extract).

Extracts for in vivo imaging

Based on the results of the individual extracts on NF-κB activity in U937 cells, foods with a strong inhibitory effect on NF-κB activity were combined to make an extract for *in vivo* imaging in mice. The food extracts used in the *in vivo* experiments were made as described above, but instead of dissolving in DMSO, corn oil was used. The final extract consisted of green tea (0.2 g/ml), black tea (0.2 g/ml), red wine (2 g/ml) and dark chocolate (2 g/ml).

5.2 *In Vitro* Experiments

5.2.1 Cell Culture

The U937 cell line is a human monoblast cell line isolated from a person with histiocytic lymphoma[84]. These cells have characteristic features similar to cells in the monoblastic stage. They can be induced to terminal monocytic differentiation, and are thus similar to monocytes. Monocytes are a type of white blood cells made in the bone marrow. These are released into the bloodstream, where they circulate approximately 24 hours until they migrate into tissues and develop into macrophages. Both monocytes and macrophages are attracted to sites of inflammation and infection, and help combat foreign bodies.

The U937 3xκB-LUC cell line is a subclone of the U937 cell line. The U937 cell line has been stably transfected with a luciferase gene coupled to a promoter of three NF-κB- binding sites (**Figure 5.1**). Luciferase activity thus reflects the activity of NF-κB. The U937 3xκB-LUC cell line also contains a plasmid (pMEP4) with the hygromycin resistance gene. Hygromycin B is an antibiotic that kills bacteria, fungi and higher eukaryotic cells. Addition of hygromycin B secures that only cells with the hygromycin resistance gene, and thus the stably transfected cells, are selected.

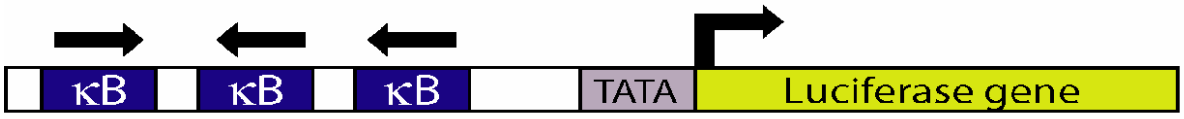


Figure 5.1 Illustration of the luciferase gene coupled to a promoter of three NF-κB binding sites stably transfected in the U937 3xkB-LUC cells. By courtesy of I. Paur.

The cells are stored frozen in liquid nitrogen with DMSO to prevent crystal formation during storage. Cells were quickly thawed in a water bath at 37°C, and DMSO was removed by changing the medium. The cells were kept in culture at least one week after thawing, before they were used in experiments. The cells were cultured at 37°C and 5 % CO_2 in RPMI 1640-medium supplemented with 10 % fetal bovine serum

(FBS), L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 mg/ml) and hygromycin (75 μg/ml).

5.2.2 Cell Counting

Every second to third day, the cell culture medium was changed. To maintain growth, the cells were counted and diluted to 0.4 million cells per ml. A haemocytometer was used to count the cells visually. The haemocytometer has two chambers, each containing nine squares. Each square is 1x1 mm, and the height of liquid when filled is 0.1 mm. The total volume of one square is 1 mm^3. Average number of cells in each square multiplied with 10^4 will therefore give the total number of cells per ml.

5.2.3 Experimental Outline

One day prior to the experiments, the cells were diluted to 0.4 million cells per ml in order to be in exponential growth phase for the experiment. The day of experiment, the old medium containing 10 % FBS was changed to new RPMI 1640-medium containing 2% FBS. The cell suspension was seeded in 24 well plates, with one ml cell suspension in each well.

Extracts were used in three different concentrations. To dilute the original extract of 2 g/ml, RPMI-1640 medium with 2% FBS was used. The medium was added DMSO in the same amount as in the extracts.

For measurements of basal NF-κB activity cells were incubated with extract in different concentrations or vehicle control for 6.5 hours. Lipopolysaccharide (LPS) induced NF-κB activity was also measured. LPS is an endotoxin, - a component of the outer membrane of Gram-negative bacteria, and will induce a strong response from the immune systems of human cells. LPS can bind to LPS-binding protein (LBP), which again will bind to the CD14/TLR4/MD2 receptor complex, and activate the NF-κB signalling pathway. For LPS-induced NF-κB activity, cells were pre-incubated with extract for 30 minutes, and then added LPS for an additional

incubation of 6 hours. Triplicates were performed of all concentrations and vehicle control, with and without LPS.

5.2.4 Luminescence

Six and a half hours after treatment of the cells with various extracts, luminescence was measured. Bioluminescence is light produced by living organisms, such as fireflies and marine algae among others. Before detection of luminescence, 10 µl luciferin was added to each well and incubated for five minutes. Luciferin is a small-molecule substrate for the enzyme luciferase. An activation of NF-κB will cause firefly luciferase to catalyze the reaction of luciferin to oxyluciferin in the presence of magnesium, ATP and oxygen. This will lead to emission of yellow light with peak intensity at 560 nm.

Luciferase has a high sensitivity as a reporter gene, and is therefore often used to analyze up- and down-regulation of promoters, such as NF-κB. The luminescence was measured by a Synergy 2 instrument, which measures relative luminescence units (RLU) in each well for the integration time (10 sec), and then reports the average data of these. The measured luminescence reflects the NF-κB activity.

5.2.5 Cell Viability

The method of trypan blue exclusion was used to measure cytotoxity. The cell membranes of non-viable cells are permeable to trypan blue. To 1 ml of cell suspension with extract, which had been incubated for 6.5 h with LPS, 50 µl trypan blue was added. The non-viable cells were then dyed blue, and hence could be distinguished from the viable cells.

The total number of viable and non-viable cells was counted, using a haemocytometer as explained above. The cut-off value was set to 10 % non-viable cells. Green and black tea showed high cytotoxicity at 3 mg/ml, hence these were tested at 0.15, 0.3 and 1.5 mg/ml. The unhydrolysed form of oat "Hurdal" was also toxic at concentrations of 30 and 15 mg/ml, and was tested at 0.3, 1.5 and 3 mg/ml. All other extracts were tested at 3, 15 and 30 mg/ml, except the hydrolysed grain extracts which were tested at 0.3, 1.5 and 3 mg/ml due to the high content of DMSO in the extract.

5.2.6 Preconditioning with LPS

It is possible that small episodes of increased NF-κB activity protect against later and stronger insults. This is a term called pre-conditioning, which is based on the theory that if cells are exposed to small insults, they will be protected against damaging effects when bigger insults occur. NF-κB has been shown to be involved in pre-conditioning effects, observed in sepsis[70], cerebral diseases[71] and coronary heart disease[69]. To see whether this is the case for NF-κB in the U937 3xκB-Luc cells, cells were incubated with different concentrations of LPS before NF-κB activation was assessed.

Cells were diluted to 0.4 million cells/ml and transferred to three 75 cm^3 cell culture flasks. To the first flask, no LPS was added, flask two was added 0.01 μg/ml LPS, and flask three 1.0 μg/ml LPS (**Figure 5.3**). The pre-conditioning was done over night (12 hours). The following day, the medium containing 10 % FBS was changed to medium containing 2 % FBS in all three flasks. Cells were seeded in 24-well plates, 1 ml in each well. From each flask, triplicates were added no LPS, 0.01 μg/ml LPS or 1.0 μg/ml LPS. The cells were incubated for 6 h before luminescence was measured as described in 5.2.4.

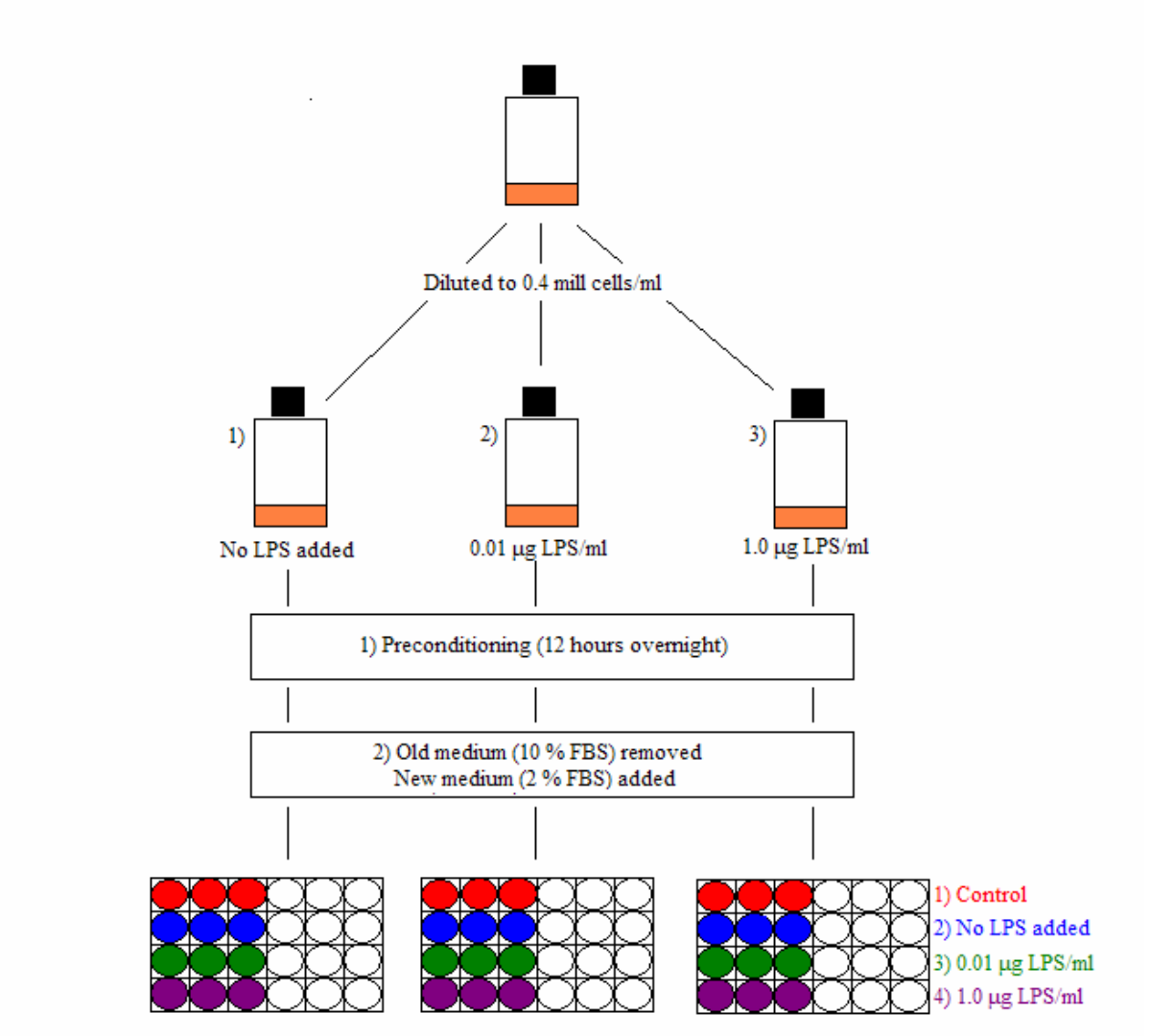


Figure 5.3 Pre-conditioning with LPS. Three different flasks of U937 3xκB-LUC cells were treated over night with either no LPS, or LPS at 0.01µg/ml or 1.0 µg/ml and then added LPS at different concentrations for additional 6 h before NF-κB activity was measured.

In Vivo Experiments

5.2.7 Transgenic Mice

Even though cell cultures are excellent tools for initial experiments, screening purposes and mechanistic experiments, conclusions regarding processes that occur in an intact organism cannot be drawn on the basis of these results. To extend the knowledge from *in vitro* results, the use of transgenic reporter mice is a good way to investigate regulation of genes. Non-invasive imaging provides the opportunity to perform several measurements of NF-κB activity in the same animal, and hence the animal can serve as its own control. This minimizes the number of animals needed for one experiment, and it reduces the effect of differences between animals.

For the *in vivo* experiments, transgenic mice carrying a transgene with three binding sites for NF-κB coupled to the luciferase gene were used. These mice have previously been described by Carlsen *et al*.[46]

5.2.8 Experimental Outline

Mice were fasted for 2 hours prior to the start of the experiment. Before imaging (-3 hours), mice were tube fed with 300 µl of a combination extract containing green tea (0.2 g/ml), black tea (0.2 g/ml), red wine (2 g/ml) and dark chocolate (2 g/ml). Three hours later, at 0 hours, *in vivo* imaging was again performed as previously described, whereupon the mice were injected with LPS (2.5 mg/kg) in 100 µl PBS subcutaneously on the back near the tail. *In vivo* imaging was thereafter performed 2, 4 and 6 hours after LPS injection, and the mice were sacrificed after imaging at 6h. Tissues were excised and intestine imaged *ex vivo* before the organs were transferred to separate tubes for storage at -70^{o}C for later analysis, as shown in **Figure 5.4**.

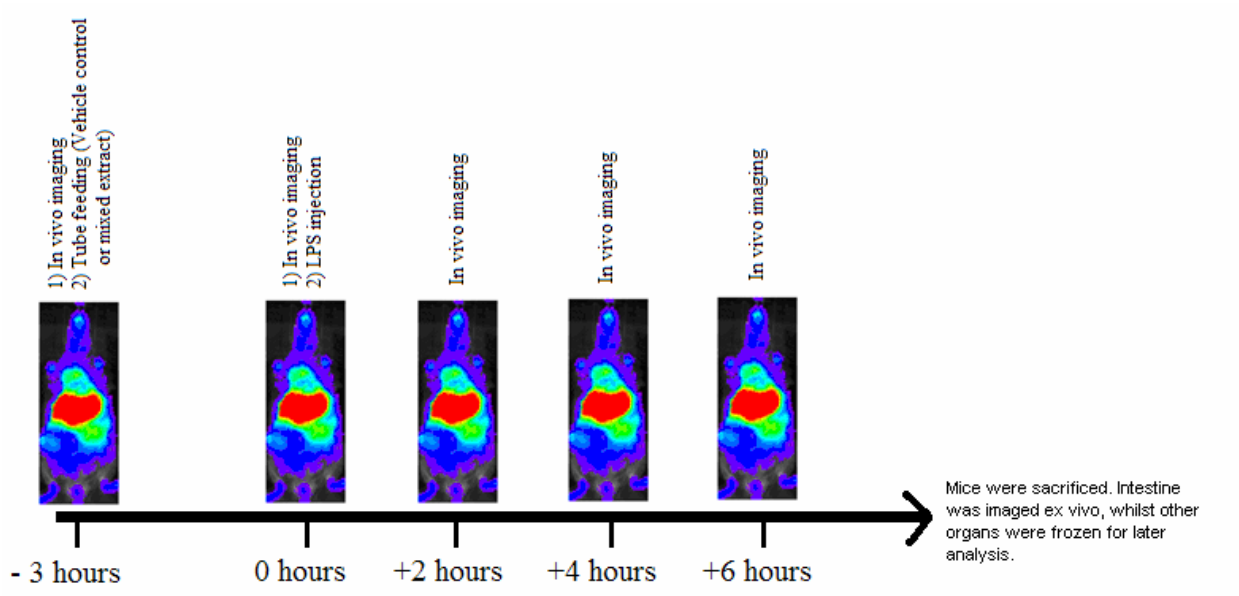


Figure 5.4 Experimental outline for *in vivo* imaging. Mice were fasted for 2 h prior to the experiment, before *in vivo* imaging. The mice were tube fed with vehicle control (corn oil) or mixed extract (green- and black tea, red wine and dark chocolate) 3 h prior to basal imaging. After basal imaging (0 h), mice were injected with LPS, and further imaged at 2, 4 and 6 h after LPS injection. Mice were after 6 h sacrificed and organs collected for *ex vivo* imaging and luciferase measurement in homogenates.

5.2.9 *In Vivo* Imaging

The mice were anesthetized with 2.5 percent isoflurane and shaved in the abdominal region before imaging. D-luciferin (160 mg/kg) was injected intraperitoneally 5 minutes before imaging. The mice were placed in a light-proof chamber. Imaging was performed in an IVIS 100 System, and the luminescence emitted from the mice was recorded for 1 minute. Isoflurane anesthesia was maintained inside the imaging chamber.

5.2.10 Luciferase Activity in Tissues

Luciferase activity was also measured in homogenates of organs in addition to the *in vivo* imaging.

Frozen organs were transferred to 5 ml tubes, and added 1 ml 1x Reporter lysis buffer each. To homogenize the tissues, ultra thorax cleaned with lysis buffer between each organ was used. The organs were constantly kept on ice. After being transferred to

Eppendorf tubes, the homogenates were centrifuged at 4^{o}C 13000 rpm for 15 minutes.

For luciferase measurement, 20 µl supernatant of each organ was collected, and then added 100 µl room tempered luciferase substrate solution (**Table 5.1**). The mixture was vortexed for 3 seconds before measuring luminescence in a TD 20/20 luminometer.

Table 5.1 Luciferase substrate solution

Reagent	Amount	Comments
ATP	52.1 mg	
Coenzyme A	20.7 mg	
Tricine	358.4 mg	
$MgSO_4$ x 7 H_20	92.2 mg	
DTT	513.5 mg	
EDTA 0.5 mg	15 µl	Final [0.075 mM]
dd H_2O	80 ml	
		pH adjusted to 7.4
Luciferin (20 mg/ml)	650 µl	Final [0.13 mg/ml]
		Volume adjusted to 100 ml

5.2.11 Protein Concentration

In order to normalize the luciferase activity to the total amount of protein in the tissue, protein concentration was measured. The method used is based on the Bradford protein assay. When the acidic solution of Coomassie Brilliant Blue binds to a protein, primarily basic and aromatic amino acid residues, a blue color with an absorbance maximum at 595 nm will be produced.

Triplicates from each organ and standards of known albumin concentrations were added to a 96-well plate, 10 µl in each well. One well contained a blank of MQ-water. Bio-Rad protein-measurement solution (200 µl) was added to each well. Absorbance was measured at 595 nm in a Titertek microplate reader.

Protein concentrations of the unknown samples were quantitative measured by comparison with the standard curve made from the protein standards of 0.041, 0.061, 0.091, 0.273 and 0.410 mg albumin/ml.

5.3 Effects of Food Extracts on Recombinant Luciferase

The luciferase reporter gene assay is a commonly used tool for measuring the activation of promotors or promoter regions, such as the NF-κB-DNA binding site, as activation is directly linked to the photons emitted by the luciferase reaction. There have been some reports that compounds may inhibit the activity of the luciferase enzyme itself, rather than the activity of the promoter to which it is coupled[85]. Resveratrol, for example, a phytochemical found in grape e.g., has been proposed to act directly on the enzymatic reaction, although other ways of inhibition may occur.

Recombinant luciferase was diluted to 1 ng/ml in RPMI-1640 medium containing 2 % FBS, L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 mg/ml). Extracts of green tea, black tea, red wine, cocoa 100 %, dark chocolate, apple, banana, Brussels sprouts, broccoli, Buckwheat "Lileja", hydrolysed wheat "Mjølner", hydrolysed barley "Olve" and hydrolysed oat "Hurdal" were diluted in the recombinant luciferase – RPMI-1640 solution to the same three concentrations used in the *in vitro* experiments.

To 20 µl of the sample, 100 µl room tempered luciferase assay solution (see **Table 5.1**) was added. The samples were vortexed for 3 seconds before luminescence was detected in a TD 20/20 luminometer.

5.4 FRAP Assay

The method of ferric reducing ability of plasma (FRAP), developed by Benzie and Strain[86], was used to measure the total reducing capacity of the extracts. The FRAP method measures the ability of a sample to reduce Fe^{3+} to Fe^{2+}, and thus is a more direct measurement of reducing capacity, compared to other tests.

At low pH in the presence of a reducing agent, such as an antioxidant, the TPTZ-Fe^{3+} complex will be reduced to TPTZ-Fe^{2+}. The measurement of reducing capacity is based on changes in absorbance, as the latter complex has an intense blue colour, with a maximum absorbance at 593 nm. Fe^{3+} is used in excess, so that the rate limiting step in the reaction is the reducing ability of the sample. The FRAP method is hence not specific to phytochemicals, as any substance with a reduction potential, will reduce Fe^{3+} to Fe^{2+}. Change in absorbance is thus directly related to the total reducing ability of the sample.

For measuring FRAP in the samples, a Technicon RA 1000 system was used. 10 µl of the sample was added to FRAP reagent (300 µl) (**Table 5.2**) that had been heated to 37^{o}C. Each sample was measured in triplicates. The mixture of sample and FRAP reagent was incubated for 4 minutes at 37^{o}C before absorbance was measured at 600 nm. An aqueous solution where the Fe^{2+} concentration was known, was used to calibrate the FRAP assay.

Table 5.2 FRAP reagents

Reagents	Component	Volume in FRAP
Acetate buffer	300 mM acetate buffer, pH 3.6 + $C_2H_4O_2$ 16 ml/l buffer solution	25 ml
HCl	40 mM	2.4 ml
$FeCl_3 \cdot 6\ H_20$	20 mM	2.5 ml
TPTZ solution	250 mg in 5 ml methanol	155 µl

5.5 Statistical Analysis

Data in this thesis are presented as mean ± SD when normally distributed or median (min-max) if data were not normally distributed.

To analyse data from cell culture experiments, One-Way ANOVA was used due to using more than two concentrations. Differences were identified using Dunnett comparisons. Statistically significant difference was defined as $P<0.05$.

In animal experiments the non-parametric Mann-Whitney statistical analysis was used as data were not normally distributed, and statistically significant different was defined as $P<0.05$.

Correlation between FRAP value and modulation of NF-κB: The parametric Pearson Correlation was used, and significance was set to $P<0.05$.

6. Results

6.1 *In Vitro* Experiments

6.1.1 Effects of Different Food Groups on NF-κB Activity

A screening of the effect of dietary plant extracts on NF-κB activation in U937 3xκB-LUC cells has previously been performed by the research group of Rune Blomhoff[25]. To increase the knowledge of food items with the ability to modulate NF-κB activity, a number of foods and beverages common in the Norwegian diet were chosen, and extracts were made. The effects of extracts were tested both on basal and LPS-induced NF-κB activity. Each extract was tested at three different concentrations.

Unless otherwise noted, the figures represents the mean of three different experiments (n=3). The figures present percentages of the control, where control cells were treated with vehicle only in the experiments of basal NF-κB activity, or with vehicle and LPS in the experiments of LPS-induced NF-κB activity.

Fruits and vegetables

The group of fruits and vegetables consisted of apple, banana, broccoli, brussels sprouts and sesame seed bought in grocery stores in Oslo. The extracts were tested in three different concentrations, 3, 15 and 30 mg/ml, and compared to vehicle control.

Apple

Apple (**Figure 6.1 A**) significantly induced basal NF-κB activity at two out of three concentrations. At 15 mg/ml the activity was increased to 130 % (P=0.027) of the control cells incubated with vehicle only, and at 30 mg/ml the NF-κB activity had raised to 139 % (P=0.007).

Apple gave a reduction in LPS-induced NF-κB activity at both 15 mg/ml (77 %) (P=0.001) and 30 mg/ml (60 %) (P<0.001).

Banana

Banana (**Figure 6.1 B**) increased basal NF-κB activity at the two highest concentrations. An increase to 161 % (P<0.001) compared to control was seen at 15 mg/ml, and an increase to 164 % (P<0.001) could be seen at 30 mg/ml.

No statistically significant modulation of LPS-induced NF-κB activity was found when incubating cells with extract of banana.

Broccoli

Basal NF-κB activity increased when incubating U937 3xκB-LUC cells with broccoli extract (**Figure 6.1 C**). This increase was however only significantly different from control cells, incubated with vehicle only, at the highest concentration of 30 mg/ml (136 % increase) (P=0.017).

An increase in LPS-induced NF-κB activity was seen when adding broccoli at the lowest concentrations. The increase was the same at 3 and 15 mg/ml (151 %) (P=0.001 for both). At 30 mg/ml, however a strong reduction in NF-κB activity, to 49 % compared to control (P=0.001), was detected.

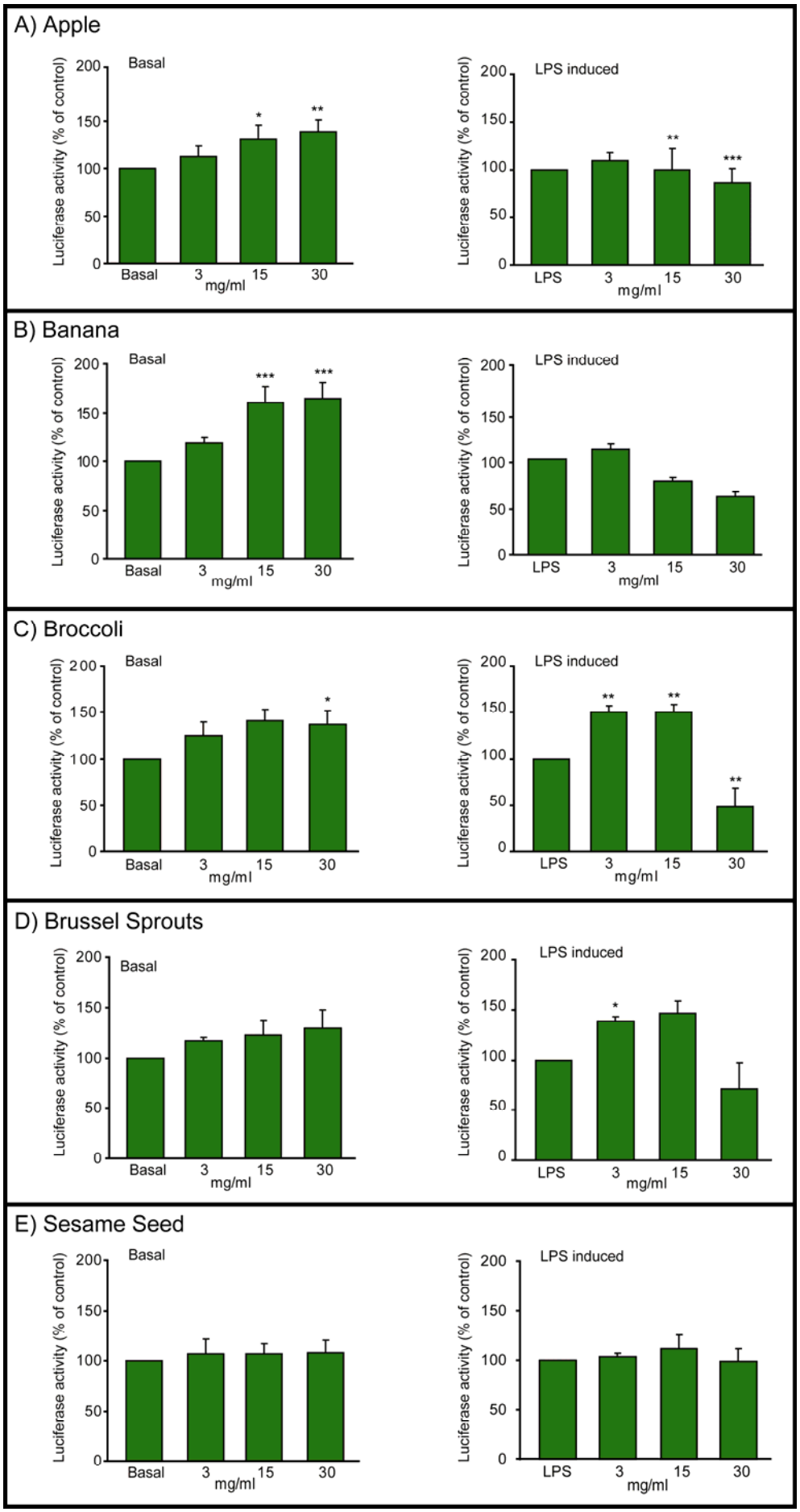

A) Apple
Basal
LPS induced
Luciferase activity (% of control)
200
150
100
50
0
*
**
***
Basal
LPS
3
15
30
mg/ml
B) Banana
C) Broccoli
D) Brussel Sprouts
E) Sesame Seed

Figure 6.1 Effect of extracts of fruits and vegetables on basal and LPS induced NF-κB activity. U937 3xkB-LUC cells were treated with extracts of various fruits and vegetables and incubated for 6.5h to see whether a modulation of basal NF-κB activity occurred. Cells incubated with extracts for 0.5h, then added LPS, were used to see whether the extracts modulated LPS induced NF-κB activity. Each bar represents mean ± SD of 3 experiments (n=3) run in triplicates ±SD, except for banana (n=4). * = P<0.05, ** = P<0.01, *** = P<0.001

Brussel sprouts

Incubation with extracts of brussel sprouts (**Figure 6.1 D**) did not significantly modulate basal NF-κB activity in U937 3xκB-LUC cells.

When cells were co-incubated with extract of brussel sprouts and LPS, an elevated NF-κB activation of 139 % of control levels was found at 3 mg/ml (P=0.027). The highest concentration of brussels sprouts extract seemingly decreased NF-κB activation to 71 % of controls, however this reduction was not statistically significant (P=0.098).

Sesame seed

No significant changes in basal or LPS induced NF-κB activity was seen when treating cells with the extract of sesame seed (**Figure 6.1 E**).

Beverages and liquids

The group of beverages comprised of green tea, black tea, red wine, skimmed milk (0.1 % fat) and whipping cream (38 % fat). Red wine, milk and whipping cream were tested at 3, 15 and 30 mg/ml. The extracts of green and black tea were tested at concentrations of 0.15, 0.3 and 1.5 mg/ml, due to cytotoxicity at 3, 15 and 30 mg/ml.

Green tea

No significant changes in basal NF-κB activity was seen when U937 3xκB-LUC cells were treated with the green tea extract (**Figure 6.2 A**).

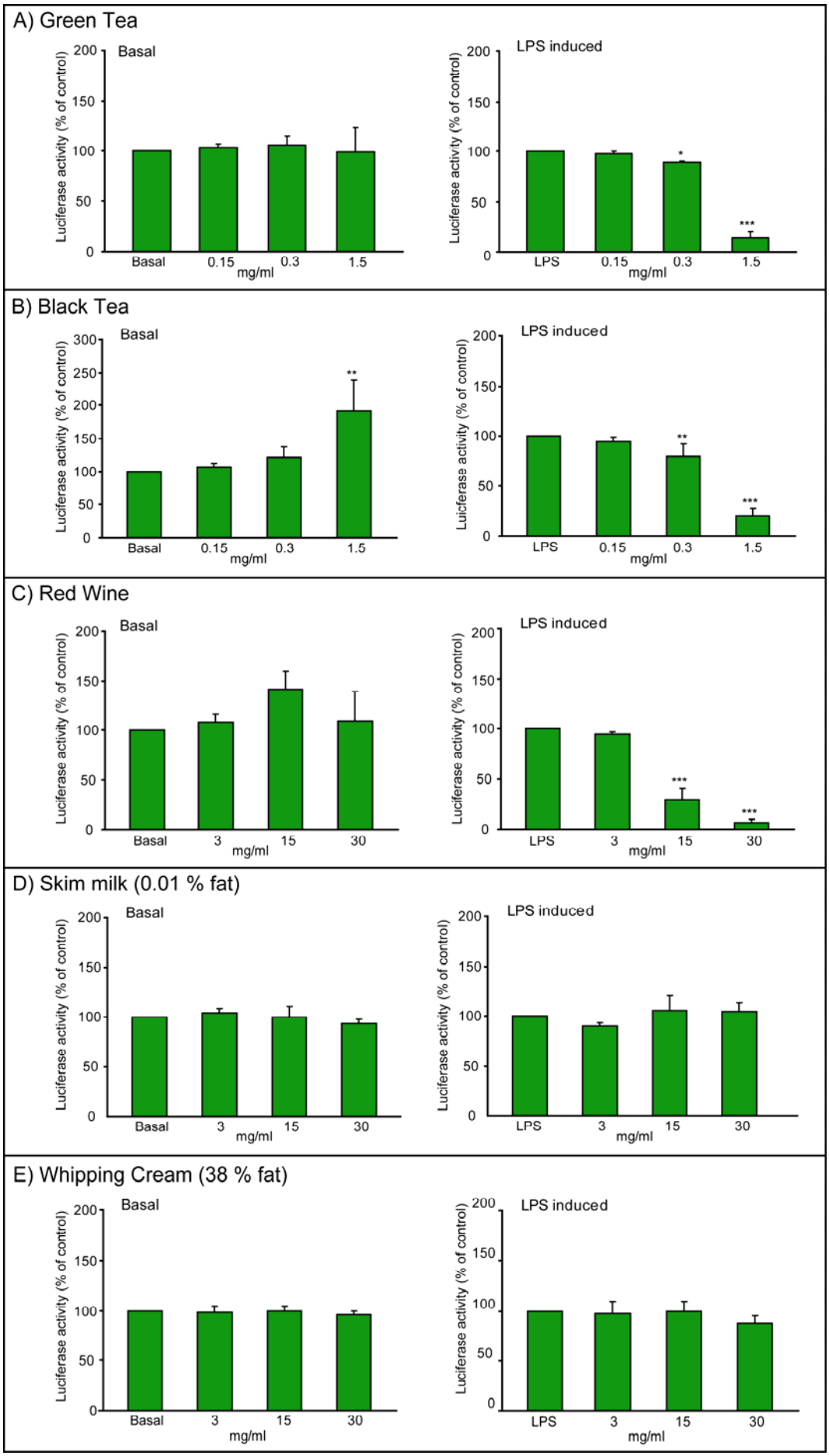

A) Green Tea
Basal
LPS induced
Luciferase activity (% of control)
200
150
100
50
0
Basal
0.15
0.3
1.5
LPS
mg/ml
*
***
B) Black Tea
300
250
**
C) Red Wine
3
15
30
D) Skim milk (0.01 % fat)
E) Whipping Cream (38 % fat)

Figure 6.2 Effect of extracts of beverages and liquids on basal and LPS induced NF-κB activity. U937 3xkB-LUC cells were incubated with extracts of various beverages and liquids for 6.5h to see whether a modulation of basal NF-κB activity occurred. Extracts were tested for their effect on LPS induced NF-κB activity by incubating cells with extracts for 0.5h and then added LPS for 6 additional hours. Each bar represents mean ±SD of 3 experiments (n=3) run in triplicates, except for green (n=6) and black tea (n=4). * = P<0.05, ** = P<0.01, *** = P<0.001

Extract of green tea gave a significant decrease in LPS-induced NF-κB activation to 88 % (P=0.015) of control at 0.3 mg/ml. At 1.5 mg/ml a further decrease in LPS induced NF-κB activity was detected, with an activity of only 14 % (P<0.001) compared to vehicle control cells incubated with LPS and vehicle only.

Black tea

At the two lowest concentrations, the black tea extract (**Figure 6.2 B**) did not significantly affect basal NF-κB activity in U937 3x-kB-LUC cells. At 1.5 mg/ml a significant increase to 192 % (P=0.001) of control cells was detected.

A decrease in LPS-induced NF-κB activity was seen at the two highest concentrations of black tea extract. At 0.3 mg/ml LPS-induced NF-κB activity reduced to 79 % (P=0.006) of control, and a strong inhibitory ability was seen at 1.5 mg/ml, where LPS-induced NF-κB activity was only 19 % (P<0.001) compared to controls.

Red wine

No statistically significant modulation of basal NF-κB activity was seen when cells were incubated with the red wine extract (**Figure 6.2 C**).

Red wine did not affect LPS-induced NF-κB activity at the lowest concentration, however the extract exhibited a strong ability to inhibit NF-κB activity induced by LPS at the two highest concentrations. At 15 mg/ml LPS-induced NF-κB activity was reduced to 30 % (P<0.001), and at 30 mg/ml activity further decreased to only 7 % (P<0.001) compared to controls.

Skim milk (0.1 % fat)

When cells were incubated with the skim milk (0.1 % fat) extract (**Figure 6.2 D**), no statistically significant changes were achieved concerning modulation of basal or LPS-induced NF-κB activity in the U937 3xkB-LUC cells.

Whipping cream (38 % fat)

As with the skim milk (0.1 % fat), no statistically significant changes in basal or LPS-induced NF-κB activity was seen when the cells were treated with extract of whipping cream (38 % fat) (**Figure 6.2 E**).

Fish and meat

The fish and meat group included white meat (chicken), red meat (beef), cod and salmon. The extracts were tested at 3, 15 and 30 mg/ml.

Chicken

The chicken extract (**Figure 6.3 A**) did not significantly modulate basal or LPS-induced NF-κB activity in the U937 3xκB-LUC cells at any concentrations.

Beef

Incubation with the beef extract (**Figure 6.3 B**) did not significantly modulate basal NF-κB activity in the cells.

At the two lowest concentrations, the extract of beef did not affect LPS-induced NF-κB activity, however a statistically significant modulation was seen at 30 mg/ml, where LPS-induced NF-κB activity was 85 % (P=0.017) compared to control cells treated with LPS and vehicle.

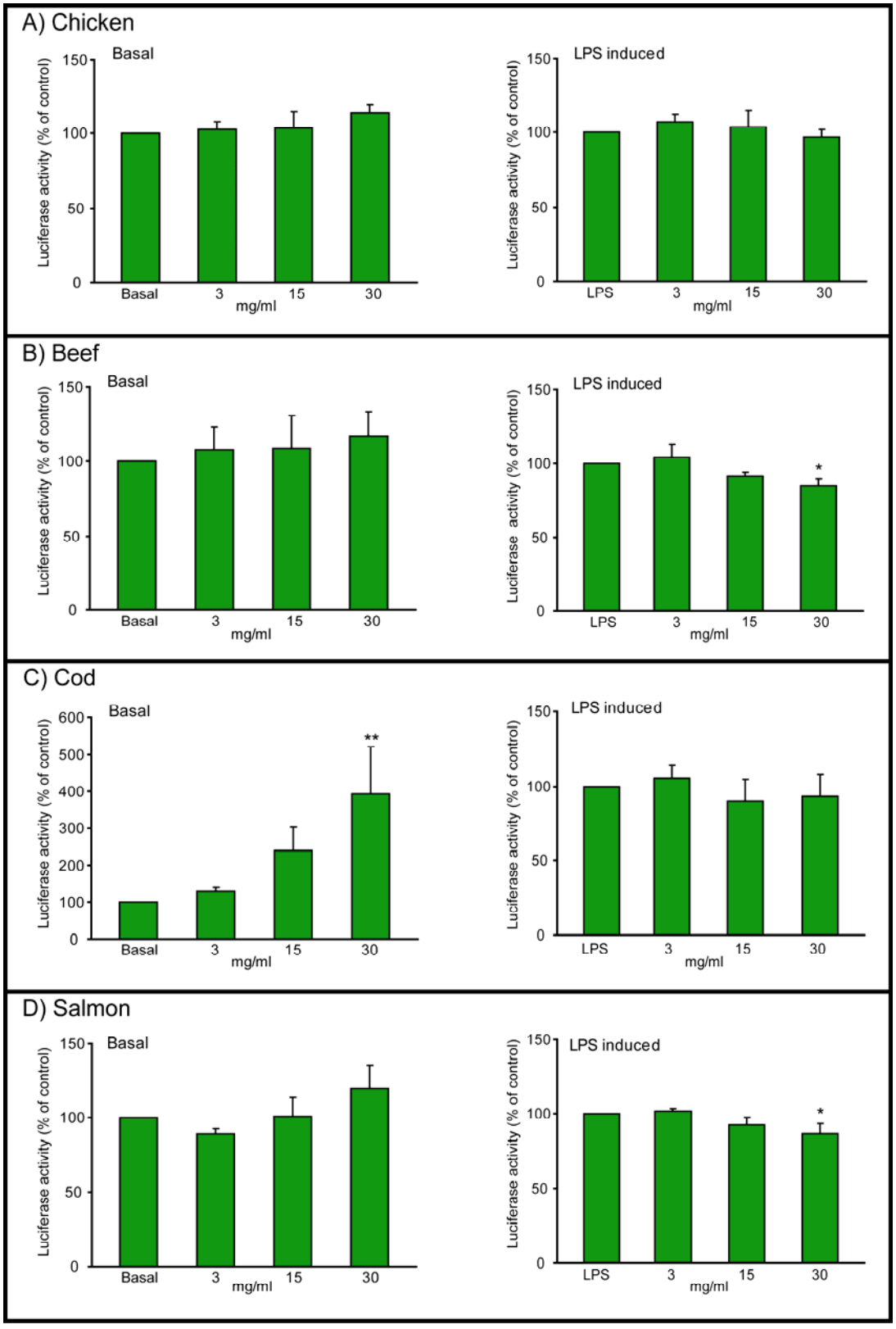


Figure 6.3 Effect of extracts of meat and fish on basal and LPS induced NF-κB activity. U937 3xkB-LUC cells were treated with extracts of various meat- and fish products and incubated for 6.5h to see whether a modulation of basal NF-κB activity occurred. Cells incubated with extracts for 0.5h, then added LPS, were used to see whether the extracts modulated LPS induced NF-κB activity. Each bar represents mean ± SD of 3 experiments ran in triplicates. * = P<0.05, ** = P<0.01, *** = P<0.001

Cod

Cod (**Figure 6.3 C**) increased basal NF-κB activity at all concentrations, however this increase was only statistically significant at 30 mg/ml, with an increase to 393 % (P=0.003) compared to controls.

No statistically significant modulation of LPS-induced NF-κB activity was seen when treating cells with the cod extract.

Salmon

Salmon (**Figure 6.3 D**) did not significantly affect basal NF-κB activity in the U937 3xκB-LUC cells at any concentration.

The highest concentration of salmon extract however reduced LPS-induced NF-κB activity significantly to 86 % (P=0.016) compared to control cells treated with LPS and vehicle.

Chocolate and cocoa products

The group of chocolate and cocoa products included two types of cocoa powder; a regular baking cocoa and a pure cocoa powder (cocoa 100 %), in addition to a dark chocolate with a cocoa content of 70 %. All extracts were tested at 3, 15 and 30 mg/ml.

Baking cocoa

Baking cocoa (**Figure 6.4 A**) increased basal NF-κB activity in the U937 3xκB-LUC cells, however this increase was only significant at 15 mg/ml, at which activity was 293 % (P=0.011) compared to control cells.

A significant reduction in LPS-induced NF-κB activity was seen with the two highest concentrations of the baking cocoa extract. The activity was 66 % (P=0.025) and 27 % (P<0.001) of control at 15 and 30 mg/ml respectively.

Pure cocoa (100 %)

A significant increase in basal NF-κB activity was seen at all three concentrations of the pure cocoa (100 %) extract (**Figure 6.4 B**). At the lowest concentration (3 mg/ml), NF-κB activity was 187 % (P=0.005) of control, and at 15 mg/ml there was a further increase to 312 % (P<0.001). At 30 mg/ml NF-κB activity was 188 % (P=0.004) compared to control cells.

As with baking cocoa, no significant reduction in LPS-induced NF-κB activity in the U937 3xκB-LUC cells was seen at the lowest concentration of pure cocoa. A statistically significant reduction in LPS-induced NF-κB activity was however detected at 15 and 30 mg/ml, where activity was 24 % (P<0.001) and 8 % (P<0.001) respectively of control cells treated with LPS and vehicle.

Dark chocolate

Dark chocolate (**Figure 6.4 C**) increased basal NF-κB activity to 167 % (P=0.018) of control at 3 mg/ml and to 169 % (P=0.016) at 15 mg/ml. At the highest concentration (30 mg/ml) the increase was not significant.

The dark chocolate extract had a remarkably strong ability to inhibit LPS-induced NF-κB activity. At 3 mg/ml activity was reduced to 83 % (P=0.024) compared to controls, however the NF-κB activity further decreased to 14 % (P<0.001) at 15 mg/ml and 4 % (P<0.001) at 30 mg/ml.

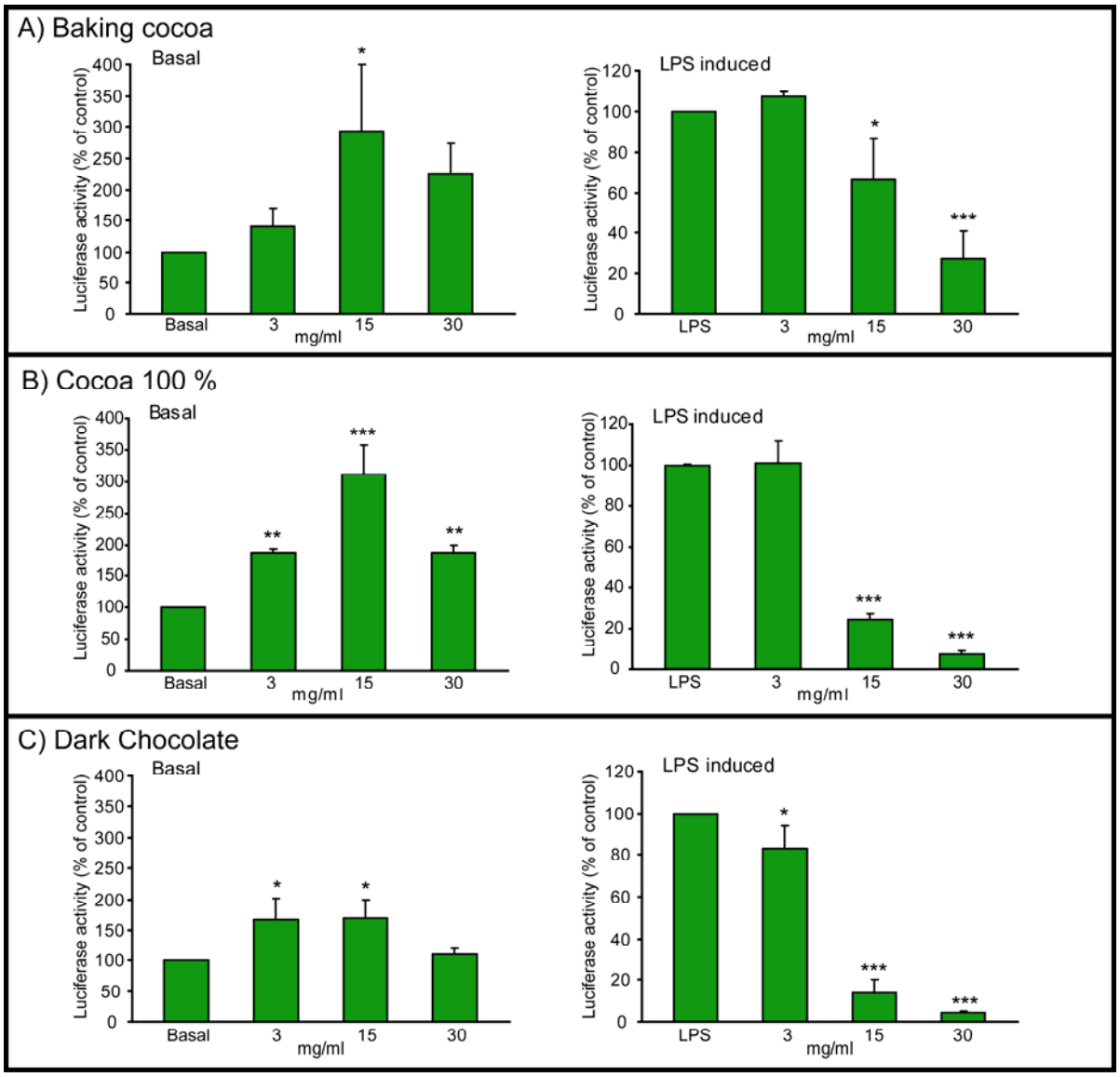


Figure 6.4 Effect of extracts of chocolate and cocoa products on basal and LPS induced NF-κB activity. U937 3xkB-LUC cells were treated with extracts of chocolate and various cocoa products and incubated for 6.5h to see whether an effect on basal NF-κB activity occurred. Modulation of LPS induced NF-κB activity was tested by incubating cells with extracts for 0.5 h, then adding LPS for additional 6 h. Each bar represents mean ± SD of 3 experiments run in triplicates. * = P<0.05, ** = P<0.01, *** = P<0.001

Grains

A substantial portion of the phytochemicals in grains will not be extracted by a methanol:H_2O extraction, however these "bound phytochemicals" can be extracted by hydrolysis. Both unhydrolysed and hydrolysed extracts of grains were thus tested for effects on NF-κB activity in U937 3xκB-LUC cells. Five types of grains were tested; wheat "Standard", wheat "Mjølner", buckwheat "Lileja", barley "Olve" and oat "Hurdal".

All unhydrolysed extracts, except oat "Hurdal", were testet at 3, 15 and 30 mg/ml. Oat "Hurdal" showed cytotoxicity at the two highest concentrations, hence this extract was tested at 0.3, 1.5 and 3 mg/ml. The hydrolysed extracts contained high concentrations of DMSO and were tested at 0.3, 1.5 and 3 mg/ml to not exceed 1.5 ‰ DMSO in the final cell culture.

Wheat "Standard"

The unhydrolysed extract of wheat "Standard" (**Figure 6.5 A**) increased basal NF-κB activity in U937 3xκB-LUC cells, however a significant increase was only seen at 15 and 30 mg/ml with an increase of 134 % (P=0.018) and 138 % (P=0.009) respectively compared to controls.

No significant modulation of LPS-induced NF-κB activity was seen with the unhydrolysed wheat "Standard" extract.

Also the hydrolysed extract of wheat "Standard" (**Figure 6.5 B**) increased basal NF-κB activity at the two highest concentrations, with a basal NF-κB activity of 169 % (P=0.003) at 1.5 mg/ml and 163 % (P=0.006) at 3 mg/ml compared to control cells incubated with vehicle only.

The hydrolysed wheat "Standard" extract significantly increased LPS-induced NF-κB activity at 1.5 mg/ml, with an increase of 132 % (P=0.044) compared to control cells.

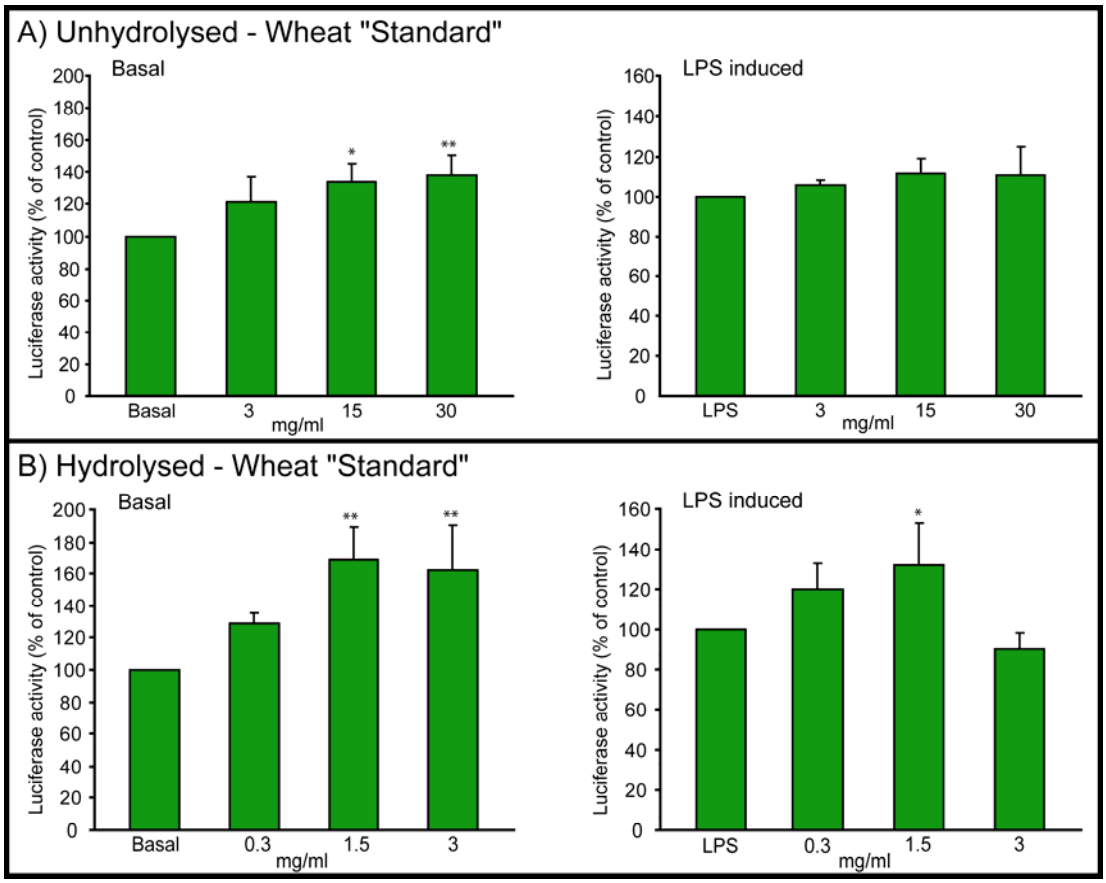


Figure 6.5 Effect of extracts of wheat "Standard" on basal and LPS induced NF-κB activity. Extracts of wheat "Standard" was added to U937 3xkB-LUC cells and incubated for 6.5 h to measure effect on basal NF-κB activity. Cells incubated with extract for 0.5 h, then added LPS, were used to see whether the extracts modulated LPS induced NF-κB activity. A) Unhydrolysed wheat "Standard", B) Hydrolysed wheat "Standard". Each bar represents mean ± SD of 3 experiments run in triplicates. * = P<0.05, ** = P<0.01, *** = P<0.001

Wheat "Mjølner"

Basal NF-κB activity increased significantly to 143 % (P=0.009) compared to control cells when treated with the unhydrolysed extract of wheat "Mjølner" (**Figure 6.6 A**) at 30 mg/ml.

No significant modulation of LPS-induced NF-κB activity was seen with the unhydrolysed form of wheat "Mjølner".

The hydrolysed extract of wheat "Mjølner" (**Figure 6.6 B**) increased basal NF-κB activity at the two highest concentrations. At 1.5 mg/ml basal NF-κB activity was 149 % (P=0.002) compared to control cells, and at 3 mg/ml the increase was 132 % (P=0.024).

A strong modulation of LPS-induced NF-κB activity was seen at the highest concentration (3 mg/ml) of the wheat "Mjølner" extract, as activity decreased to 67 % (P=0.016) of control cells. No effect was seen at the two lowest concentrations.

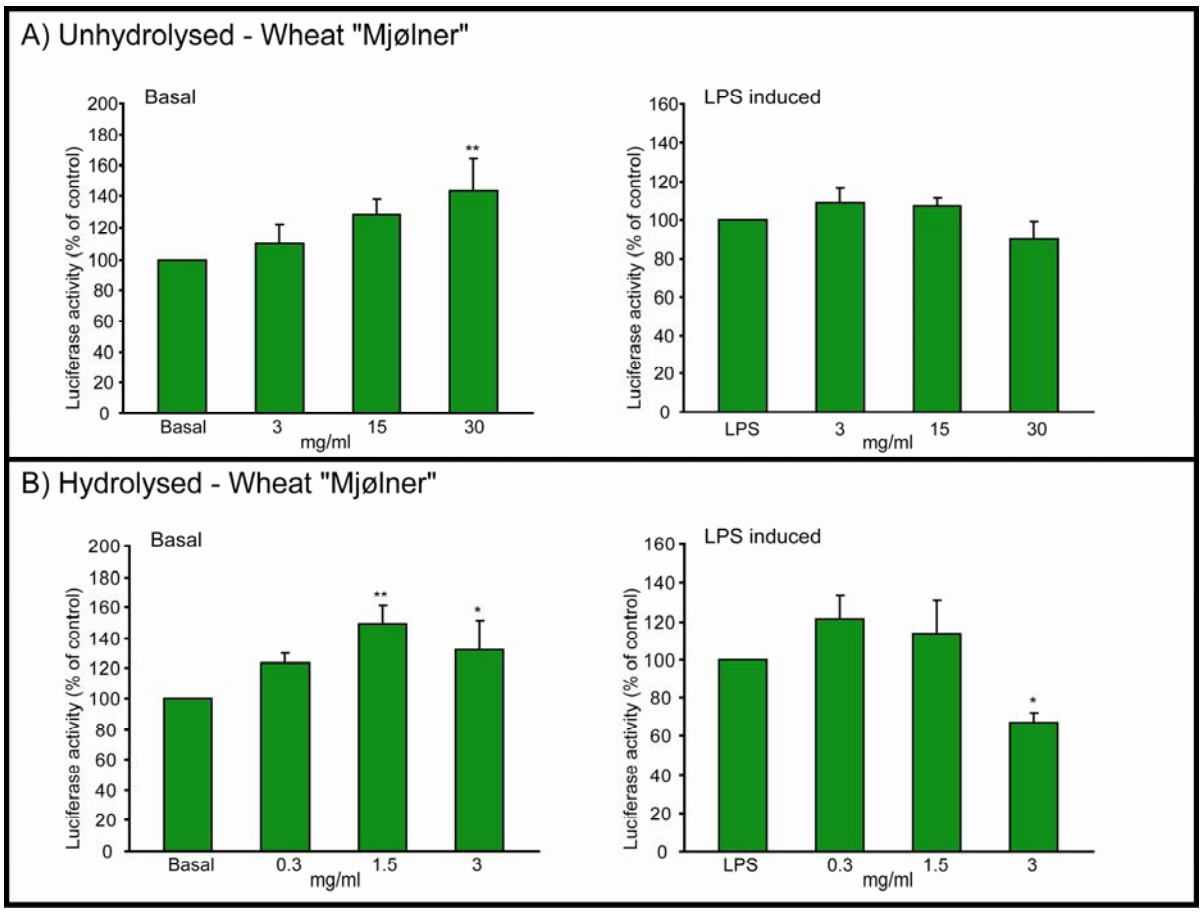


Figure 6.6 Effect of extracts of wheat "Mjølner" on basal and LPS induced NF-κB activity. U937 3xkB-LUC cells were treated with extracts of wheat "Mjølner" and incubated for 6.5 h to see whether a modulation of basal NF-κB activity occurred. Cells incubated with extracts for 0.5 h, then added LPS for additional 6 h, were used to see whether the extracts modulated LPS induced NF-κB activity. A) Unhydrolysed wheat "Mjølner", B) Hydrolysed wheat "Mjølner". Each bar represents mean ± SD of 3 experiments run in triplicates. * = P<0.05, ** = P<0.01, *** = P<0.001

Buckwheat "Lileja"

No statistically significant modulation of basal NF-κB activity was seen with the extract of the unhydrolysed buckwheat "Lileja" (**Figure 6.7 A**).

An increase in LPS-induced NF-κB activity was detected with the unhydrolysed form of buckwheat "Lileja" at 15 mg/ml, with an activity of 106 % (P=0.046) compared to control cells. At 30 mg/ml however a decrease in LPS induced NF-κB activity, to 70 % (P<0.001) of control cells, was seen.

The hydrolysed form of buckwheat "Lileja" (**Figure 6.7 B**) did not modulate neither basal or LPS induced NF-κB activity in the U937 3xκB-LUC cells.

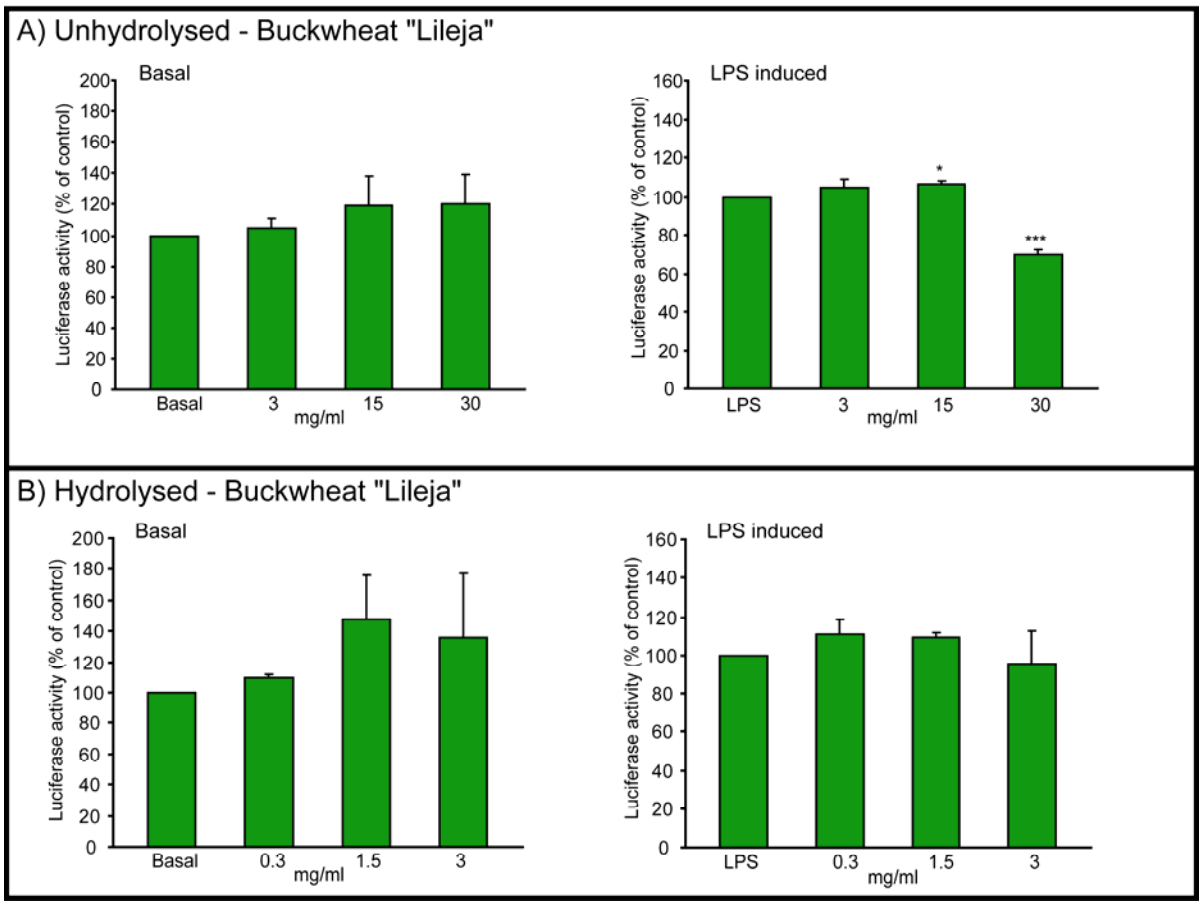


Figure 6.7 Effect of extracts of buckwheat "Lileja" on basal and LPS induced NF-κB activity. Extracts of buckwheat "Lileja" was added to U937 3xkB-LUC cells for 6.5 h before basal NF-κB activity was detected. Cells incubated with extracts for 0.5h, then added LPS, were used to see whether the extracts modulated LPS induced NF-κB activity. A) Unhydrolysed buckwheat "Liljea", B) Hydrolysed buckwheat "Lileja". Each bar represents mean ± SD of 3 experiments run in triplicates. * = P<0.05, ** = P<0.01, *** = P<0.001

Barley "Olve"

Incubation with extracts of the unhydrolysed barley "Olve" (**Figure 6.8 A**) increased basal NF-κB activity at the two highest concentrations, to 209 % (P=0.004) and 282 % (P<0.001) at 15 and 30 mg/ml respectively.

Unhydrolysed barley "Olve" also increased LPS-induced NF-κB activity at the two highest concentrations. At 15 mg/ml LPS-induced NF-κB activity was 118 % (p=0.034), and at 30 mg/ml activity was 119 % (P=0.022) of cells treated with LPS and vehicle.

When cells were treated with the hydrolysed form of barley "Olve" (**Figure 6.8 B**), no statistically significant modulation was seen on either basal or LPS-induced NF-κB activity.

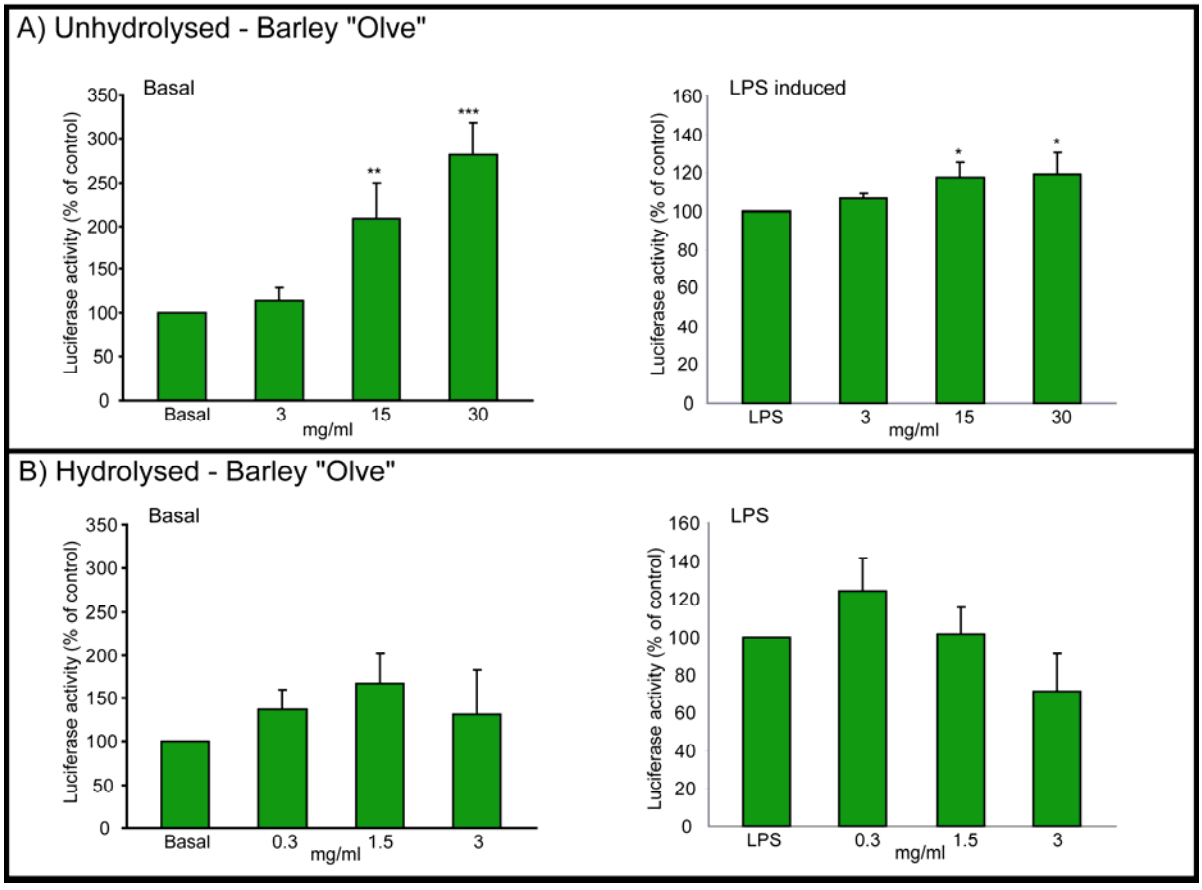


Figure 6.8 Effect of extracts of barley "Olve" on basal and LPS induced NF-κB activity. U937 3xkB-LUC cells were treated with extracts of barley "Olve" and incubated for 6.5h to see whether a modulation of basal NF-κB activity occurred. Cells incubated with extracts for 0.5 h, then added LPS, were used to see whether the extracts modulated LPS induced NF-κB activity. A) Unhydrolysed barley "Olve", B) Hydrolysed barley "Olve". Each bar represents mean ± SD of 3 experiments run in triplicates. * = P<0.05, ** = P<0.01, *** = P<0.001

Oat "Hurdal"

Unhydrolysed extract of oat "Hurdal" (**Figure 6.9 A**) significantly increased basal NF-κB activity to 132 % (P=0.003) and 145 % (P<0.001) at 1.5 and 3 mg/ml respectively.

An increase in LPS-induced NF-κB activity was seen when treating cells with the two highest concentrations of the unhydrolysed oat "Hurdal"extract, to 125 % (P=0.020) and 127 % (P=0.008) at 1.5 and 3 mg/ml respectively, compared to control cells.

No significant modulation of basal NF-κB activity in the U937 3xκB-LUC cells was seen when treating the cells with the hydrolysed form of oat "Hurdal" (**Figure 6.9 B**). A reduction in LPS-induced NF-κB activity was seen with the highest concentration (3 mg/ml) of the hydrolysed oat "Hurdal" extract to 39 % (P=0.127) of control cells, however this reduction was not statistically significant.

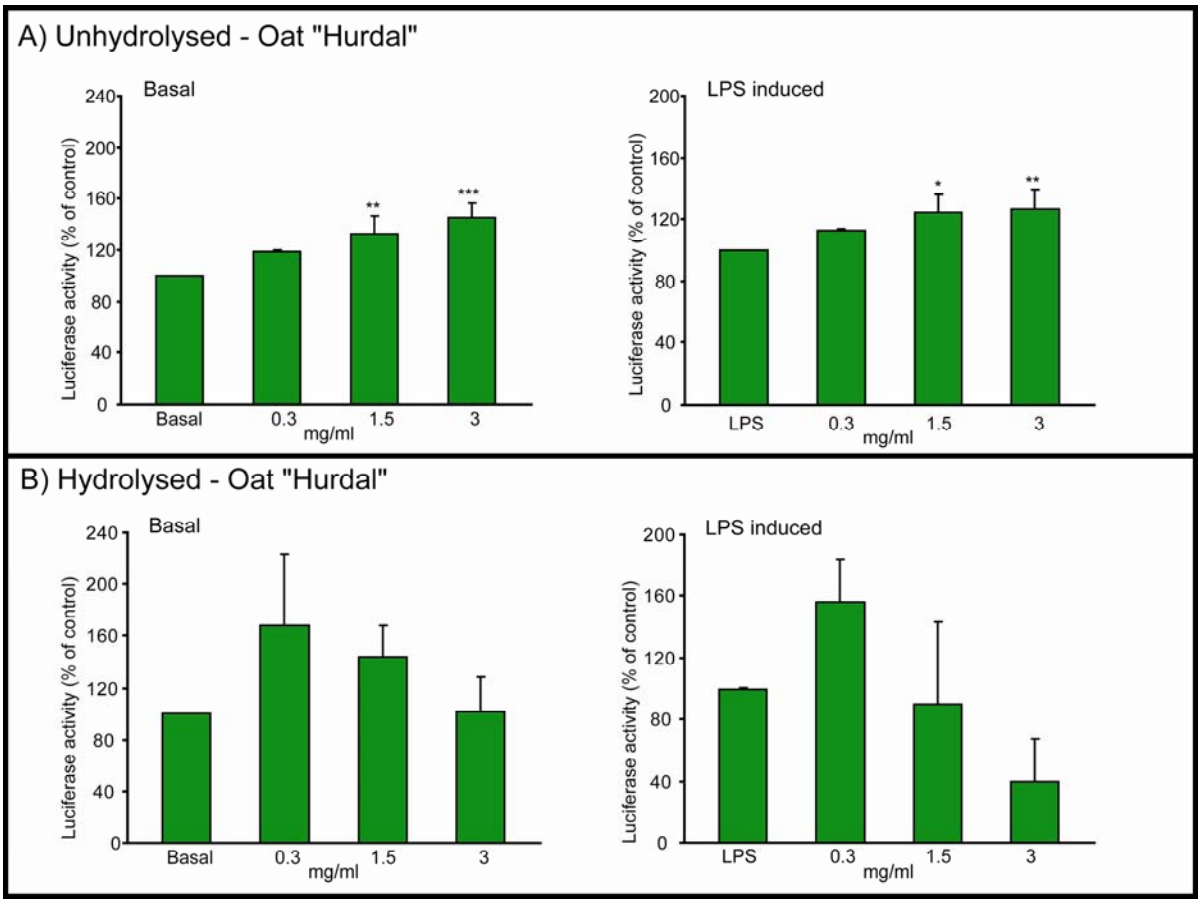


Figure 6.9 Effect of extracts of oat "Hurdal" on basal and LPS induced NF-κB activity. U937 3xkB-LUC cells were treated with extracts of oat "Hurdal" and incubated for 6.5h to before detection of basal NF-κB activity. Effect on LPS induced NF-κB activity was measured by incubating cells with extracts for 0.5 h, then adding LPS for additional 6 h. A) Unhydrolysed oat "Hurdal", B) Hydrolysed oat "Hurdal". Each bar represents mean ± SD of 3 experiments run in triplicates. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$

Mixed extract

The mixed extract consisted of foods whose extracts showed a significant reduction in LPS-induced NF-κB activity in the *in vitro* experiments above. The extract hence consisted of green tea (0.2 g/ml), black tea (0.2 g/ml), dark chocolate (2 g/ml) and red wine (2 g/ml), and was tested at several concentrations to investigate whether a dose-response effect on LPS-induced NF-κB activity could be seen. Cytotoxicity was detected at concentrations above 3 mg dark chocolate and red wine, and 0.3 mg/ml green and black tea/ml, hence the mixed extract was tested at 0.1, 0.3, 1, 1.5 and 3 mg/ml (referring to the concentration of dark chocolate and red wine).

The mixed extract (**Figure 6.10**) remarkably reduced LPS-induced NF-κB activity at the three highest concentrations. At 1 mg/ml LPS-induced NF-κB activity was reduced to 67 % (P=0.012) compared to controls. Activity further decreased to 45 % (P<0.001) and 24 % (P<0.001) at 1.5 and 3 mg/ml respectively, compared to control cells incubated with LPS and vehicle.

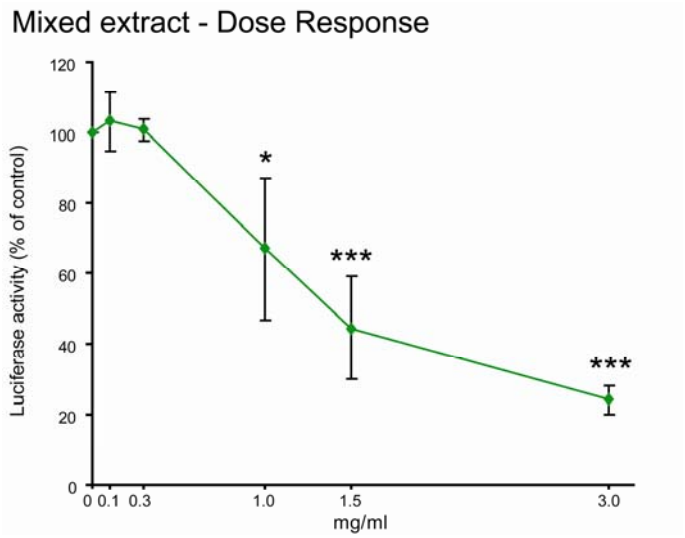


Figure 6.10 Dose-response relationship on LPS induced NF-κB activity after incubation with mixed extract. A mixed extract of red wine (2 g/ml), dark chocolate (2 g/ml), green tea (0.2 g/ml) and black tea (0.2 g/ml) was added to U937 3xkB-LUC cells in different concentrations (0, 0.1, 0.3, 1, 1.5 and 3 mg/ml). After 30 min incubation, LPS was added to the cells and incubated additionally 6 h before luminescence was measured to see whether a dose-response effect on NF-κB occurred. Each bar represents mean ± SD of 3 experiments run in triplicates. * = P<0.05, ** = P<0.01, *** = P<0.001

6.1.2 Pre-conditioning with LPS

Many of the extracts tested above increased basal NF-κB activity in U937 3xkB-LUC cells. To test whether a small increase in NF-κB activity may protect against later stronger insults, a pre-conditioning experiment was carried out. Cells were diluted and split into three flasks, where the first flask received no LPS, whilst the two others were added 0.01 μg LPS/ml and 1 μg LPS/ml respectively. The cells were incubated overnight (12h). Cell culture medium was changed and cells from each 12h treatment were consequently added no LPS, 0.01 μg LPS/ml or 1 μg LPS/ml and incubated for an additional 6h. Cells without pre-incubation and not treated with LPS the following day are referred to as control.

The cells that had not been treated with LPS overnight significantly increased their NF-κB activity in a dose-dependent manner when LPS was added for the last 6h (**Figure 6.11 Light grey bars**), with a fold increase of 2047 ± 292 % ($P<0.001$) and 2904 ± 235 % ($P<0.001$) respectively for 0.01 and 1 μg LPS/ml. This is in concordance with results in other studies[25].

Within the two batches of cells pre-incubated with either 0.01 (**Figure 6.11 Dark grey bars**) or 1 μg/ml (**Figure 6.11 Black bars**) LPS overnight, no significant differences in NF-κB activity were seen, even though these cells were incubated with no LPS, 0.01 or 1 μg/ml LPS for the final 6h of incubation. The cells that were pre-incubated with the lowest LPS concentration (0.01 μg/ml) however had significantly lower NF-κB activity than all variants of the cells pre-incubated with LPS at 1 μg/ml ($P<0.001$ for all three differences).

For the batches of cells incubated with no LPS for the final 6h (**Figure 6.11 First set of bars**) a significant difference in NF-κB activity was detected between the cells pre-incubated with 1 μg/ml LPS and the cells pre-incubated no or 0.01 μg/ml LPS concentration ($P<0.001$ for both). No significant increase in NF-κB activity was seen in the cells pre-incubated with the lowest LPS concentration (0.01 μg/ml) compared to cells incubated with no LPS overnight.

The most interesting finding was that cells pre-incubated with LPS at both 0.01 and 1 µg/ml had dramatically lower LPS induced NF-κB activity compared to cells pre-incubated with no LPS (**Figure 6.11, 2nd and 3rd set of bars**). Also, independent of treatment for the final 6h, cells pre-incubated with LPS at 0.01 µg/ml had significantly lower NF-κB activity than cells pre-incubated with LPS at 1 µg/ml. Cells incubated with 0.01 µg LPS/ml for the first 12 h and 1 mg/ml for the final 6 h only increased their NF-κB activity to 684 ± 127 % ($P<0.001$) compared to 2904 ± 235 % ($P<0.001$) in the cells pre-incubated with no LPS, and added LPS at 1 µg/ml for the final 6 h. For the highest LPS-concentration of pre-incubation (1 µg/ml) the increase in NF-κB activity was 1247 ± 97 % ($P<0.001$). Similar results were achieved when cells were pre-incubated with LPS at 0.01 µg/ml.

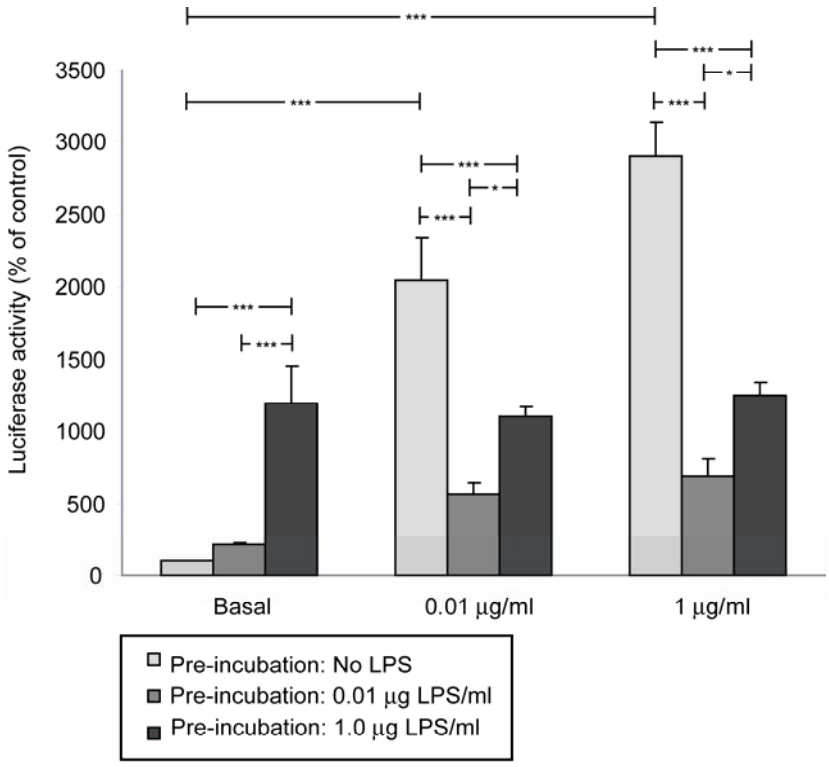


Figure 6.11 Preconditioning with LPS. U937 3xκB-LUC cells were pre-incubated with no LPS or LPS at 0.01 and 1 µl/ml for 12h, medium was changed, each overnight batch dicvided in three and cells were incubated an additional 6h with either no LPS, or LPS at 0.01 or 1 µg/ml. LPS-induced NF-κB activity was measured in a Synergy 2 Plate-reader. Bars represent mean ± SD. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$. Pre-conditioning refers to the first 12 h of incubation, while the text under the bars represents the last 6 h of incubation.

6.2 *In Vivo* Experiment

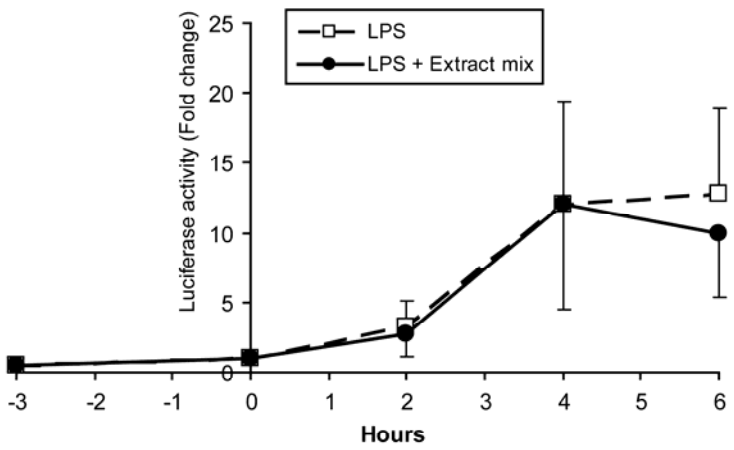
Next we investigated whether the mixed extract could modulate NF-κB activation *in vivo* in NF-κB-luciferase reporter mice.

6.2.1 *In Vivo* Imaging

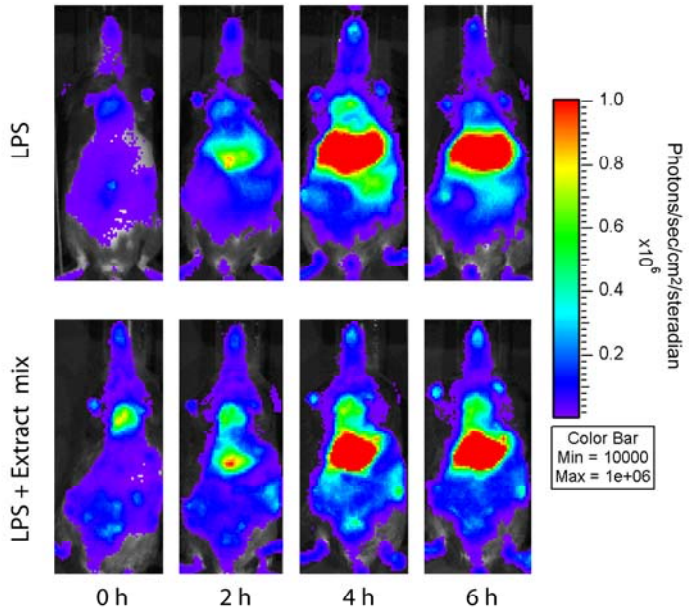
Transgenic NF-κB luciferase mice were given the mixed extract containing green tea (0.2 mg/ml), black tea (0.2 mg/ml), red wine (2 g/ml) and dark chocolate (2 g/ml) or vehicle control (corn oil) 3 h prior to s.c. LPS-injection. *In vivo* images were taken before tube feeding, at the time of LPS injection (0h) and at 2, 4 and 6 h after injection. LPS-induced NF-κB activity in these transgenic mice to a maximum of 12.8 ± 6.1 fold increase at 6 h compared to 0 h (**Figure 6.12 A**). In vivo images taken from one representative control and extract mice at 0, 2, 4 and 6 h are shown in **Figure 6.12 B.**

The area under the curve (AUC) of the fold change of photons emitted from the whole mouse from 0 to 6 h after LPS injection was calculated (**Figure 6.12 C**). This gives a measure of the total response to the treatments over the entire time period. For the mice receiving the mixed extract, the median AUC was 34.23 [16.01 - 86.84], compared to 42.48 [16.86 - 82.21] for the mice fed vehicle control prior to LPS injection. Though a slight reduction in AUC was seen, the difference was not statistically significant (p=0.753).

A


LPS
LPS + Extract mix
Luciferase activity (Fold change)
25
20
15
10
5
0
-3
-2
-1
0
1
2
3
4
5
6
Hours

B


LPS
LPS + Extract mix
1.0
0.8
0.6
0.4
0.2
x10^6
Photons/sec/cm2/steradian
Color Bar
Min = 10000
Max = 1e+06
0 h
2 h
4 h
6 h

C

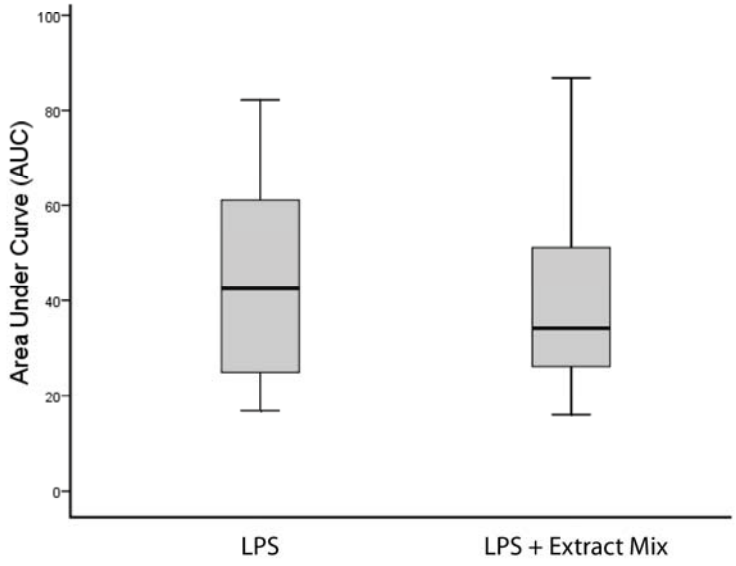

Area Under Curve (AUC)
100
80
60
40
20
0
LPS
LPS + Extract Mix

Figure 6.12 In vivo imaging of mice tube fed with vehicle control (corn oil) or mixed extract (green- and black tea, red wine and dark chocolate). A) Transgenic mice were tube fed with control or extract 3 h prior to LPS injection. The fold increase in NF-κB activity compared to 0 h is shown for both groups. Mean ± SD B) In vivo image of one representative mouse from each group at 0, 2, 4 and 6 h. Photons/sec/cm^2/steradian C) Box plot for the area under the curve (AUC) based on the fold change of photons/sec/cm^2/steradian for mice in the control (n=8) or extract (n=8) group.

6.2.2 *Ex Vivo* Imaging

The *in vivo* images only represent the luciferase activity in the whole mouse and can not distinguish between the different organs. Hence the *in vivo* imaging was combined with *ex vivo* imaging of the intestine, as well as measurement of luciferase activity in tissue homogenates.

To examine luciferase activity in separate organs, mice were sacrificed 6 h after LPS-injection and the intestines were imaged *ex vivo*.

The luminescence was quantified for the small intestine (**Figure 6.13 A**) and revealed that NF-κB activation was only 45 ± 17 % (p=0.059) compared to that in control mice receiving corn oil only, however these results were only close to statistically significant. *Ex vivo* images of one representative intestine from mice receiving LPS and mixed extract and mice receiving LPS and control vehicle only, are shown in **Figure 6.13 B**.

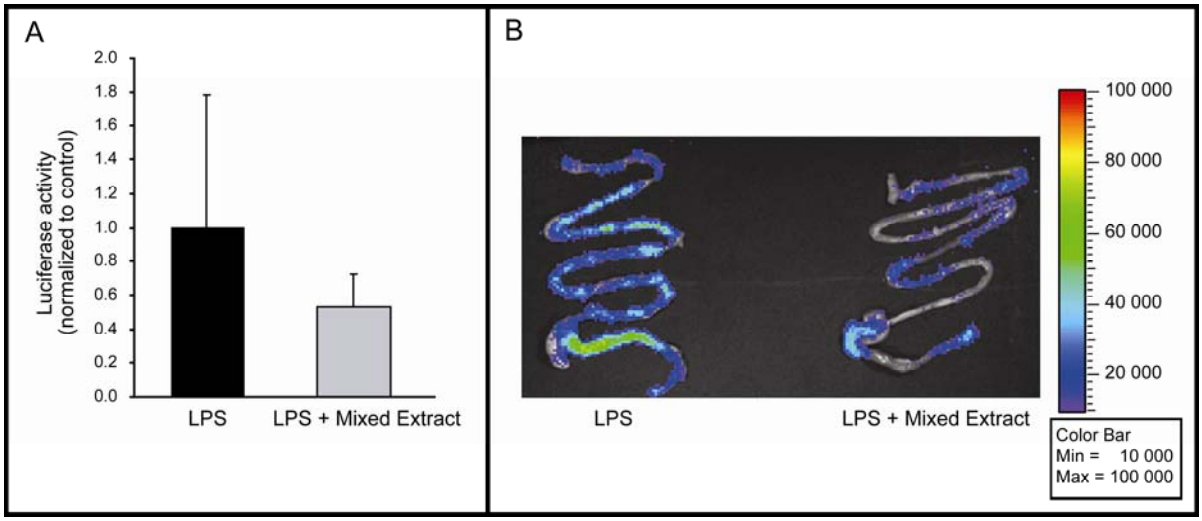


Figure 6.13 *Ex vivo* imaging of intestine. Six hours after LPS injection, mice were sacrificed. A) *Ex vivo* luminescence in small intestine was compared between the mice receiving LPS and mixed extract (n=8) and mice receiving LPS and control vehicle only (n=8) Mean ± SD.B) Ex vivo image of one representative intestine from each group. Photons/sec/cm^2/steradian

6.2.3 NF-κB Activity in Tissue Homogenates

Organs that had been stored at -70°C after the mice were sacrificed, were homogenized for measurement of NF-κB activity. The NF-κB activity was adjusted to the total protein content in each homogenate.

NF-κB activity in ovary (**Figure 6.14**) from mice receiving the mixed extracts was decreased to 48 % (P=0.345) relative to ovaries from control mice receiving vehicle only, however this was not statistically significant. All other organs of mice fed the extract mix expressed NF-κB activity close to the activity of organs of control mice, and neither of these changed significantly.

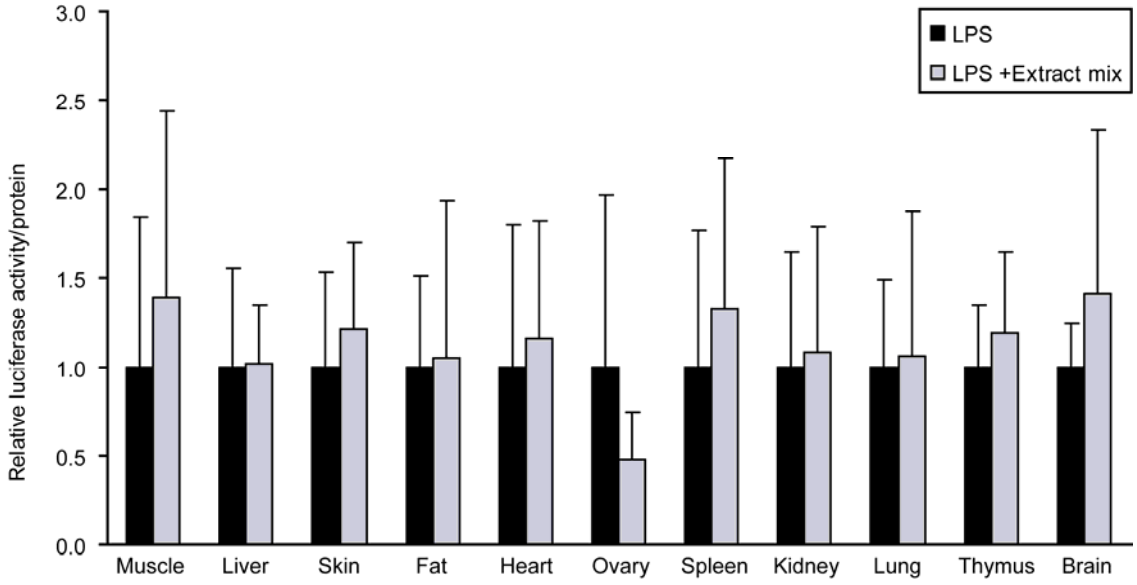


Figure 6.14 NF-κB activity in tissue homogenates. Luciferase activity was measured in tissue homogenates. Luciferase activity is presented as % of controls. Bars represent mean ± SD (n=8).

6.3 Effects of Food Extracts on Recombinant Luciferase

In the U937 3xκB-LUC cells, NF-κB activity is reported through the production and activity of the enzyme luciferase, leading to the conversion of luciferin to oxyluciferin and emission of photons. To test whether the extracts used could affect the activity of the luciferase enzyme itself, recombinant luciferase was utilized.

Thirteen extracts, the most potent inhibitors from the cell culture screening, were tested in the same concentrations as used in the cell culture experiments.

All tested extracts exhibited the ability to decrease recombinant luciferase activity in a dose dependent manner (**Figure 6.15**). The most prominent inhibitors were green- and black tea, red wine, pure cocoa (100 %) and dark chocolate, with nearly no detectable luminescence at the highest concentrations of extract. Even at the lowest concentrations (0.15 mg/ml), addition of green- and black tea extracts produced only 9.6 % and 8.1 % respectively of luminescence compared to control of recombinant luciferase only. Red wine, pure cocoa (100 %) and dark chocolate produced respectively 12.0 %, 21.5 % and 16.8 % of the luminescence compared to controls at 3 mg/ml, which was the lowest concentration tested for these extracts. The hydrolysed forms of barley “Olve” and oat “Hurdal” affected recombinant luciferase activity to a lesser extent, here the production was respectively 74.5 % and 75.3 % compared to control at 0.3 mg/ml. All extracts, except broccoli, were significantly reduced compared to control at all three concentrations ($P<0.001$). Broccoli was reduced to 84.7 % ($P=0.005$) and 76.0 % ($P=0.047$) at 3 and 30 mg/ml respectively, however the reduction seen at 15 mg/ml was not significant. It should be noticed, that only recombinant luciferase, and not intact cell cultures, was used in this experiment.

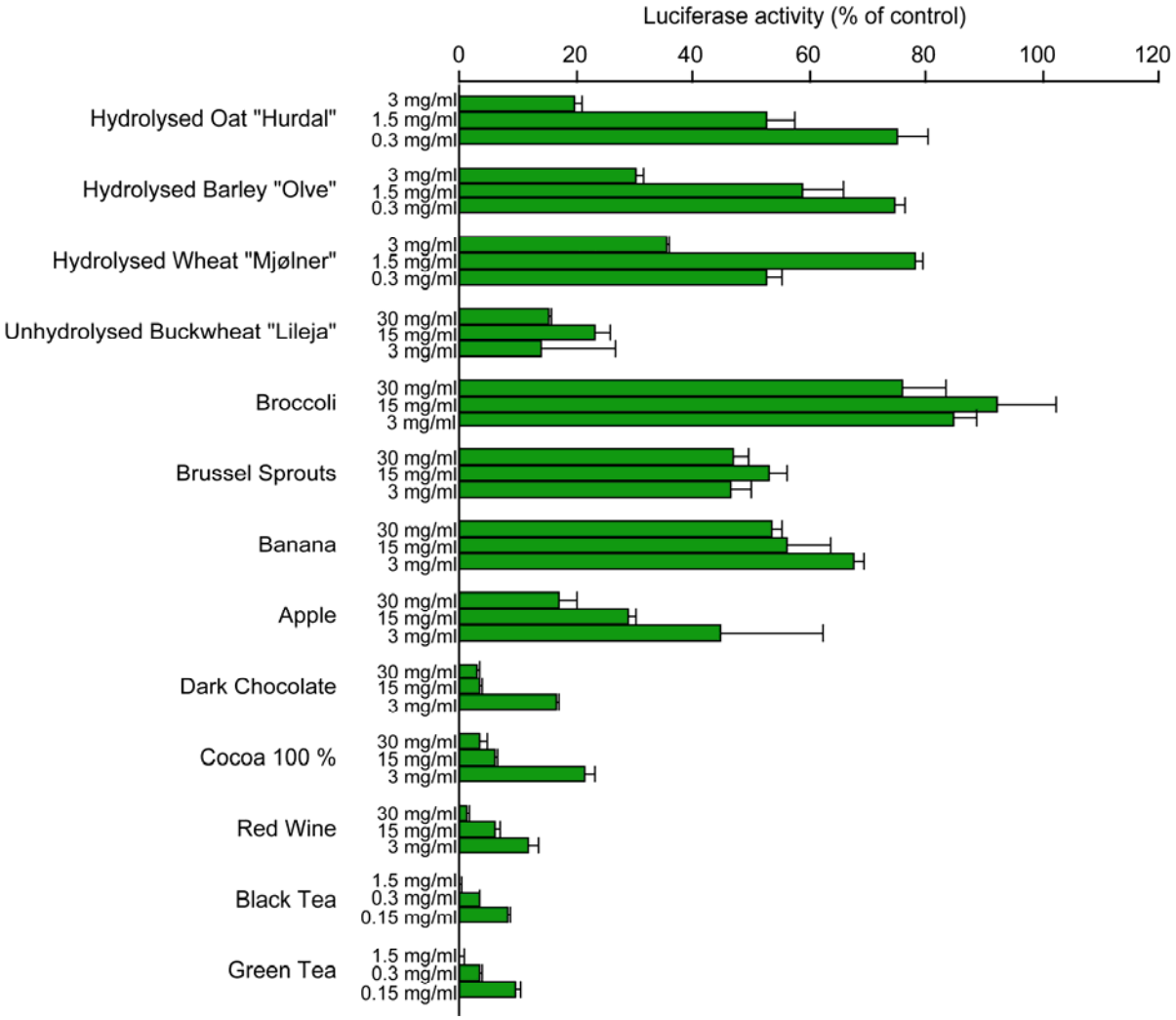


Figure 6.15 Effects of food extracts on recombinant luciferase. Extracts of the most potent inbibitors of LPS induced NF-κB activity was tested on recombinant luciferase in the same concentrations as used in the in vitro studies. All extracts had significantly reduced activity compared to control, except broccoli at 15 mg/ml.

6.4 FRAP Measurements

Total antioxidant content in fruits, vegetables, berries, herbs and cereals among others have previously been screened[14,25,81,87]. These results have revealed a big difference in total antioxidant content among food items. Here extracts of foods representative of different food groups in the Norwegian diet have been screened for total antioxidant content, and investigated to see whether there is a correlation between the total antioxidant content and the ability to modulate NF-κB activity.

The FRAP content of all extracts, except the grains and the mixed extracts, are listed in **Table 6.1**. There is more than a 1000-fold difference in the FRAP-values between the extracts. The food group with the lowest FRAP-values is the group containing meat and fish, here almost no reducing capacity was detected. As a large number of antioxidants in foods are phytochemicals, the low antioxidant capacity of meat and fish might not be surprising, since phytochemicals are plant and fruit derived compounds.

The vegetables broccoli and brussels sprouts had the highest FRAP-values in their group, with FRAP-values of 0.77 ± 0.01 and 0.67 ± 0.01 mmol/100 g respectively.

The beverages and liquids group showed large variations in FRAP-values. Almost no reducing capacity was detected in the extracts of skimmed milk (0.1 % fat) and whipping cream (38 % fat), however green- and black tea were the food items with the highest FRAP value tested, with 79.17 ± 8.87 and 56.18 ± 1.35 mmol/100 g respectively. Also red wine had high FRAP-values, with 3.37 ± 0.06 mmol/100 g. It should be noted, however that green- and black tea were the only food items tested in dry weight, i.e. 10 g of dried black- and green tea were used to make the extract, compared to 10 g red wine from the bottle.

The group of chocolate and cocoa products also revealed high FRAP-values, with pure cocoa (100 %) as the item with the highest value at 12.24 ± 0.27 mmol/100 g.

Table 6.1 FRAP values of extracts of fruits and vegetables, beverages and liquids, fish and meat, and chocolate and cocoa products.

Extract	FRAP (mmol/100 g) Mean (n=3)	SD (mmol/100 g)
Fruits and vegetables		
Apple	0.36	0.01
Banana	0.16	0.00
Broccoli	0.77	0.01
Brussels sprouts	0.67	0.01
Sesame seed	0.13	0.00
Beverages and liquids		
Green tea	79.17	8.87
Black tea	56.18	1.35
Red wine	3.37	0.06
Milk (0.1 % fat)	0.01	0.00
Cream	0.03	0.00
Fish and meat		
White meat	0.06	0.01
Red meat	0.03	0.00
Cod	0.03	0.00
Salmon	0.04	0.00
Chocolate and cocoa products		
Baking cocoa	8.48	0.61
Cocoa 100 %	12.24	0.27
Dark chocolate	7.62	0.28

The grains used in this thesis were donated from the Norwegian Food Research Institute (Matforsk). Both hydrolysed and unhydrolysed extracts were tested for antioxidant capacity (**Table 6.2**). The FRAP values of pure flours had previously been tested, and are also shown in **Table 6.2** with the other results. For all species, the hydrolysed extracts' antioxidant capacity were higher than the unhydrolysed ones, furthermore there was a slight tendency that also the pure flours had higher FRAP values than the unhydrolysed extracts. Buckwheat "Lileja" and oat "Hurdal" in

their hydrolyzed forms were the extracts with the highest reducing capacity, with a FRAP value of 6.63 ± 0.12 and 4.82 ± 0.12 respectively. Wheat "Standard" was not tested for antioxidant capacity as a flour, only as extracts.

Table 6.2 FRAP values of extracts of grains.

Grain		FRAP (mmol/100 g) Mean (n=3)	SD (mmol/100 g)
Wheat "Standard"	Unhydrolysed	0.09	0.00
	Hydrolysed	0.41	0.04
	Flour	-	-
Wheat "Mjølner"	Unhydrolysed	0.17	0.00
	Hydrolysed	2.09	0.15
	Flour	0.17	0.04
Barley "Olve"	Unhydrolysed	0.60	0.02
	Hydrolysed	3.22	0.09
	Flour	1.93	0.05
Buckwheat "Lileja"	Unhydrolysed	1.12	0.01
	Hydrolysed	6.63	0.12
	Flour	2.52	0.05
Oat "Hurdal"	Unhydrolysed	0.17	0.00
	Hydrolysed	4.82	0.12
	Flour	0.23	0.04

Based on the results from the *in vitro* screening, mixed extracts were made. The extracts consisted of red wine and dark chocolate (2 g/ml of each), and green and black tea (0.2 g/ml). Two different kinds of the extract was made – one solved in DMSO for *in vitro* testing, and one solved in corn oil to see whether the extract could modulate LPS induced NF-κB activity *in vivo*. Despite the same concentration of foods, the extract solved in DMSO had a higher FRAP value (31.65 ± 2.38) than the extract for in vivo testing (23.77 ± 1.51), however the reason is unclear (**Table 6.3**).

Table 6.3 FRAP value of the mixed extract.

Extract	FRAP (mmol/100 g) Mean (n=3)	SD (mmol/100 g)
Mixed extract for cells	31.65	2.38
Mixed extract for	23.77	1.51

6.4.1 Correlation Between FRAP and NF-κB Activity in U937 3xκB-LUC Cells

Many of the compounds shown to modulate NF-κB activity, have a high antioxidant capacity[25], however it is uncertain whether the antioxidant content is the main contributor to NF-κB modulation. In this thesis, both modulation of NF-κB activity and FRAP values were measured, hence a possible relationship between these two parameters was examined.

A linear correlation was used, making scatter plots of FRAP values and the extracts' ability to modulate NF-κB activity. Both correlation between basal and LPS induced NF-κB activity versus FRAP values were investigated.

No correlation was found between FRAP and the ability to modulate basal NF-κB activity ($R=0.117$, $P=0.561$) (**Figure 6.16 A**), however there was a significant inverse correlation between the ability to modulate LPS induced NF-κB activity and FRAP ($R=-0.739$, $P<0.001$) (**Figure 6.16 B**).

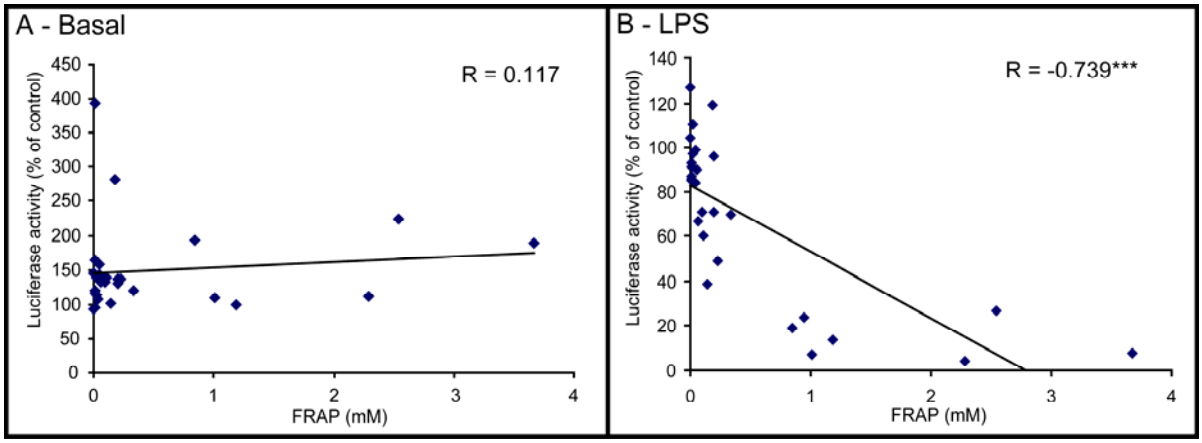


Figure 6.16 Correlation between FRAP and NF-κB activity. FRAP values versus A) basal or B) LPS induced NF-κB activity. r = Pearson correlation coefficient. *** = P<0.001.

7. Discussion

Epidemiological studies have shown that there is a link between chronic inflammation and chronic diseases, such as cancer[67]. It seems that NF-κB, a transcription factor important for immunity and inflammation, plays a pivotal role in the development of chronic diseases. Hyperactivation of NF-κB is often seen in several forms of cancer, including pancreatic and prostate carcinomas[65].

As aberrant NF-κB activity often is related to chronic and neurodegenerative diseases, a number of inhibitors of NF-κB activity have been identified[47]. At the same time, no compounds designed specific for modulating NF-κB activity are yet in clinical use, as these substances are not tissue or cell specific, and hence might interfere with NF-κB where proper NF-κB activity is needed.

Of the many compounds shown to modulate NF-κB activity are several phytochemicals, substances of plant origin[16]. It has been proposed that the high content of phytochemicals is one of the factors why a diet rich in fruits and vegetables is associated with a reduction in the risk of developing chronic diseases. However, even though single compounds of fruits and vegetables have shown to inhibit NF-κB activity, few have tested the effects of whole food items.

In this thesis, the aim was to screen foods from several food groups for their ability to modulate NF-κB activity, by using a cell line stably transfected with the luciferase gene coupled to a NF-κB promoter. This screening is based on whole food items, and hence is more related to general nutrition than previous experiments studying single compounds. An initial screening by Paur *et al.*[25] focused on dietary plants rich in phytochemicals, whereas the food items for this thesis are chosen to represent different food groups in a Nordic diet. Another purpose of this screening was to further test the most potent food extracts in transgenic mice, to study the effects *in vivo*.

Even though persistent up-regulated NF-κB activity is associated with the development of several chronic diseases, small, repetitive insults of NF-κB activity may protect against later, stronger insults[69-71,88]. Hence, whilst inhibitors of persistently elevated NF-κB activity might be the main interest in the pharmacological industry, foods with the ability to up-regulate basal NF-κB activity and/or inhibit over activation of NF-κB may be of interest in nutrition and in prevention of disease.

The results from the screening showed that there was a vast difference between the food items in their ability to modulate NF-κB activity, both on basal and LPS-induced NF-κB activity. None of the extracts decreased basal NF-κB activity, however half of them increased basal activity. Some extracts showed the ability to decrease LPS induced NF-κB activity, with green- and black tea, red wine and different cocoa products as the most potent inhibitors. On the other hand, a few extracts increased LPS induced NF-κB activity.

7.1 Discussion of Methods

7.1.1 Luciferase as a reporter

Bioluminescence emitted from luciferase can be detected externally, thus luciferase was used as a reporter to detect the activity of NF-κB. This is achieved by making a DNA construct containing a promoter with NF-κB responsive elements coupled to the luciferase gene. Such a construct will lead to the production of luciferase whenever the NF-κB promoter is activated.

The use of luciferase as a reporter for gene expression has several advantages. First, it has high sensitivity, and will be detected even at low levels of gene expression. The luminescence is virtually background-free and the light produced is linearly correlated over a wide range of luciferase concentrations. Secondly, the translation of luciferase is very rapid, and its maximum activity will occur short time after

transcription initiates[89]. The enzyme has a high turn-over rate, with a half-life of approximately 2h, and hence has the ability to detect dynamic changes of NF-κB activity. Other reporters, like β-galactosidase and green fluorescent protein do not share this advantage[90].

Alternative methods for measuring NF-κB activity is the use of DNA binding assay (i.e. Electrophoretic Mobility Shift Assay (EMSA) and Trans AM assay), and immuno-localization of NF-κB in the nucleus. However, even though NF-κB binds to the DNA, it often needs to be further modulated to activate transcription of its target gene. Furthermore, protein inhibitors can interfere with NF-κB when the transcription factor is bound to DNA, hence inhibiting transcription. By using luciferase as a reporter gene, whose activity is dependent on both NF-κB binding and promoter activation, these problems will be circumvented.

The reaction where oxyluciferin is made from luciferin in the presence of ATP, O_2 and Mg^{2+}, is an oxidative reaction. It is possible that a high presence of antioxidants may inhibit this reaction, and thus light emission, to occur. This has been seen with resveratrol, a compound in red wine[85]. Other compounds may also act as chromophores, hence absorbing photons, or directly interfere with the luciferase enzyme, such as β-naphtoflavone[91]. This will be further discussed in section 7.1.3.

7.1.2 U937 3xκB-LUC cells

Several chronic and neurodegenerative diseases have been associated with a dysregulation of the transcription factor NF-κB. NF-κB plays a pivotal role in inflammatory responses in the body, and has been found to be modulated by several dietary compounds. Hence, NF-κB was chosen as a response element in this thesis, enabling screening of dietary foods with a view on inflammation.

The U937 3xκB-LUC cells have Toll-like receptors that bind LPS, leading to activation of NF-κB. LPS is a part of the outer membrane of Gram negative bacteria, and hence also will be present in the blood of people having a bacterial infection.

Thus, monocytes *in vivo* can be affected both by LPS and circulating compounds from foods such as phytochemicals.

The U937 3xκB-LUC cells can be grown in sufficient amounts, they are cost efficient and the effect on NF-κB activity can be tested with a wide range of stimuli. Hence, they are an excellent model system for screening purposes. For the results to have relevance to an intact organism, it is important that the responses of the cells to different forms of stimuli resemble responses of an intact organism. The increased NF-κB activity related to increasing LPS concentrations (**Figure 6.11**) suggests that such properties are present.

7.1.3 NF-kB luciferase mice

To extend the knowledge from the *in vitro* results, transgenic mice with the NF-κB-LUC construct were used. These mice were first made by Carlsen et al. in 1997[46]. The use of such mice can give valuable information, as it enables investigation of NF-κB activity in a whole body. Compared to the *in vitro* experiments, the transgenic mice visualize the complex biological networks of an intact animal in a realistic manner. The use of in vivo imaging of luciferase transgenic mice is non-invasive, thereby making it possible to detect NF-κB activity at different time points in the same animal. This allows each animal to serve as its own control, which decreases differences caused by variations between animals, and hence reduce the number of mice needed in the experiment.

The D-luciferin was injected intraperitoneally, which results in a longer response compared to venous injection. The luciferin will rapidly be distributed to all organs and oxidized to oxyluciferin with the emission of light where luciferase is synthesized. However, photons will be scattered and absorbed by the tissues, the major limitation of bioluminescence reporters. Studies have shown that for each centimetre tissue depth, as much as 90 % of the light is attenuated[90]. Especially haemoglobin is a strong absorber of luminescence, which leads to absorption of light in a tissue specific manner[92,93]. Due to this, luminescence from deeper sources is

difficult to detect *in vivo*, and the light emitted is likely produced in the upper layers of the body. *In vivo* imaging should therefore be combined with ex vivo imaging and measurements of luciferase activity in tissue homogenates, as done in this thesis, which gives a more tissue specific view on NF-κB activity.

7.1.4 Cell Cytotoxicity

Cell viability was measured by the use of trypan blue. This dye will not penetrate intact, living cells, and hence non-viable cells can be distinguished from the viable by their blue colour. Another method commonly used is the lactate dehydrogenase (LDH) assay, based on the measurement of extra-cellular activities of LDH[94]. LDH has reducing capacity, and viability is measured by colour change when reduction occurs. However, many of the extracts tested have a high content of substances with antioxidant capacity that will produce the same colour change as LDH, and hence this method is not suited.

Hoechst staining of cells, another commonly used method, can be useful to differentiate viable, apoptotic and necrotic cells, furthermore viability can be detected by the use of measuring protein- or DNA synthesis by the use of radioactivity. These methods can i.e. be useful in characterization of cells, however the method of trypan blue was determined to be adequate for the work in this thesis.

7.1.5 FRAP

The antioxidant capacity of the extracts was tested by the use of the FRAP method, which has the ability to determine the total amount of antioxidants or reductants in a sample, expressed as the concentration of all electron-donating agents[86]. Compared to other tools to determine antioxidant capacity, such as the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC) and the oxygen radical absorbance capacity (ORAC), the FRAP method directly quantifies antioxidants and reductants in a sample. The TEAC and ORAC assays measure the ability of antioxidants and reductants in a sample to neutralize

reactive species, hence they are indirect methods of measuring antioxidant capacity, and the result strongly depends on the reactive specie being used[14].

7.1.6 Extracts

The extraction procedure of the foods is based on the method by Halvorsen *et al.* developed for the FRAP analysis[14]. This method uses aqueous/methanol solvents, hence also most fat soluble substances are extracted. This extraction method circumvents the disadvantage of FRAP, which originally only detect water soluble substances[14,86]. It should be noted that the original extraction procedure was further evolved. The extracts were concentrated by the use of heat, and dissolved in DMSO, and both steps may affect the antioxidant capacity of the sample.

Both hydrophilic and most hydrophobic substances can be extracted by the procedure used, however the distribution of substances in each extract is not known. Such a characterization can be done by the use of high performance liquid-chromatography (HPLC) analyses[95] and liquid chromatography mass spectroscopy[96], and can help characterize the compounds of foods responsible for the effect on NF-κB activity. This screening is however time-demanding, and is thus not suited for screening purposes.

7.1.7 Recombinant luciferase

To study whether the extracts used in the *in vitro* experiments could affect the luciferase enzyme activity, thirteen of the most potent inhibitors of LPS-induced NF-κB activity were tested with a recombinant luciferase enzyme. The extracts were tested for their ability to affect the production of light by this enzyme. The same concentrations of extracts as in the cell culture experiments were used and luciferase activity was tested right after addition of extract.

The antioxidant capacity of the extracts may inhibit the oxidation of luciferin to oxyluciferin, and hence light production decreases. The major part of the extracts

tested did inhibit recombinant luciferase activity. It should be kept in mind, however that the reduction in light production due to luciferase inhibition, may not occur in living cells. Intact cells have several defence systems that can buffer the antioxidant load, and direct influence on luciferase activity may not occur in the same degree as seen in this experiment. Also, it should be kept in mind, that only a low percentage of the plant compounds are expected to be taken up by the cells, and would thus not be in direct contact with the luciferase enzyme in an intact cell.

The ability to inhibit luciferase should however be further elucidated. Knudsen[97] has previously studied the effect of extracts on the luciferase enzyme by in vitro experiments. These experiments were set up as usual, as described in 5.2.3, however the extracts were added to the cell culture directly before luminescence was measured. The results showed that the extracts did not affect luciferase activity in intact cells as they did with recombinant luciferase. Similar studies should be a part of the future work with extracts, however, due to the time limit, this was not done in this thesis.

7.2 General Discussion

7.2.1 Effects on NF-κB Activity in U937 3xκB-LUC Cells by Extracts of Foods.

An effect on NF-κB activity by phytochemicals and other bioactive compounds in foods may be an important factor in preventing the development of several diseases. In this thesis, LPS was used as an inducer of inflammation, to see whether extracts are able to inhibit LPS-induced NF-κB activity. However, even though persistent aberrantly up-regulated NF-κB activity is associated with the pathophysiology of many diseases, small, repetitive episodes of NF-κB activation may also play a role in the prevention of disease. This term, called pre-conditioning, has earlier shown to be protective against sepsis[70], coronary heart disease[69], cerebral diseases[71] and hepatic ischemia injury[88]. As shown in **Figure 6.11**, pre-incubation with LPS over night

reduced NF-κB activity compared to control when cells were added LPS for additionally 6 h, suggesting that such a pre-condiditoning in monocytes might occur. However, this pre-conditioning, mediated by LPS, increased NF-κB activity in a more extensive way than any of the extracts of foods did with basal NF-κB activity. Although suggesting that foods can be beneficial due to pre-conditioning effects, as seen with the LPS pre-conditioning experiment, further research should be done to investigate this topic.

Modulation of basal NF-κB activity

Few studies have studied the effect of food items on basal NF-κB activity. One exception is Paur *et al.*[25], who found that a number of extracts of dietary plants, such as spices and berries, and some single phytochemicals, had the ability to increase basal NF-κB activity in U937 3xκB-LUC cells.

Of the 27 extracts tested in this thesis, 12 extracts significantly increased NF-κB activity (**Table 7.1**). The highest increase in NF-κB activity was seen with cod, which had an NF-κB activity of 393 % compared to control cells. Other potent NF-κB inducers were unhydrolysed barley "Olve", pure cocoa (100 %), unhydrolysed wheat "Standard" and banana. The increase in basal NF-κB activity was not dose-dependent for all extracts, suggesting that several factors determine the ability to modulate NF-κB activity.

All food groups had at least one food item that significantly induced basal NF-κB activity however the fruit and vegetables and the grains had several inducers of NF-κB. A diet with a high consumption of fruits, vegetables and wholegrain has shown to have protective effects against a number of chronic diseases[7,82], which might be partly explained by a pre-conditioning effect caused by inducing basal NF-κB activity, as seen in these results. Furthermore, most of the grain extracts did not differ in their

Table 7.1 Modulation of basal and LPS induced NF-κB activity of extracts of foods.

Food group	Extract	Basal NF-κB activity	LPS induced NF-κB activity
Fruits and vegetables	Apple	++	---
	Banana	+++	
	Broccoli	+	--
	Brussels sprouts		
	Sesame seed		
Beverages and liquids	Green tea		---
	Black tea	++	---
	Red wine		---
	Skim milk (0.1 % fat)		
	Whipping cream (38 % fat)		
Fish and meat	Chicken		
	Beef		-
	Cod	++	
	Salmon		-
Chocolate and cocoa products	Baking cocoa		---
	Cocoa 100 %	++	---
	Dark chocolate		---
Grains	Wheat "Standard" unhydrolysed	++	
	Wheat "Standard" hydrolysed	++	
	Wheat "Mjølner" unhydrolysed	++	
	Wheat "Mjølner" hydrolysed	+	-
	Buckwheat "Lileja" unhydrolysed		---
	Buckwheat "Lileja" hydrolysed		
	Barley "Olve" unhydrolysed	+++	+
	Barley "Olve" hydrolysed		
	Oat "Hurdal" unhydrolysed	+++	++
	Oat "Hurdal" hydrolysed		

Extracts were tested for their effect on basal or LPS induced NF-κB activity in U937 3xκB-LUC cells. The table presents the effect on NF-κB in the highest concentration tested for each extract. +++/--- = $p<0.001$, ++/-- = $p<0.01$, +/- = $p<0.05$, where + indicates increase in NF-κB activity, and - a decrease. Empty squares means no significant modification occurred.

effect on basal NF-κB activity based on if they were hydrolysed or not. The only exceptions were barley "Olve" and oat "Hurdal", where the unhydrolysed forms significantly increased basal NF-κB activity, whilst the hydrolysed forms did not.

None of the other extracts tested reduced basal NF-κB activity (**Table 7.1**).

Modulation of LPS induced NF-κB activity

A variety of substances has been shown to inhibit NF-κB activity, however most studies have focused on single compounds, and not whole food items. Curcumin, capsaicin, gingerol and genistein in i.e. turmeric, chilli, ginger and soy respectively, are among the phytochemicals shown to inhibit NF-κB activity[17].

A great variety in effects on LPS induced NF-κB activity was detected with the extracts of foods (**Table 7.1**). Of the 28 extracts tested, 12 reduced the NF-κB activity, whilst two further increased activity. Of the identified inhibitors of LPS induced NF-κB activity, extracts of green- and black tea, red wine, baking cocoa, pure cocoa (100 %) and dark chocolate gave the strongest reductions in NF-κB activity to 14 %, 19 %, 7 %, 27 %, 8 % and 4 % respectively compared to control. All these products have a high antioxidant capacity[13]. Green tea contains large amounts of polyphenols, of which the epigallocatechin-3-gallate (EGCG) is the most abundant and frequently studied[76]. It has previously been shown that EGCG inhibits IKK, thereby avoiding translocation of NF-κB to the nucleus[76]. Sun *et al.* [98]showed that resveratrol, a phytochemical in red wine, downregulated NF-κB *in vitro*, furthermore several flavonoids in chocolate are potent NF-κB modulators[99]. Whether the phytochemical content in these foods is the main factor to the major inhibition in LPS induced NF-κB activity as seen here, is possible, but however not clear, as few studies have been done with complete food extracts.

Of the fruits and vegetables, only the extracts of apple and broccoli were able to significantly inhibit NF-κB activity. Sulforaphane in broccoli has earlier been shown to inhibit LPS induced inflammation[73], and might be part of the effect detected in this

thesis. The reduction in NF-κB activity by treating cells with the apple extract is in concordance with previous work. Apple is one of few foods that previously has been tested as a whole food extract, and has revealed to inhibit NF-κB activity in breast cancer cells[100].

Only half of the grain extracts tested modulated LPS induced NF-κB activity, and furthermore the only extracts found to increase LPS induced NF-κB activity in their highest concentrations were in this group – the unhydrolysed forms of barley "Olve" and oat "Hurdal". Gliadin, a glycoprotein in several grains, has been found to increase NF-κB[83], however, wheat contains large amounts of this protein, and as neither wheat "Standard" nor wheat "Mjølner" increased LPS induced NF-κB activity, gliadin is less likely to be the cause of the effects seen by the unhydrolysed extracts of oat "Hurdal" and barley "Olve". Furthermore, it seems like the unhydrolysed extracts in general are more potent modulators of NF-κB than the hydrolysed extracts, as extracts of barley "Olve" and oat "Hurdal" did modulate NF-κB activity as unhydrolysed extracts, but not as hydrolysed. This may be explained by the extraction method used for hydrolysed grains, which might break down some of the phytochemicals, both the bound and unbound. Still, the FRAP values were higher in the hydrolysed extracts than in the unhydrolysed (see **Table 6.2**), suggesting that maybe other compounds that do not inhibit LPS induced NF-κB activity also are present in a higher amount in the hydrolysed extracts. Furthermore, the hydrolysed extracts were tested at lower concentrations, due to a high content of DMSO, which might explain the differences in effects.

The meat and fish group had low antioxidant capacities, still both beef and salmon were able to slightly inhibit LPS-NF-κB activity. The mechanisms behind the effect of beef remain to be elucidated. Salmon contains docosahexaenoic acid (DHA) that has an anti-inflammatory effect, and has previously been shown to modulate NF-κB activity[78]. These results indicate that several components in foods, not only phytochemicals, have the ability to affect NF-κB, and that the screening done in this

thesis is important for further knowledge of single foods' modulation of NF-κB activity.

Several of the tested extracts did not modulate NF-κB in a dose-dependent manner, i.e. broccoli and unhydrolysed buckwheat "Lileja" significantly increased NF-κB activity at lower concentrations before a reduction occurred at a higher dose. This might indicate that several compounds in foods affect NF-κB, and that the concentrations of several substances might be important for the effects on NF-κB. It is possible that some of the components in foods increase NF-κB activity until a certain level of inhibitory NF-κB components is reached.

7.2.2 Effect on LPS induced NF-κB activity in transgenic mice

The next step in the screening of whole foods would be to test these extracts in a more physiological setting, such as in NF-κB-luciferase reporter mice, to study whether the *in vitro* results are reproducible *in vivo*. The luciferase-reporter mice make it possible to look at NF-κB activity in a whole organism and in separate tissues, and it is possible to follow individual mice over time.

The mixed extract was made of green- and black tea, red wine and dark chocolate, which all potently reduced LPS induced NF-κB activity *in vitro*. Whilst there have been few studies with whole food extracts *in vitro*, more studies have been performed *in vivo*. Cocoa did show to increase flow-mediated dilation in a study by Sies *et al.*[79], however whether this was due to modulation of NF-κB is not known. Also green tea extract has shown to be beneficial against hepatic injury in rats with hepatic ischemia, an effect partly explained by attenuating NF-κB activity[101]. Furthermore, resveratrol in red wine has been widely studied, and this compound can i.e. reduce NF-κB activity in induced colitis[102], however resveratrol has shown to inhibit inflammation independently of NF-κB modulation as well[103].

In this thesis, the mixed extract did not significantly inhibit LPS induced NF-κB activity, neither in whole mice, *ex vivo* imaging of intestine or tissue homogenates.

However, there was a slight reduction in AUC for mice fed with the extract (34.2) compared to control mice (42.5). Although not statistically significant, the NF-κB activity in small intestine and ovary was reduced compared to controls, with an activity of 45 % and 48 % of control respectively. This trend, with lowered AUC and NF-κB activity in ovary, may indicate that bioactive compounds with the ability to inhibit NF-κB activity, such as phytochemicals, are absorbed in the gastrointestinal tractus, and further circulated and taken up by various organs where they can modulate gene expression.

A local action of phytochemicals, even if they are not absorbed, can be beneficial in the intestine, as a vast number of oxidizing agents may lead to inflammatory diseases and cancer in the intestine [15,16]. Being close to significant, the reduction in NF-κB activity in small intestine as seen here, might suggest a strengthening of this hypothesis, which may be part of the mechanisms for the inverse association between a high intake of fruit and vegetables and colon cancer[104]. Thus further work should be performed to study whether this inhibited NF-κB activation in the intestine can be confirmed with a larger number of animals.

7.2.3 FRAP

Earlier screening have revealed that the food groups highest in antioxidant capacities have been the spices, fruits and vegetables, berries, chocolate and chocolate products, cereals, nuts and seeds[81]. The FRAP-values for the extracts in this thesis are hence in concordance with previous findings. The measurements of antioxidant capacity however revealed enormous differences between the extracts.

Previous measurements of antioxidant content of fruits and vegetables have shown that the reducing capacity varies both within and between classes. In the Norwegian diet, fruits contribute to approximately 43.6 % and vegetables to 8.9 % of total antioxidant intake[14]. Here reducing capacity did not greatly vary within the fruits and vegetable group, and neither of the items chosen had a very high total antioxidant content.

The extracts of green- and black tea, red wine, baking cocoa, pure cocoa (100 %) and dark chocolate had the highest FRAP values, which is in concordance with previous results[13,14,81]. Furthermore several of the grains had high FRAP values, with the highest values detected in the hydrolysed extracts, suggesting that many of the compounds with antioxidant properties are indeed released by hydrolysis. Antioxidant capacity of meat- and dairy products were low, as previously mentioned[13].

Earlier screenings have been on whole foods and not extracts, and despite correcting the FRAP values of the extracts to original weight of the product, the detected values were lower than previous findings[13,14]. This can be due to ripeness, storage or processing of the product, however the most likely reason is that the extended preparation of the extracts leads to some loss of antioxidant capacity.

7.2.4 Correlation between FRAP and NF-κB activity in U937 3xκB-LUC cells.

To investigate whether the effect on basal and LPS induced NF-κB activity of certain extracts were associated with their antioxidant content, a correlation test was done between the measured FRAP values and the modulation of NF-κB.

No correlation was found between FRAP values and the ability to modulate basal NF-κB activity, which may suggest that several compounds in foods can increase basal NF-κB activity, thereby causing a pre-conditioning effect that might be beneficial. There was however a correlation between the antioxidant capacity and the ability to inhibit NF-κB activity. This might indicate that foods with high antioxidant content are the most potent inhibitors of NF-κB, hence the correlation found here might only be a result of differences between food groups in the ability to modulate NF-κB. Still, some foods low in antioxidant capacity, such as salmon and beef, did also reduce LPS induced NF-κB activity, suggesting that not all effects by foods can be explained by antioxidant content. This area hence needs further investigation.

7.2.5 Possible mechanisms of regulation of NF-κB activity

As previously mentioned, inhibition of NF-κB can occur at several steps in the pathway, including modulation upstream of IKK, ubiquitination and other processes in the cytoplasma, NF-κB translocation and DNA binding[47]. Several antioxidants have the ability to inhibit NF-κB activity, leading to the hypothesis that NF-κB is redox regulated[57]. Some studies have shown that ROS, such as H_2O_2 and UV light, activate NF-κB by stimulating degradation of IκB[57], however other studies have found different results[58,59]. H_2O_2 will for example only activate NF-κB in certain cells, and have shown to have the opposite effect in most cells[59].

Hayakawa *et al.*[58] revealed that antioxidants act on the NF-κB activation pathway independently of antioxidant function. N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), two potential antioxidants, inhibited NF-κB activation by different mechanisms. NAC inhibited binding of TNF-α, one of the major NF-κB activators, to cell surface receptors, whilst PDTC avoided IκB polyubiquitination.

Several phytochemicals modulate NF-κB activity, and they seem to do so in different ways. Surh[17] found that curcumin down-regulated IKK, thereby inhibiting IκB degradation. Furthermore, genistein might inhibit NF-κB activity by avoiding phosphorylation of p65. Other molecules may reduce NF-κB activity by preventing translocation to nucleus, by maintaining high levels of IκB in the cytoplasma, either by up-regulating synthesis of IκBα, or avoid ubiquitination and degradation by IκBα[47]. Possible mechanisms for modulation of NF-κB are shown in **Figure 7.1**.

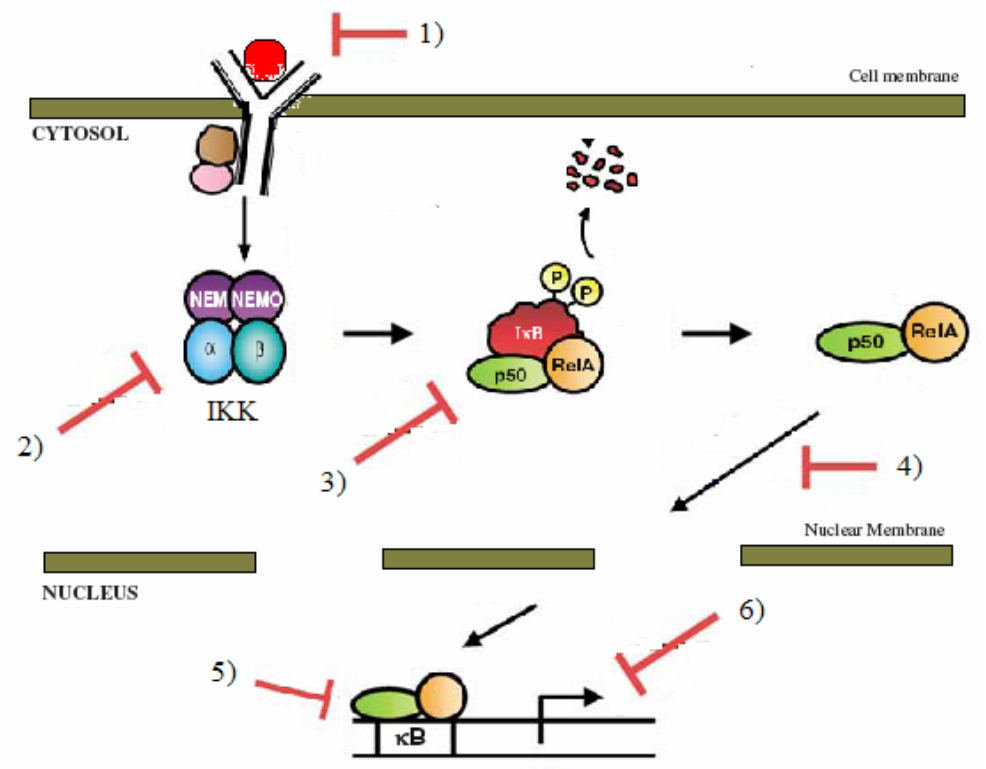


Figure 7.1 Levels of NF-κB inhibition. Inhibition can occur by: 1) Signal binding (Upstream of IKK), 2) IKK activation and IkB ubiquitination, 3) Proteasomal degradation of IkB, 4) Translocation of NF-κB to the nucleus, 5) NF-κB DNA binding and 6) Transactivation. Adapted from Gilmore and Herscovitch[47].

Even though phytochemicals and other antioxidants may modulate NF-κB activity independently of their antioxidant content, NF-κB is closely related to endogenous antioxidants. The expression of genes related to the endogenous antioxidant defense system, such as SOD, is up-regulated by NF-κB, and hence the organism can better combat reactive species[68].

7.3 Future perspectives

Many single compounds have been tested for their inhibitory effect on NF-κB, however few whole food items have so far been screened. Although the most potent inhibitors of LPS induced NF-κB activity had a high content of phytochemicals, also other foods showed to have effects on NF-κB. Further testing of other common foods should therefore be done. If a more extensive screening is performed, such information can be utilized in epidemiological studies to look for possible associations between health and intake of foods with the ability to modulate NF-κB activity.

To understand the mechanisms behind the effect of specific food items, a screening to reveal the content and distribution of single compounds will be of importance. This screening will however not tell how several components interact, as the effect seen here most likely is a result of the combination of single compounds. Therefore possible synergistic effects of foods and food compounds should also be investigated.

In addition the beneficial effects of pre-conditioning should be further examined, as many foods up-regulate basal NF-κB activity in this work. It remains to be elucidated whether foods can be protective in a model of pre-conditioning.

Even though extracts of foods modulate NF-κB activity *in vitro*, further *in vivo* testing should be done, as bioavailability can be low for several substances. Transgenic mice are an important tool, as these allow us to see effects of NF-κB over time, and enables investigation of uptake and distribution in the body.

8. Conclusions

The screening of foods, to study effects of NF-κB, is important to further understand the relationship between diet and health. In this thesis food from 5 food groups were tested and a huge variation in the ability to modulate NF-κB activity between the foods was seen.

Of the 27 food extracts tested, 12 showed the ability to inhibit LPS-induced NF-κB activity. The most potent inhibitors were green- and black tea, red wine and dark chocolate, which all reduced LPS-induced NF-κB activity to less than 20 % of controls. Interestingly, also extracts of beef, salmon, apple, broccoli, and some grains showed the ability to inhibit NF-κB activation.

Furthermore, 12 of the extracts did increase basal NF-κB activity, which might be beneficial to health by a pre-conditioning effect, however this should be further studied.

The most potent inhibitors of NF-κB, mentioned above, were combined to make a mixed extract that was tested *in vivo* on transgenic mice. This was done to see whether the effects seen *in vitro* could be reproduced *in vivo*. No significant effects were found, even though there was a trend towards lower NF-κB activity in whole mice, as well as in intestine and ovary. This may suggest that bioactive compounds in food, such as phytochemicals, are absorbed and distributed throughout the body.

The screening of foods common in the Norwegian diet should be further extended to increase knowledge of foods with the ability to modulate NF-κB activity, and to further understand the relationship between diet and health.

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