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Synergy research: Vitamins and secondary plant components in the maintenance of the redox-homeostasis and in cell signaling

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Abstract

The maintenance of the redox-homeostasis is an essential task of antioxidants. Reactive oxygen species (ROS) formed during oxidative stress can potentially damage the normal cellular functions and support pathological processes like atherosclerosis in vessels or malignant growth in other tissues, but also the aging process. However, recent findings link ROS also to cell survival and/or proliferation, which revolutionises the age-old dogmatic view of ROS being exclusively involved in cell damage and death. Low concentrations of hydrogenperoxide e.g. are involved in cell signaling and can activate mitogen-activated kinases (MAPK) to initiate cell growth. Nutritional antioxidants like vitamin C or E can promote endothelial cell growth, but can also inhibit growth of muscle cells, and influence MAPK. Thus, keeping the redox-homeostasis in a steady state especially in the context of tissue regeneration appears to be more important than previously known and seems to be a controlled synergistic action of antioxidants and ROS. The present review summarizes the properties and functions of ROS and nutritional antioxidants like the vitamins C and E, and polyphenols in redox-homeostasis. Their relevance in the treatment of various diseases is discussed in the context of a multitarget therapy with nutraceuticals and phytotherapeutic drugs.

Keywords: Antioxidants; Reactive oxygen species; Atherosclerosis; Redox-homeostasis; Cell signaling

Introduction

Reactive oxygen species (ROS) have been implicated in the pathophysiology of various diseases being as diverse as the group of rheumatic diseases, respiratory infections, diabetes type 2, malignancies or cardiovascular diseases (CVD). A common phenomenon in all is an imbalance between the rates of production and elimination of ROS. In CVD for example, an increase of ROS contributes to early vascular dysfunction and initiates the inflammatory cascade. In rheumatic diseases ROS promote the inflammatory process.

Recent findings link the presence of ROS also to cell survival or proliferation signals. This has resulted in a paradigm shift from the age-old dogma implicating ROS

Abbreviations: Bcl-2, intracellular protein (Bcl2-family) that either promotes or inhibits apoptosis by regulating the release of cytochrome c and other mitochondrial proteins from the intermembrane space into the cytosol; CHD, coronary heart diseases; c-Jun, stress-induced MAP-kinase; CVD, cardiovascular diseases; GTE, green tea phenolic extract; ECs, endothelial cells; ERK, extracellular-regulated kinases; ETC, electron transport chain; GSH, glutathione; IL, interleukin; MAPK, mitogen-activated kinases; p, phosphorylated; p38, stress-induced MAP-kinase; PDGF, platelet-derived growth factor; PI-3-kinase, phosphoinositide-3-kinase; SOD, superoxide dismutase; VSMC, vascular smooth muscle cells; ROS, reactive oxygen species.

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exclusively in cell damage and death (Pervaiz and Clement, 2007). The underlying mechanism(s) that allow ROS to support survival signaling on one hand and facilitate cell death on the other, are becoming elucidated (Pervaiz and Clement, 2007). At the same time, their opponents – the antioxidants – appear in a new light. Their task may not be solely the suppression of ROS, but rather a modulatory function on the survival and death signaling of ROS.

Interplay of reactive oxygen species and antioxidants

ROS levels are kept under tight control by the enzymatic activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase and GSH reductase, by non-enzymatic compounds such as α-tocopherol, β-carotine, ascorbate and GSH (Droge, 2002; Halliwell and Gutteridge, 1990), and by the action of low-efficiency ROS scavengers like free amino acids, peptides and proteins (Droge, 2002; Stadtman, 1993). In addition, polyphenols in vegetables and other plants can influence ROS. In healthy living cells, one or several of these redox regulatory mechanisms are activated in response to a transient increase in intracellular ROS to prevent oxidative stress (Pervaiz and Clement, 2007). A disturbance in the tight balance of ROS production and elimination, either via augmentation of ROS generation or defective/deficient antioxidant defenses for their elimination, results in an increase of intracellular ROS and can lead to persistent changes in signal transduction and gene expression, thereby giving rise to oxidative stress-related pathological states (Pervaiz and Clement, 2007) like endothelial dysfunction or inflammation.

The intracellular generation of ROS is part of the normal cellular metabolism and occurs from stimulus-induced activation of membrane-bound enzyme systems like the NADPH oxidase complex or the mitochondrial electron transport chain (ETC) (Pervaiz and Clement, 2007). The NADPH oxidase complex primarily generates ROS. Evidence that the ETC is a primary source for ROS in cells is the ability of isolated mitochondria to produce O_2^- either by auto-oxidation of the flavin component of complex I (NADH hydrogenase) and/or by auto-oxidation of the ubisemiquinone at complex III (Halliwell and Gutteridge, 1990; Pervaiz and Clement, 2007). During the transfer of electrons through the earlier components of the ETC a few electrons leak out directly onto O_2 and generate O_2^- (Valko et al., 2007). The superoxide (O_2^-) and the hydroxyl ($^{\bullet}OH$) radical, as well as non-radical derivates of oxygen (O_2), such as hydrogen peroxide (H_2O_2) are the main ROS (Fridovich, 1978). Probably the highly reactive $^{\bullet}OH$-radical accounts for most of the oxidative damage attributed to ROS (Halliwell and Gutteridge, 1990).

Another group is the reactive nitrogen species (RNS). Peroxynitrite, peroxynitrous acid and nitrogen dioxide are generated from an iron-independent reaction involving the interaction of $O_2^{\bullet -}$ and nitric oxide (NO). Both are e.g. generated in large amounts during ischemia-reperfusion (Venardos et al., 2007; Nicolls et al., 2007). However, in physiological conditions NO is continuously formed and deliberated in endothelial cells (ECs). It induces vasorelaxation (Frick and Weidinger, 2007) and inhibits aggregation of thrombocytes, adhesion of leukocytes and the proliferation of vascular smooth muscle cells (VSMC) (Metzner and Ulrich-Merzenich, 2001).

Nutritional antioxidants

Central nutritional antioxidants are vitamin E, vitamin C, but also polyphenols. Their intake is primarily recommended due to their antioxidant activity, even though their activity spectrum is much more complex and in all consequences not yet fully understood.

Vitamin E

Vitamin E is especially present in parts of plants which serve as oil sources such as wheat germs, rape seeds, sun flower seeds, maize and soy, but also in hazelnuts. The term vitamin E stands for a family of eight molecules with related structures each consisting of a chromanol ring with an alipathic side chain. Based on the side chain two groups are distinguished – tocopherols and tocotrienols with four isoforms in each group (α,β,γ,δ). All members have vitamin E activity, but the naturally occurring R,R,R-α-tocopherol has the highest bioavailability (Rodrigo et al., 2007). This is thought to be a consequence of the expression of the hepatic α-tocopheryl transfer protein (α-TTP), which is highly selective for α-tocopherol, but has a low or very low affinity for the other tocopherols (Sato et al., 1991; Azzi, 2007). The fat soluble vitamin E is located on the cell membrane, both intra- and extracellularly (Rodrigo et al., 2007). It acts chemically as a free radical chain-breaking molecule by inhibiting lipid peroxidation by its conversion into an oxidized product, α-tocopheroxyl. α-Tocopherol is restored by reduction of the α-tocopheroxyl radical with redox-active reagents like vitamin C or ubiquinol (Rodrigo et al., 2007; Shi et al., 1999). Recently a novel water soluble form of vitamin E, α-tocopheryl phosphate, has been discovered. Its role is presently investigated (Gianello et al., 2005) and

proposed to be the ultimate molecule, which specifically interacts with receptors or transcription factors and modulates cell functions (Azzi, 2007; Negis et al., 2005, 2006). α-Tocopherol has been shown to inhibit VSMC proliferation, to preserve endothelial integrity, and to inhibit monocyte–endothelial adhesion and aggregation, monocyte ROS and cytokine release, and platelet adhesion and aggregation (Azzi, 2007), altogether actions helpful to counteract the process of atherosclerosis and thereby the development of CVD. Concentrations of 30 μM are recommended for prevention (Biesalski, 1995; Ulrich-Merzenich et al., 2002a, b).

There is an ongoing controversy whether the actions described above are non-antioxidant or antioxidant-related actions (Brigelius-Flohé and Davies, 2007). Azzi (2007) proposes that the modulation of VSMC proliferation, the inhibition of the monocyte–endothelium adhesion and the cytokine release are controlled by non-antioxidative properties of vitamin E. Presumably cell pathways involving protein kinase C (PKC), 5-lipoxygenase, phospholipase A2, and diacylglycerol are inhibited (Rodrigo et al., 2007). Vitamin E thereby specifically modulates gene expression, but is also seen as a key redox-sensor. On the contrary, Traber and Atkinson (2007) interpret all effects on enzymatic signaling cascades or gene expression to be the result of changing lipid peroxidation. They postulate the bioactive lipids to be the crucial signaling molecules. Their changing amounts or their loss due to oxidation or their oxidation products are proposed to be the key cellular events that cells respond to. In addition to the controversy on the mechanistic aspects of vitamin E, results of clinical studies on the effect of vitamin E in different diseases are not uniform (Table 1).

Vitamin C

Excellent sources for vitamin C are sea buckthorn, sweet pepper, blueberries, fennel, broccoli or citrus fruits (DGE et al., 2000). Vitamin C is a 6-carbon lactone that readily oxidizes in aqueous solutions to its di-keto form, dehydroascorbic acid (Levine et al., 2006). Both forms contribute to the biological activities of vitamin C. It effectively quenches free radicals thereby protecting cell membranes and proteins from oxidative damage. It is an essential cofactor for the formation of collagen and plays a role in the glycation of proteins. It can contribute to an enhancement of carnitine synthesis and the reduction of the plasma-triacylglycerol concentration (Okamoto and Uensby, 2006; Ulrich-Merzenich et al., 2002a, b). Vitamin C participates in the biosynthesis of norepinephrine from dopamine and modulates the tyrosine metabolism (Rodrigo et al., 2007). In the vascular wall it acts like an enzyme modulator exerting upregulation of endothelial NO-synthase (eNOS) and downregulation of NADPH oxidase (Rodrigo et al., 2007; Ulker et al., 2003). It enhances the utilization of other nutrients e.g. it facilitates the duodenal absorption of iron, regenerates vitamin E and enhances the utilization of calcium into bones (Webb and Villamor, 2007). Vitamin C inhibits the effects of endothelin-1 (ET-1), thereby impairing endothelium-dependent and endothelium-independent vasodilation, and the stimulation of interleukin-6 (IL-6) release in humans *in vivo* (Rodrigo et al., 2007; Böhm et al., 2007). Physiological concentrations promote the proliferation of ECs, but inhibit the same in VSMC (Ulrich-Merzenich et al., 2002a, b). This is of special interest in atherosclerosis and CVD; repair processes after microlesions are promoted. Concentrations of 60 μM are regarded as protective for the vessel wall (Benzie, 1999; Ulrich-Merzenich et al., 2002a, b).

In the context of the innate immune system vitamin C supplementation was shown to enhance the motility of neutrophil leukocytes, and their chemotactic and post-phagocytic metabolic activity and to neutralize phagocytic-derived oxidants (Webb and Villamor, 2007). However, results of clinical studies with vitamin C are like for vitamin E contradictory (Table 1).

Polyphenols

Polyphenols are abundant secondary plant constituents in our diet. They are generally associated with health-promoting effects due to their antioxidant activities. They scavenge free radicals, inactivate metal catalysts by chelation, reduce hydroperoxides to stable hydroxyl derivatives, and interact synergistically with other reducing compounds (Frankel and Finley, 2008). About one third of the total polyphenol intake are phenolic acids, flavonoids account for the remaining two third (Scalper and Williamson, 2000). As the most frequently encountered phenolic acids are regarded caffeic acid and, to a lesser extent, ferulic acid (Scalbert and Williamson, 2000). The flavonoids are divided according to the degree of oxidation of the oxygen heterocycles: flavones, flavonols, flavanols, flavanonols, isoflavones, proanthocyanidins and anthocyanins (Scalbert and Williamson, 2000). The most abundant flavonoids in the diet are flavanols (catechins plus proanthocyanidins), anthocyanidins and their oxidation products. The total intake is ~1 g/day. The consumption of 10–100 mg of a single phenolic compound results in plasma concentrations rarely exceeding 1 μM (Scalbert and Williamson, 2000). Thus, bioavailibility is a central and an often neglected issue for judging the effect of polyphenols *in vivo*. Several authors have demanded that *in vitro* models should use physiologically relevant

Table 1. Trials evaluating vitamin supplementation in the prevention of cardiovascular diseases and atherosclerosis.

Study/year	Cohort	Duration (y)	Supplementation	Results
Nurses Health Study Stampfer et al. (1993)	US-Nurses Age: 34–59 y ($n = 87\,245$)	8	Vitamin E	CHD (non-fatal MI or death) RR: 0.87 (95% CI:0.7–1.07)
Nurses Health Study Rimm et al. (1998)	US-Nurses See above ($n = 80\,082$)	14	Multivitamins (4–7 tablets/week)	CHD (non-fatal MI or death) RR:0.76 (95% CI:0.65–0.90)
Physicians Health Study Muntwyler et al. (2002)	Physicians Without CVD ($n = 83\,639$)	4	Vitamin C Vitamin E Multivitamins	CVD and CHD mortality Vitamin C: RR: 0.88/RR: 0.86 Vitamin E: RR: 0.92/RR: 0.88 Multivitamins: RR: 1.07, RR: 1.02

Study/year	Study collective	Study design	Duration	Supplementation	Results
Primary and secondary prevention					
CHAOS Stephens et al. (1996)	CAD, M, F Mean age: 61.8 y ($n = 2002$)	p,r,p-c	1.5 y	Vitamin E 800 or 400 IU	RR:0.53 (95% CI: 0.34, −0.58) MI non-lethal
ATBC Virtamo et al. (1998)	Healthy persons, smokers, M Mean age: 50–69 y ($n = 27\,271$)	p,r,p-c	6.1 y	Vitamin E 50 mg β-Carotene 20 mg	Lethal CHD, RR: 0.92 (95% CI: 0.81–1.05) Non lethal and lethal MI RR: 0.96 (95% CI: 0.88–1.04)
GISSI-Prev. GISSI-Prevention Investigators, 1999	Patients with MI, M, F mean age: 59.4 y ($n = 3658$)	p,r	3.5 y	Vitamin E 300 mg	Cardiovascular endpoint: RR: 0.88 (95% CI: 0.85–1.16) Lethal CVD: RR: 0.80 (95% CI: 0.65–0.99)
HOPE Trial, Yusuf et al. (2000)	CHD/Diabetes, M, F Aged $\geqslant$ 55 y ($n = 9541$)	p,r,p-c	4.5 y	Vitamin E 400 IU	No effect on cardiovascular endpoint: MI,CVI, death due to CHD
SPACE Boaz et al. (2000)	End stage renal disease M, F, aged 40–75 y	p,r,p-c	1.4 y	Vitamin E	Reduction of the combined endpoints: AMI ischaemic stroke, PAD, unstable angina
HATS Brown et al. (2001)	CHD with low HDL M < 63 y, F > 70 y ($n = 146$?) (79/67)	r,p-c,db	3.0 y	Vitamin E 800 IU Vitamin C 1000 mg Selenium 100 µg β-Carotene 25 mg	Change in coronary MLD (angio) No benefits on death, MI, stroke, revascularisation
SECURE Lonn et al. (2001)	CVD or high risk CVD M, F aged $\geqslant$ 55 y ($n = 732$) (349/344)	r,p-c,db	4.5 y 12 y	Vitamin E 400 IU	No change in mean maximum carotic IMT (US)

Table 1. (*continued*)

Study/year	Study collective	Study design	Duration	Supplementation	Results
Fang et al. (2002)	Cardiac transplant (16/21)	r,p-c,db	1.0 years	Vitamin E 800 IU Vitamin C 1000 mg	Change in coronary maximum intimal thickness (IVUS)
Co PPP (2001)	High risk for CVD, M,F Aged 50–58 y, $n = 4495$	r	3.6 y	Vitamin E	No beneficial effect
VEAPS Hodis et al. (2002)	High LDL but no Clinical CVD, M, F Aged $\geqslant 40$ y ($n = 353$) (162/170)	r,p-c,db	3.0 y	Vitamin E 400 IU	No change in IMT carotid (US)
WAVE Waters et al. (2002)	Postmenopausal women with coronary disease (156/164)	r,p-c,db	2.8 y	Vitamin E 800 IU Vitamin C 1000 mg	Change in coronary MLD (angio)
MRC/BHD Heart Protection study (2002) MRC/BHF Heart Protection study (2002)	Healthy subjects $n = 20500$	p,r	5.0 y	Vitamin E 650 mg Vitamin C 250 mg β-Carotine 20 mg	No effect on cardiovascular risk
ASAP Salonen et al. (2000) Salonen et al. (2003)	Hypercholesteremia M, F, aged 45–69 y ($n = 440$) (335/105)	r,p-c,db	6.0	Vitamin E 272 IU Vitamin C 500 mg	Change in mean maximum carotid IMT (US)
SU.VI.MAX Zureik et al. (2004)	Apparently healthy M: 45–60 y, F: 30–60 y $n = 13017$	r,p-c,db	7.2 y	Vitamin E 33 IU Vitamin C 120 mg Selenium 100 µg β-Carotene 6 mg Zinc 20 mg	Carotid IMT (US)
Physicians Health study Up to 2007	Apparently healthy M: >55 y, $n = 15000$	r,p-c	8.0 y	Vitamin C: 500 mg Vitamin E: 400 IU β-Carotene: 50 mg (every second day)	Ongoing until 2007

p: prospective; r: randomized; p-c: placebo controlled; y: year; CHAOS: Cambridge Heart Antioxidant Study; db: double-blind, ATBC: Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (1994); HOPE: Heart Outcomes Prevention Evaluation Study; AMI: acute myocardial infarct, PAD: peripheral artery disease, CHD: coronary heart disease, CVD, coronary vascular disease, M: male, F: female, CVI: cerebro-vascular insult, RR: relative risk, CI: confidence interval, IU = 0.67 mg, angio: angiography, IMT: intima-media thickness, IVUS: intra vascular ultrasound, MLD: minimal lumen diameter, NA: not available, US: ultrasound, (summarized from Bleys et al., 2006; Brown and Hu, 2001; Cherubini et al., 2005; Morris and Carson, 2003; Siekmeier et al., 2006).

flavonoids and their conjugates in appropriate concentrations (Kroon et al., 2004). But the identification of the relevant physiological polyphenols is also still ongoing. E.g. the bioavailability is particularly low for quercetin and rutin, a glycoside of quercetin (0.3–1.4%), but higher for catechins in green tea, isoflavones in soy, flavanones in citrus fruits or anthocyanidins in red wine (3–26%). Interindividual variations were observed (Fuhr and Kummer, 1995, in Scalbert and Williamson, 2000). The least well-absorbed polyphenols are the proanthocyanidins, the galloylated tea catechins, and the anthocyanins (Manach et al., 2005). Besides absorption, also the plasma kinetics differ (~1.5 to ~5.5 h). Therefore, it is often argued that the bioavailability of

polyphenols in target tissues is to low for antioxidative actions (Azzi, 2007). The effective concentration range is stated to vary *in vitro* from <0.1 to $>100\,\mu mol/l$, while physiological concentrations presumably do not exceed $10\,\mu mol/l$ (Williamson and Manach, 2005a, b). But the total plasma phenol concentration is thought to be substantially higher due to the presence of metabolites. Indeed, the absorption of polyphenols is accompanied by extensive conjugation and metabolism (Williamson and Manach, 2005a, b). The plasma concentrations of total metabolites range according to Manach et al. (2005) from 0 to $4\,\mu mol/l$ with an intake of 50 mg aglycone equivalents. A number of recent reviews have addressed this issue (Williamson and Manach, 2005a, b; Manach et al., 2005; Kroon et al., 2004; Frankel and Finley, 2008). These metabolites are supposed to contribute to the observed effects. An indirect evidence that the polyphenols do contribute to the antioxidative activity *in vivo* is the increase in the antioxidant capacity of the plasma after consumption of polyphenol-rich foods (Scalbert and Williamson, 2000; Van het Hof et al., 1997; Duthie et al., 1998; Young et al., 1999). Plasma concentrations of the intact parent polyphenols are too low (overview Scalbert and Williamson, 2000) to account alone for this increase. That metabolites can contribute has been shown e.g. for quercetin. The antioxidant activity of quercetin conjugates was shown to be on average about half of that of the aglycone, but there was a substantial variation according to the position of the conjugation (Day et al., 2000, in Kroon et al., 2004). E.g. quercetin-3-glucuronide, one of the three major plasma quercetin conjugates, could significantly delay the Cu^{2+}-induced oxidation of human LDL *ex vivo*, but quercetin-3′-sulfate and 3′-methyl-quercetin-3-glucuronide were largely ineffective (Janisch et al., 2004; Kroon et al., 2004).

Whitehead et al. (1995) measured the contribution of polyphenol-rich red wine to the total antioxidant capacity of plasma by comparing the latter with that of vitamin C. The consumption of 300 ml of red wine (containing ~500 mg of polyphenols) increased the plasma antioxidant capacity similar to that of 1 g of vitamin C (Scalbert and Williamson, 2000). The consumption of 1 g of vitamin C increased the plasma concentration to $\sim 75\,\mu M$ (Levine et al., 1995). Taking into account the relative reducing powers of vitamin C and gallic acid (Singleton and Rossi, 1965), the concentration of total polyphenols in plasma after the ingestion of 500 mg of polphenols would be $50\,\mu M$ (Scalbert and Williamson, 2000). Similar calculations were made by Duthie et al. (1998). The concentration of $50\,\mu M$ after ingestion of ~500 mg of red wine polyphenols is on an average 10 times higher than the peak concentration of the parent flavonoids, which suggests that the metabolites contribute substantially to the antioxidant capacity (Scalbert and Williamson, 2000).

The maintenance of a high plasma polyphenol concentration requires often their repeated ingestion. This has been observed with volunteers consuming tea every 2 h (Van het Hof et al., 1997) or with the consumption of soy and the subsequent genistein and daidzein plasma concentrations (Watanabe et al., 1998). In addition to bioavailability another question needs to be tackled: polyphenols are by itself multifunctional (Frankel and Finley, 2008) and even more in combination. Thus, interactions of different aglycons and their metabolites will occur *in vivo* and need to be investigated.

Babu and Liu (2008) summarized the properties of green tea catechins recently: (a) catechins have antioxidant activities by scavenging free radicals, chelating redox-active transition-metal ions, inhibiting redox-active transcription factors, inhibiting pro-oxidant enzymes and inducing antioxidant enzymes, (b) they inhibit key enzymes involved in lipid biosynthesis and reduce intestinal lipid absorption, thereby improving blood lipid profile, (c) they regulate vascular tone by activating endothelial NO, (d) they prevent vascular inflammation probably through inhibiting transcriptional NF-κB, (e) they inhibit proliferation of smooth muscle cells and (f) they suppress platelet aggregation. These activities are essential for the prevention of CVD, which demonstrates that catechins, like all polyphenols, are ‘‘multitargeting’’ molecules.

Clinical studies with polyphenols

Most clinical studies with polyphenols are undertaken in the context of their potential cardioprotective, anticancerous or anti-inflammatory activities. Some examples which illustrate their potential, will be quoted. Troxerutin, a rutoside derivative, is known as an antioxidant with radical scavenger activity. It may promote the healing of capillary endothelial defects (Siegers et al., 2008). A recent randomized controlled trial demonstrated that a fixed combination of troxerutin and aescin (versus pentoxyfyllin) was useful in the treatment of inner ear perfusion problems of different etiology. Both drugs showed a synergistic effect (Siegers et al., 2008). Synergistic effects of components of *Ginkgo biloba* extracts (GBE) are well established (Wagner, 2006). Wu et al. (2008) demonstrated recently, that GBE, increases coronary blood flow in healthy elderly people which might relate to the improved endothelium-dependent vasodilatory capacity. The most important mechanism of action was the scavenging of oxygen-free radicals (Wu et al., 2008).

Epidemiological observations in Southeast Asia indicate an inverse correlation between habitual consumption of green tea beverages and the incidence of

cardiovascular events (Basu and Lucas, 2007). Green tea is rich in antioxidant and anti-inflammatory catechins, especially epigallocatechin gallate (EGCg). EGCg was shown to reduce lipid peroxidation, particularly LDL oxidation and malondialdehyde concentrations (Basu and Lucas, 2007). A recent evaluation of the association between intake of green tea and blood glucose levels in a sample of elderly adults from Cyprus, Mitilini and Samothraki islands ($n = 542$, aged 65–100 years) revealed that moderate tea consumption (1–2 cps/day) was associated with 88% (95% CI 76–98%) lower odds of having diabetes among non-obese participants, irrespective of age, sex, smoking, physical activity status, dietary habits and other clinical characteristics (Polychronopoulus et al., 2008).

Summary of clinical studies

There is an apparent inconsistency in the results of the clinical studies with vitamins. Variable study designs, differences in the applied forms and amounts of the antioxidants, differences in the study populations as well as the unknown daily dietary intake of the participants are made responsible for the diverging results. It has been even argued that not the vitamins but the plant polyphenols are responsible for the protective effects e.g. in CVD, and that thereby the discrepancy between epidemiological and prospective studies might be explained. The non-uniform and often negative results of the vitamin studies have prompted the German drug administration (BfArM) as well as the American Heart Association and the US preventive services task force not to advise the preventive supplementation of these antioxidants for the healthy population (Cherubini et al., 2005; Siekmeier et al., 2006; Lichtenstein et al., 2006). However, a better knowledge of the underlying mechanisms of action(s) of antioxidants, may result in a better understanding of conflicting clinical results. Especially in the context of polyphenols more clinical studies alone or in combination with vitamins in men are needed to further substantiate their protective effects in various diseases as well as to identify their mechanisms of action. Especially while evaluating the mode of action, multitargeting comes into focus, which may be applicable to antioxidants in general. The multitarget therapy concept derives from the assumption that a complex multifactorial pathophysiology (multifactorial diseases) can be managed more effectively by the use of a correspondingly composed multidrug mixture (Wagner, 2006). Herbal medical drugs are such multidrug mixtures which have long been criticized for this characteristic and subsequently for a more or less impossible prediction of their pharmacological actions. But today, with the availability of the "omic"-technologies, investigations of complex mixtures are possible (Ulrich-Merzenich et al., 2007b) and data banks on the plant constituents and a multitude of at least preliminary data on pharmacological actions of single and multidrug preparations including their clinical use are existing. This knowledge has not yet been reasonably explored for the development of multidrug mixtures for multifactorial diseases.

Influence of ROS and antioxidants on cell signaling

For the prevention of diseases like cancer or vascular diseases, but also in the aging process the control of tissue/cell regeneration is essential. Increasing evidences demonstrate that oxidants and antioxidants influence important signal cascades such as mitogen-activated protein kinases (MAPK), which control proliferation and apoptosis and thus tissue regeneration. MAPK are serine/threonine kinases, which play an important role in the regulation of a variety of transcription factors and gene expressions. They consist of four interconnected families of MAPKs linked to different signals at the plasma membrane with different substrate specificities. They include extracellular signal-regulated kinase (ERK)-1 and -2, p38 kinases, ERK5 (big MAPK1) and c-Jun N-terminal kinases (JNKs) (Liu et al., 2000; Kyriakis et al., 1994). ERK1 and -2 are activated primarily in response to proliferative stimuli, the other MAPKs are activated primarily by inflammatory and stressful stimuli including oxidant and osmotic stresses (Liu et al., 2000; Kyriakis et al., 1994). The p38 kinases are associated with the inhibition of cell growth in some cell types (Kyriakis and Avruch, 2001). ERK5 was shown to be activated by oxidative stress and hyperosmolarity, but also by receptor tyrosine kinases (Kyriakis and Avruch, 2001) and may play a role in the angiogenesis of the heart muscle. C-Jun is a component of the transcription factor AP-1 involved in a separate mitogenic and stress-induced signaling pathway (Kyriakis and Avruch, 2001). The MAPK are modulated by ROS and antioxidants and thereby form a link between the oxidative status of the plasma and the intracellular transcription factor activation.

Influence of ROS on MAP-kinases

Mild elevation in intracellular O_2^- or H_2O_2 can stimulate growth responses in a variety of cell types (Brand et al., 2001). Recently, ROS including O_2^- and H_2O_2 were shown to be produced in the VSMC, ECs and cardiomyocytes (Venardos et al., 2007; Abe and Berk, 1998; Griendling et al., 2000; Yoshizumi et al.,

2001a; Kyaw et al., 2004). Growth promotion in response to ROS requires the activation of ERK1/2. ERK1/2, however, are known to play a dual role. The ultimate result of activation – growth promotion or inhibition – depends on the cell type, the simultaneous activation of the different signal transduction kinases and apparently the concentrations of the stimulators and duration of activation. For example high concentrations of H_2O_2 activate ERK1/2 permanently (Fig. 1), leading to apoptosis whereas the transient activation as in case of a strict cell regulation leads to proliferation (Brand et al., 2001). In tumor cells a mild increase in the cellular ROS does not trigger proliferation but inhibits apoptosis, irrespective of the trigger (Pervaiz and Clement, 2007). The presence of O_2^- or relatively low concentrations of H_2O_2 augment T cell activation (Droge, 2002; Venardos et al., 2007). Venardos et al., (2007) propose a "pro-life" activity of ROS, since NADPH-dependent generation occurs upon growth factor stimulation or cytokine receptor activation e.g. in cases of PDGF, TNF-α, IL-1β, IL-6, IL-3, fibroblast growth factor (FGF-2), TGF-α, and insulin (Droge, 2002; Sauer et al., 2001). They propose superoxide and hydrogen peroxide as the main "players". High amounts of superoxide lead to cell survival whereas a tilt in favor of hydrogen peroxide facilitates apoptosis. Even though this model relates to oncogenesis, the balance between the two and the antioxidants is regarded to determine cell fate.

Influence of the vitamins C and E on MAP-kinases

Antioxidants can influence the MAPK-pathway via ROS-modulation. But we recently showed that e.g. vitamin C can also activate MAPK directly (Ulrich-Merzenich et al., 2007a, b). To exclude an interference of H_2O_2, which can potentially be formed by the oxidation of vitamin C, we stimulated EC with vitamin C in the presence of catalase (CAT), which eliminates H_2O_2. Vitamin C activated ERK-1/2 in a concentration-dependent manner (Fig. 2). The stress kinases p38 and c-jun were not activated by vitamin C, but by H_2O_2. The activation of ERK1/2 was also stable in the presence of CAT thus indicating a H_2O_2 independent action. Besides ERK1/2, also ERK5 was activated by vitamin C during proliferation (Fig. 3) and thus may be related to growth promotion in EC in general (Ulrich-Merzenich et al., 2007a, b). The latter is supported by recent findings in ERK 5 null mice. Their ECs which line the developing myocardium and embryonic blood vessels were disorganized and failed to mature (Regan et al., 2002). Further ERK5 activation by steady laminar blood flow is proposed to be atheroprotective by inhibiting EC apoptosis (Pi et al., 2004).

These results give an additional mechanistic explanation for earlier findings that vitamin C promotes proliferation of several cell types (Ulrich-Merzenich et al., 2002a, b, 2007a, b). *In vivo* it was shown (Nunes et al., 1997) that the combination of vitamins C and E promoted vessel remodeling. In addition, the combination of α-tocopherol, β-carotine and vitamin C or α-tocopherol (α-Toc) alone attenuated myocyte apoptosis and improved cardiac function in CHF via changes in the MAPK-pathways involving the ERK1/2 signaling and the c-JUN and p38 pathways (Qin et al., 2003).

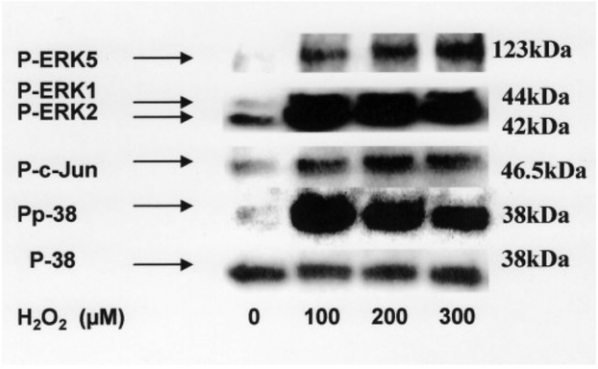

Fig. 1. Panel of MAP-kinases (ERK 1,2 and 5, p38, c-Jun) induced by H_2O_2 in endothelial cells. (Ulrich-Merzenich et al., 2007a).

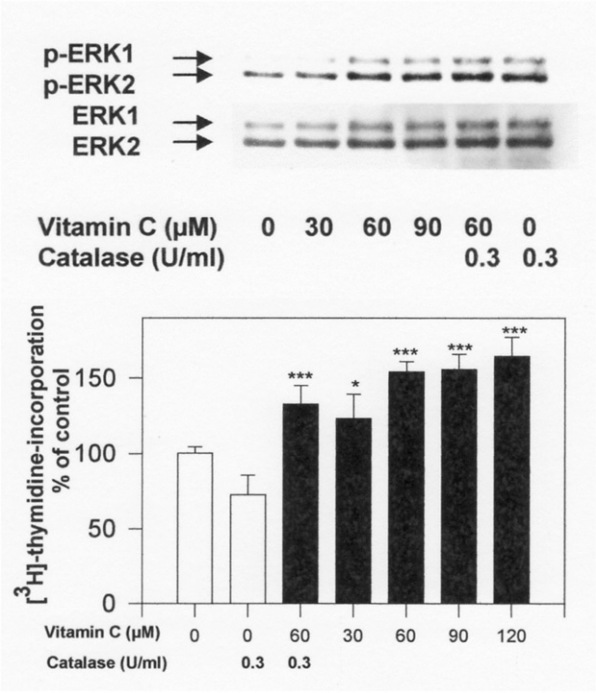

Fig. 2. Figure showing vitamin C as signaling molecule, which can stimulate the non-stress-induced MAP-kinase ERK 1 and 2 in the presence of catalase. In parallel experiments the proliferation of endothelial cells is shown (Ulrich-Merzenich et al., 2007a).

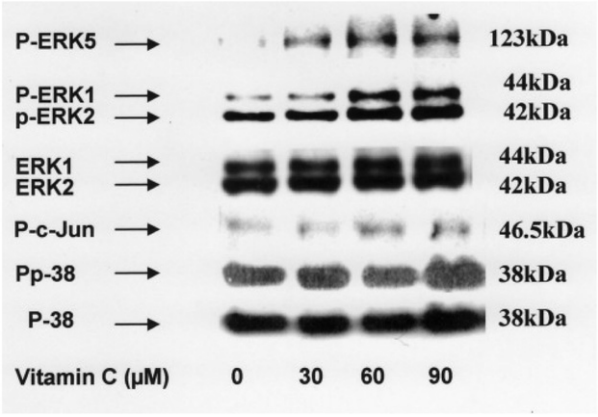

Fig. 3. Shows that vitamin C induces the non-stress-induced MAP kinase 1,2 and 5, but not the stress-induced MAP-kinases p38 and c-Jun in endothelial cells (Ulrich-Merzenich et al., 2007a).

CHF-animals exhibited increased oxidative stress as evidenced by a decreased GSH/GSSG ratio, an increased total and phosphorylated c-Jun NH2-terminal protein kinase and a phosphorylated p38 kinase as well as a decreased phosphorylated ERK1/2 (Qin et al., 2003). These changes were counteracted by the combination of α-Toc, β-carotine and vitamin C, but also by α-Toc alone. α-Toc modulated the proliferation in several cell types like promoting EC growth and inhibiting VSMC growth. Furthermore α-Toc reproducably increased the neutrophil count in humans in respiratory infections (Webb and Villamor, 2007). Thus, vitamins C and E can modulate proliferation, in some case directly via MAP-kinase activation.

Influence of polyphenols on MAP-kinases

Quercetin was shown to inhibit JNK activation induced by angiotensin II in rat aortic smooth muscle cells while ERK1/2 and p38 were not affected (Yoshizumi et al., 2001a, b). In a human hepatoma cell line quercetin induced apoptosis via caspase activation, the regulation of Bcl-2 and the inhibition of PI-3-kinase/Akt and the ERK pathways (Granado-Serrano et al., 2006). Quercetin-3-glucuronide, one of the three major plasma quercetin conjugates, was shown to inhibit the MAPK JNK and the transcription factor AP-1 signaling pathway in concentrations comparable to the aglycon (Yoshizumi et al., 2002).

Epigallocatechin-3-gallate (EGCg) was shown to promote hair growth in hair follicle cultures *ex vivo* and the proliferation in cultured dermal papilla cells. The growth stimulation appeared to be mediated via the upregulation of phosphorylated ERK and Akt and by an increase in the ratio of Bcl-2/Bax (Kwon et al., 2007). EGCg also promoted pro-matrix metalloproteinase-7 production (MMP-7) in HAT-29 human colorectal cancer cells. Authors suggest that the pro-MMP-7 production is mediated via O_2^- production and the activation of JNK1/2, c-JUN, c-FOS and AP-1 (Kim et al., 2005).

Oral administration of the green tea polyphenolic extract (GTE) attenuated the ethanol-induced expression of COX-2 and iNOS. Inactivation of MAPKs, especially p38 and ERK1/2, by GTE were proposed to be responsible (Lee et al., 2005). Also the proanthocyanidin prodelphinidin B-4 3′-*O*-gallate was shown to be involved in the inhibition of COX-2 and iNOS via the downregulation of TAK1–NF-κB pathway (Hou et al., 2007).

Black tea polyphenols (PBPs)-fractions were able to decrease 12-*O*-tetradecanoylphorbol-13-acetat (TPA) induced cell proliferation by decreasing activation of signaling kinases (c-Jun, ERK, p38 and Akt) in the epidermis of skin of mice (Patel et al., 2008).

Red wine polyphenols (RWPs) were shown to inhibit the expression of vascular endothelial growth factor (VEGF), a major pro-angiogenic and pro-atherosclerotic factor, in VSMCs by preventing the redox-sensitive activation of p38 MAPK-pathway (Oak et al., 2003). In a study on the inhibitory potential of red wine polyphenols it was shown that anthocyanins presenting a hydroxyl residue at position 3′ are able to inhibit $PDGF_{AB}$-induced VEGF expression by preventing activation of p38 MAPK and JNK in VSMCs (Oak et al., 2006).

Signaling events in response to black tea polyphenols were investigated by Anter et al. (2005). They induced a time-dependent phosporylation of the estrogen receptor-α (ER) on Ser-118 in bovine aortic endothelial cells. Phosphorylation of ER was due to p38 MAPK activation (Anter et al., 2005).

Cocoa polyphenol (CP) fractionated from commercial cocoa powder, containing gallic-acid-equivalent phenolics and epicatechin-equivalent flavonoids, exhibited a dose-dependent free radical scavenging activity and also inhibited dose dependently xanthine oxidase activity and TPA induced superoxide anion generation in cultured human promyeolytic leukemia HL-60 cells (Lee et al., 2006). Oral administration of CP (4–200 mg/kg body weight) to ICR mice before TPA inhibited ear edema at 5 h in a dose-dependent manner. Cox-2 expression was also down regulated. Overall CP inhibited the catalytic activity of ERK 1/2 in TPA stimulated mouse skin (Lee et al., 2005).

Chlorogenic acid, the ester of caffeic acid with quinic acid is supposed to be one of the most abundant polyphenols in our diet (Feng et al., 2005). Feng et al. (2005) showed that chlorogenic acid inhibited the proliferation of A549 human cancer cells *in vitro*. The

chemoprotective effects was proposed to be through the upregulation of cellular antioxidant enzymes and the suppression of ROS-mediated NK-κB, AP-1 and MAPK activation (Feng et al., 2005). Thus, polyphenols can modulate MAP-kinases primarily via ROS.

ROS as second messengers and antioxidants as regulators of the 'steady state'

ROS as well as antioxidants can both influence signal cascades like MAPK. But that unspecific stimuli like ROS can act as second messengers and participate or even determine cell growth is not easy to imagine (Azzi, 2007). It raises the questions whether unspecific stimuli are at least to some extent involved in physiological growth and how? The apparent inconsistencies in the clinical trials with antioxidants (Table 1) seem to give hints where to look for answers. Under the assumption that the main task of antioxidants is not the elimination of 'oxidative stress', but the maintenance of a "steady state", some of the so far inexplicable results become plausible.

In several clinical studies short-term supplementation of antioxidants was shown to lead to a reduction of "oxidative stress", which was not maintained by the long-term supplementation (Richards et al., 1990; Van Tits et al., 2001; Meydani et al., 1997). Transient reductions of respiratory bursts are observed with short-term, but not with long-term supplementation (Webb and Villamor, 2007). RCTs also suggest an influence of short-term, but not of long-term vitamin E supplementation on mitogen-stimulated ROS production (Webb and Villamor, 2007).

It seems that the redox-system adapts and regulates the ROS production so that the former 'set point' (steady state) is reached again. The idea of a regulation of the ROS production is supported by the finding that in the moment the supplementation is discontinued, the "oxidative stress" in the supplemented group is higher than in the placebo group (Webb and Villamor, 2007), which may indicate that under supplementation the system is transiently set for a higher intake of antioxidants and therefore produces at this stage a higher amount of ROS for compensation. After some time the original redox state is reached again. This model has the assumptions of (1) an individual "steady state" and (2) the capability of the cell to control its own ROS production.

Indeed the so-called "redox state" of a cell is proposed to be kept within a narrow range under physiological conditions, comparable to the pH regulation (Valko et al., 2007). How the "redox state" is kept in balance is still not fully understood. The complex interplay of oxidants and antioxidants or the regenerative capacity of one single antioxidant in relation to another still requires investigations (Venardos et al., 2007; Dhalla et al., 2000). E.g. GSH is able to regenerate the most important antioxidants, vitamin C and E, back to their active forms. GSH can reduce the tocopherol radical of vitamin E directly, or indirectly via the reduction of semi-hydroascorbate to ascorbate (Valko et al., 2007). Eventually antioxidants may have their individual function in the system and are therefore not simply exchangeable. Azzi (2007) proposed in this context that the concentration of plant polyphenols provided with *in vitro* antioxidant properties is kept in the human organism very low by their limited absorption and their subsequent modification and efficient elimination (Scalbert and Williamson, 2000; Williamson and Manach, 2005a, b; Kroon et al., 2004) in order to protect the organism from excessive antioxidant intake since ROS have evolved as signaling molecules (Li et al., 2006; Schmelter et al., 2006; Bell et al., 2005; Wollin et al., 2005; Jackson et al., 2004). At the same time we have seen that the conjugates of polyphenols still possess antioxidant abilities and that obviously a combination of polyphenols and their conjugates make up for their total antioxidative potential *in vivo*.

We are still only at the beginning to unrevealing the interaction of oxidants and antioxidants. Arguments to regard their interplay to be a complex regulatory system are not only *in vivo* and *in vitro* findings that the combination of antioxidants is often superior with respect to the desired effect compared to the application of single antioxidants (Ulrich-Merzenich et al., 2002a, b; Gey, 1998; Tantcheva et al., 2003; Nunes et al., 1997), but also the generally observed discrepancy between positive results of epidemiological studies and negative results of interventional studies especially with single vitamins. This underlines the assumption that the modulation of complex regulatory systems requires complex rather than single interventions and goes along with the "multitarget" effects of polyphenols and vitamins. The goal of investigating the mode of action of "antioxidants" in future may not be the demonstration of an isolated suppression of a free radical but rather the modulation of the redox state, i.e. preserving the redox-homoestasis and the balance of tissue regeneration, in which MAPK, as signal cascades, act as mediators (Fig. 4).

Conclusion

Both, ROS and antioxidants modulate MAP-kinase activity and thereby tissue regeneration. The maintenance of this redox-system in a 'steady state' appears therefore much more important for health than originally thought and may be easier achieved by "multitarget" rather than single component interventions.

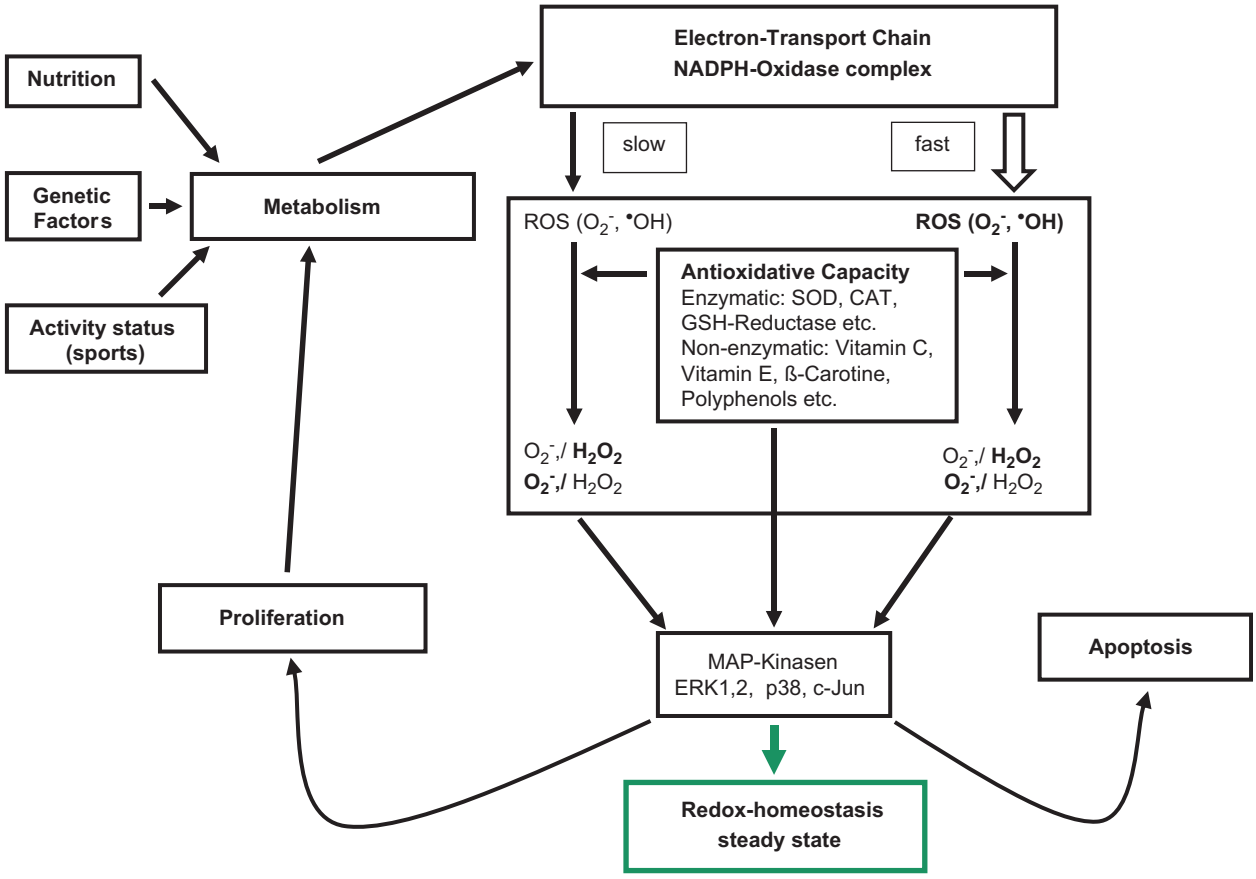

Fig. 4. Figure showing the link between energy production, oxidative stress and tissue regeneration with MAP-kinases as central downstream effectors. In case of a highly active metabolism e.g. through sports activities, the ETC/NADPH-oxidase complex runs on a high speed and produces besides energy equivalents large amounts of ROS. Those are eliminated according to the availability of the non enzymatic antioxidants and simultaneously the induction of the enzymatic antioxidants is triggered. In case high amounts of ROS remain and persist over time e.g. H_2O_2, apoptosis is induced, whereas low concentrations over a long duration can initiate proliferation. Here, the proportion of H_2O_2/O_2^- appears to be important. The redox-homoeostasis leads to a steady state. However, vitamins like vitamin C can activate MAP-kinases also directly depending on their availability. Thus, the redox-homeostasis as controlled synergistic action of "antioxidants" and ROS forms an elementary link between the energy production of the primary metabolism and the activation status of the MAP-kinases to regulate tissue regeneration.

A rational therapeutic approach would require a monitoring of the redox-state of the patient, for which adequate methods still need to be developed and established.

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