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ORIGINAL CONTRIBUTION

Gudrun Ulrich-Merzenich
Heike Zeitler
Darius Panek
Dirk Bokemeyer
Hans Vetter

Vitamin C promotes human endothelial cell growth via the ERK-signaling pathway

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G. Ulrich-Merzenich and H. Zeitler are contributed equally to this work.

G. Ulrich-Merzenich (✉) · H. Zeitler
D. Panek · H. Vetter
Medical Policlinic of the Rheinische
Friedrich-Wilhelms-University of Bonn
Wilhelmstr. 35-37
53111 Bonn, Germany
Tel.: +49-228/2872-2674
Fax: +49-228/2872-2019
E-Mail: gudrun.ulrich-merzenich@
ukb.uni-bonn.de

D. Bokemeyer
Augusta-Krankenanstalten
Bochum, Germany

Abstract *Background* Epidemiological, secondary prevention and small interventional trials suggest a preventive role of vitamin C for cardiovascular diseases (CAD), especially through improving endothelial dysfunction. Large primary prevention trials failed to confirm this. Mechanistic studies may contribute to resolve this discrepancy. *Aim of the study* We examined whether vitamin C activates mitogen-activated protein kinases (MAPK) in human umbilical cord venous endothelial cells (HUVECs) and whether reactive oxygen species (ROS) play a role in this process. *Methods* Subconfluent quiescent HUVECs were incubated with vitamin C alone or in combination with catalase (CAT) and/or hydrogenperoxide (H_2O_2). Intracellular MAPK were determined by Western blot, proliferation by cell count and DNA-synthesis by [^{3}H]-thymidine-uptake. *Results* HUVECs were incubated with vitamin C (60 μM) for 5–60 min or for 20 min (30–90 μM). A dose-dependent phosphorylation of extracellular signal-regulated-kinases (ERKs)-1 and -2 with a maximum of phosphorylation at 15–20 min was observed and inhibitable by MEK1/2-inhibitor U0126 (5–10 μM). Vitamin C (60 μM) stimulated phosphorylation of ERK5, but not of p38 and c-Jun, demonstrating a different MAPK-activation pattern compared to H_2O_2. Vitamin C (60 μM) induced proliferation and a dose-dependent [^{3}H]-thymidine-uptake (30–120 μM) within 20 h. CAT (0.3 U/ml) did neither suppress the vitamin C induced [^{3}H]-thymidine-uptake nor ERK1/2-phosphorylation. CAT (0.3 U/ml), but not vitamin C (60 μM) abrogated the inhibitory effects of H_2O_2 (100 μM) on [^{3}H]-thymidine-uptake. *Conclusion* Physiological vitamin C-concentrations promote proliferation of subconfluent ECs by activating an ERK1/2 controlled pathway. Targeting MAPK by vitamin C may improve, besides antioxidant mechanisms, endothelial dysfunction by promoting a fast regeneration of the endothelium after tissue injury, particularly required during secondary prevention and early development.

Key words antioxidants – endothelium – vitamin C – mitogen-activated proteinkinases

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Introduction

Epidemiological, secondary prevention and small interventional trials suggest a preventive role of vitamin C for cardiovascular diseases (CAD) [1]. But large primary prevention trials failed to confirm these findings [1]. In addition few investigators observed pro-oxidative actions of vitamin C [2, 3] primarily after bolus application of high dosages. The generation of reactive oxygen species (ROS) were observed through the oxidation of vitamin C by metal ions (Cu^{2+}, Fe^{3+}). Variable study designs, differences in the applied forms and amounts of vitamin C can be made responsible for diverging results but the underlying mechanisms of action(s) of vitamin C are also not yet fully understood. For vitamin C the initial point of contact with the vascular system is the endothelium. Its integrity is central to the optimal function of the vascular system. Endothelial dysfunction, one of the earliest manifestations of atherosclerosis, is promoted e.g. by ROS or by oxidized low-density lipoproteins (oxLDL). They inhibit endothelial cell growth [4, 5]; reduce the endothelial formation of nitric oxide, increase the production and release of endothelin-1 and activate endothelial cells (EC) to express cell surface adhesion molecules [6], all processes promoting the development of atherosclerotique plaques. Latest until or after lesion formation has begun, endothelial denudation begins [7]. Also oxidative reperfusion injury often complicates myocardial infarct (MI) healing [8]. In such cases injured areas need to be regenerated extremely rapidly [7] to prevent further development of atherosclerotique plaques and to retain the function of the vessels. The endothelium itself is known to be one of the most quiescent tissues of the body [9], but it is assumed that these regenerative processes occur fast *in vivo* [10]. In this context Nunes et al. [11] demonstrated that vitamin C and E promote vessel remodelling *in vivo*. Also *in vitro* studies showed that subconfluent EC proliferation can be stimulated by vitamin C [4, 12]. Thus, we hypothesized that vitamin C may be active not only as an antioxidant, but also as a mitogen.

The mitogen-activated protein kinases (MAPK) are vital for proliferation and sensible to ROS [13]. 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) [14, 15]. ERK1 and -2 are activated primarily in response to proliferative stimuli, the other MAPK are activated primarily by inflammatory and stressful stimuli including oxidant and osmotic stresses [14, 15]. The p38 kinases are associated with the inhibition of cell growth in some cell types [13]. The biological role of the more recently defined ERK5 is less clear. It was shown to be activated by oxidative stress and hyperosmolarity, but is also activated by receptor tyrosine kinases [13] and proposed to 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 [13]. Thus, we investigated in EC whether and which of these signal cascades are activated by vitamin C. To exclude the possibility that H_2O_2 potentially formed through the oxidation of vitamin C activates MAP-kinases, we stimulated EC also in the presence of catalase (CAT), which eliminates H_2O_2.

Materials and methods

Chemicals

Vitamin C (L-(+) ascorbic acid), CAT and acidic fibroblast growth factor (aFGF) were from Sigma (Germany), U0126 was obtained from Boehringer Mannheim and dissolved in DMSO. Other chemicals were of reagent grade and obtained from commercial sources. The following antibodies (Ab) were used: phospho-p44/42 MAPK (THR202/Tyr204) rabbit polyclonal Ab (1:2000), p44/42 rabbit polyclonal MAPK Ab (1:1000), phospho-p38 (Thr180/Tyr182) rabbit polyclonal Ab (1:1000), p-38 rabbit polyclonal Ab (1:1000), phospho-c-Jun (Ser73) rabbit polyclonal Ab (1:1000), phospho-ERK5 (Thr218/Tyr220) rabbit polyclonal Ab (1:1000) from Cell Signaling Technology, ERK-5 goat polyclonal Ab (1:200), anti-goat IgG-horseradish peroxidase (HRP)-conjugate from Santa Cruz Biotechnology, Anti-Map2 Kinase mouse monoclonal Ab (1:1000) (pan-ERK) from Gibco BRL, Protein A-HRP-conjugate from BioRad, anti-rabbit-IgG and anti-mouse IgG from Amersham Pharmacia Biotech UK.

Cell culture and cell proliferation assays

HUVECs were prepared and maintained as described earlier [4]. Primary EC-cultures in passages 3–5 were used. Cells were maintained on collagen coated 75 cm^2 flasks in culture medium (medium 199 supplemented with 20% foetal calf serum (FCS), penicillin 100 IU/ml, streptomycin 100 μg/ml, 10 μg/ml heparin and 30 μg/ml crude endothelial growth factor) [4]. For the assays ECs were seeded into 24 well culture plates coated with collagen (10 μg/well) and left for attachment 24 h in culture medium. Thereafter culture medium was removed, cells were washed twice with phosphate-buf-

fered saline (PBS) and quiescent medium (medium 199 without phenol red/HAM's F-10 (1:1, v/v). was added. After 4 h of incubation, cultures were exposed to the various stimuli for 20 h. Cell proliferation was assayed by cell count and DNA-synthesis by [^{3}H]-thymidine-incorporation as described [4, 12]. The MAP kinase kinase (MEK)-inhibitor U0126 (5–10 μM) or the enzyme CAT (0.15–0.3 U/ml) were added 10 min ahead of vitamin C (30–120 μM).

Western blot analysis

Subconfluent ECs were made quiescent for 8–15 h in serum-free medium (medium 199 without phenol red/HAM's F-10 (1:1, v/v). They were exposed to vitamin C (30–90 μM) or to H_2O_2 (100–300 μM) (20 min) or to vitamin C (60 μM) for various times (5–60 min). In experiments with the MEK-inhibitor U0126 (5–10 μM) or CAT (0.3 U/ml), cells were preincubated with those substances for 10 min before stimulation. In case of a combination of vitamin C and H_2O_2 cells were preincubated (10 min) with vitamin C. Cells were lysed in RIPA-buffer and lysates prepared as described [16]. Equal amounts of protein (10–40 μg) were mixed 1:4 with 5× Laemli buffer, boiled for 5 min, size-separated using 10% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked in superblocking buffer (ERK 5) or in 20 mM Tris (pH 7.6), 137 mM NaCl, 0.1% Tween-20 (TBST) supplemented with 2.5% BSA for 3 h at RT. Membranes were incubated overnight at 4°C with the primary Ab dissolved in superblocking buffer (Pierce) (ERK5) or in TBST containing 1% BSA or 5% BSA (p-ERK5). The primary Ab were detected using the respected secondary Ab and visualized by Western lightening chemiluminescense (Perkin Elmer Life Science) after extensive washing of the membranes. The intensity of the identified bands was quantified by densitometry employing the BioRAD GelDoc 100 system using the software Multi-Analyst as described earlier [17].

Statistical analysis

Values are reported as means ± SEM. *P*-values were calculated by Sigma Stat 2.0 software (Jandel, San Rafael, CA) using the Student's t-test (normally distributed data) or otherwise the Mann-Whitney U test. Western Blots shown are representative of $n \geq 4$ independent experiments if not indicated otherwise with cells originating from $n \geq 4$ different umbilical cords. A minimum of three independent cell proliferation- and [^{3}H]-thymidine-uptake- experiments were performed, each in triplicates with cells originating from ≥3 different umbilical cords.

Results

ERK1/2 MAP-kinases are activated by vitamin C

The stimulation of subconfluent quiescent HUVECs with vitamin C (60 μM) was followed by ERK1/2 phosphorylation with a maximum between 15 and 20 min after stimulation. FCS, a known stimulator of ERK 1/2, was used as positive control (Fig. 1a, b). The activation persisted until 35 min (data not shown) and was dose-dependent (Fig. 1c, d). There was some degree of variation in the strength of the induced phosphorylation by vitamin C as can be seen comparing Fig. 1a, c, relating to the different cell lines used. Superoxidedismutase (SOD) forms H_2O_2 from superoxideradicals whereas CAT converts H_2O_2 into water and oxygen and eliminates thereby oxidative stress. Under cell culture conditions heavy metals of the Ham's F-10 medium or in vivo Fe^{3+} [2] could potentially oxidize vitamin C which in turn could act as oxidant producing H_2O_2, a known stimulator of MAPK activities. In order to eliminate the possibility that MAPK are activated by H_2O_2 and not directly by vitamin C, we preincuabated HUVECs with CAT before stimulating them with vitamin C. In the presence of CAT alone (Fig. 1c, d), vitamin C still stimulated ERK1/2 phosporylation, but CAT itself also activated ERK1/2 alone (Fig. 1c, d).

We further determined the cell count and DNA-synthesis of HUVECs over a period of 20 h in response to vitamin C and CAT. Cell count and DNA-synthesis increased upon stimulation with vitamin C alone. The aFGF, a known stimulator of DNA-synthesis in ECs, was used as positive control. DNA-synthesis increased in the presence of vitamin C and CAT, but not with CAT alone (Fig. 1e, f).

U0126 inhibits vitamin C induced proliferation and ERK1/2 phosphorylation

U0126 is a selective inhibitor of MEK1 and MEK2 [18] affecting the ability of cells to subsequently activate ERK. In case of an ERK1/2 dependent proliferation, U0126 should inhibit ERK1/2 phosphorylation and proliferation of HUVECs. Indeed U0126 (5–10 μM) inhibited vitamin C-induced effects on DNA-synthesis and on ERK1/2-phosphorylation (Fig. 2a, b) completely.

Comparison of the MAPK activation patterns of vitamin C and H_2O_2

We further examined whether vitamin C or H_2O_2 influenced the phosphorylation of MAPK differen-

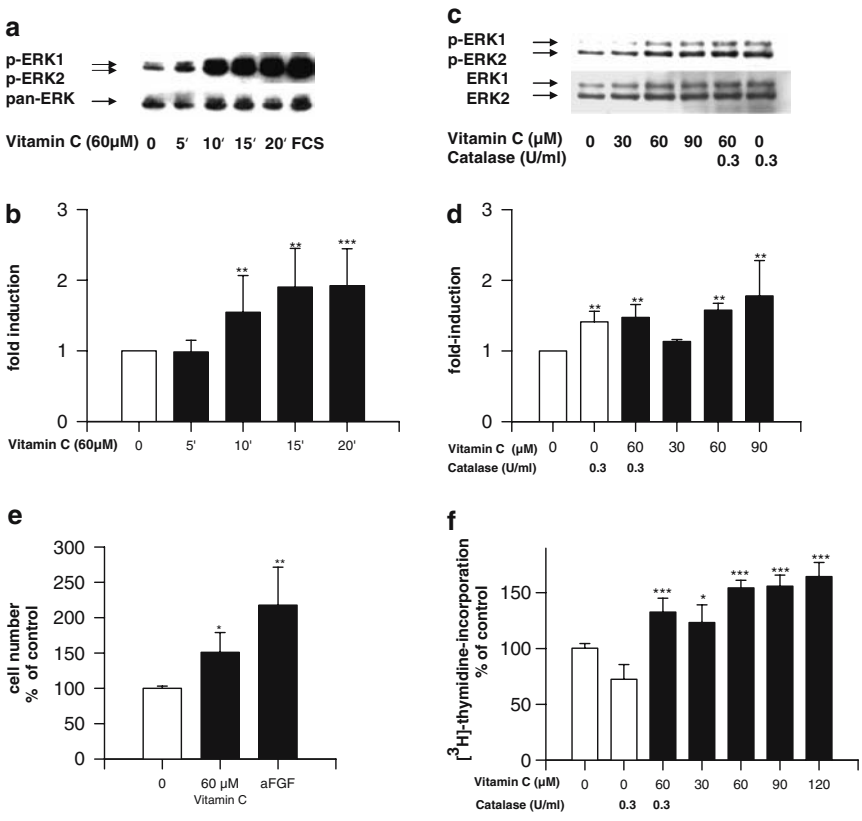

Fig. 1 Vitamin C induced cellular proliferation via ERK1/2. (**a**) and (**b**) Subconfluent quiescent HUVECs were stimulated with vitamin C (60 µM). ERK1/2 were phosphorylated. Densitometric analysis of p-ERK1/2 bands revealed a significant increase of phosphorylation after 15–20 min. Values (mean ± SEM) were normalized by arbitrarily setting the densitometry of unstimulated cells to 1.0. Loading was controlled by reprobing the same membrane with ERK1/2- or pan-ERK-antibodies (Ab). positive control: FCS ($n \geq 4$). (**c**) and (**d**) Subconfluent quiescent HUVECs stimulated with vitamin C showed a dose-dependant increase in ERK1/2-phosphorylation. This increase was also present when vitamin C was combined with catalase (CAT). CAT itself also lead to an ERK1/2 phosphorylation. The same membrane was reprobed with ERK 1/2-Ab as loading control. The densitometric analysis revealed a significant increase from 60 µM onwards ($n \geq 4$). (**a**) and (**c**) demonstrate the magnitude of variation in the activation signal between the different cell lines. (**e**) Cell count revealed a significant increase of proliferation after 20 h incubation of subconfluent quiescent HUVECS with Asc (60 µM) or with acidic fibroblast growth factor (aFGF) ($n = 4$). (**d**) [^{3}H]-thymidine-uptake revealed a dose-dependent increase of DNA-synthesis after a 20 h incubation of quiescent HUVECS with vitamin C (60 µM). DNA-synthesis also increased in the presence of additional catalase. Three independent experiments were performed, each in triplicates. ERK: extracellular-regulated kinases. HUVECs: human umbilical venous endothelial cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

tially. In response to vitamin C, the basal level of phosphorylation of ERK-5 increased whereas p38 and c-Jun remained unaffected after 20 min of stimulation. In response to H_2O_2 the phosphorylation of ERK1/2, ERK-5, p38 and the transcription factor c-Jun strongly increased. (Fig. 2c, d, e).

DNA-synthesis in the presence of vitamin C, CAT and H_2O_2

A preincubation of HUVECs with CAT (0.3 U/ml), but not with vitamin C (60 µM) could abrogate the inhibitory effect of H_2O_2 (100 µM) on DNA-synthesis (Fig. 3).

Discussion

Our data propose that vitamin C can act as mitogen for EC in concentration ≥ 60 µM *in vitro*. Commonly found plasma concentrations of vitamin C range between 37 and 121 µM [19, 20] and plasma concentrations ≥ 50 µM [21] or even ≥ 70 µM [22] have been recommended as preventive for CAD. A recent 10-years prospective study reported a 2.6 fold risk reduction for primarily ischemic stroke in hypertensive men with vitamin C levels > 65 µM compared to those with concentrations < 28.4 µM [23]. These recommended concentrations correspond to our results for the mitogenic effect in vitro. However, it was recently shown that up to 100-fold higher intracellular concentrations of vitamin C are generated during overnight culture in ECs [23] and this accumulation is assumed to take place in vivo, too. We took care of this accumulation by incubating ECs in quiescent medium before stimulation. However, this may contribute to the variation in the strength of phosphorylation between the cultures. We also do not yet know which amount of vitamin C has entered after stimulation into the ECs and in which form. At physiological pH vitamin C is largely present as ascorbate-monoanion [23]. A specific high affinity transport into ECs is assumed since the kinetics and inhibitor specifity of the ascorbate transport into ECs are similar to those observed in other cell types [23]. Our finding that the ERK1/2 activation occurred after 15–20 min of exposure to vitamin C supports this indirectly.

Even though high amounts of vitamin C can accumulate in the ECs, they appear to be insufficient if local lesions occur. Significantly lower mean plas-

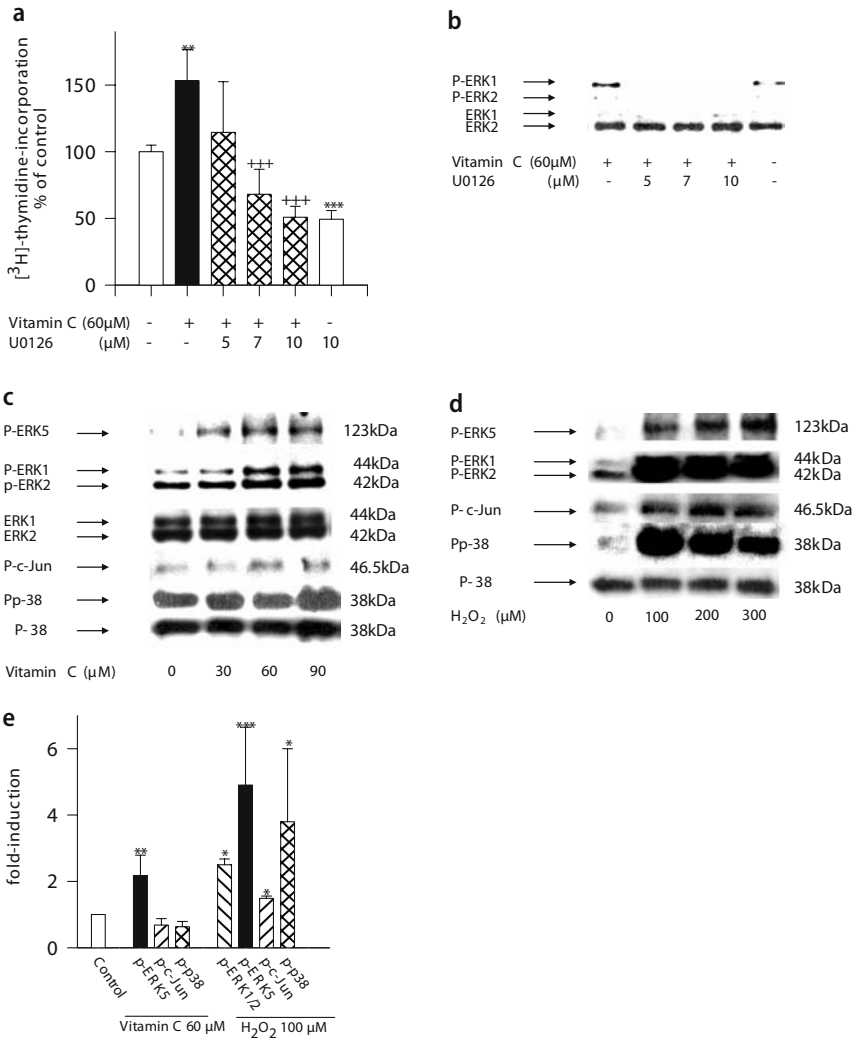

Fig. 2 (**a**) and (**b**) U0126 inhibited DNA-synthesis and ERK1/2-activation (**a**) Subconfluent quiescent HUVECs were stimulated with vitamin C for 20 h. DNA-synthesis was dose-dependently inhibited by U0126. Three independent experiments were performed, each in triplicates. (**b**) Subconfluent quiescent HUVECs were stimulated with vitamin C for 20 min. ERK1/2 activation was inhibited by U0126 ($n \geq 4$). (**c**), (**d**) and (**e**) Effect of vitamin C and H_2O_2 on MAPK-activation. (**c**) Vitamin C induced the phosphorylation of ERK5 and ERK1/2, but not of p38 or c-Jun. Reprobing with ERK1/2-antibodies (Ab) and p38-Ab were used as loading controls. (**d**) H_2O_2 strongly induced the phosphorylation of ERK1, 2 and ERK5, p38 and c-Jun. Reprobing with p38 was used as loading control (**d**) and (**e**) The densitometric analysis revealed a significant increase of phosphorylation for ERK5 by vitamin C (60 µM) whereas H_2O_2 (100 µM) stimulated phosphorylation of p38, ERK1/2, ERK5 and c-Jun. Values (mean ± SEM) were normalized by arbitrarily setting the densitometry of unstimulated cells to 1.0 ($n \geq 4$). HUVECs: human umbilical venous endothelial cells. MAPK: mitogen-activated protein kinases, ERK: extracellular-regulated kinases. *: vs. control, + vs. vitamin C. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, +++: $P < 0.001$

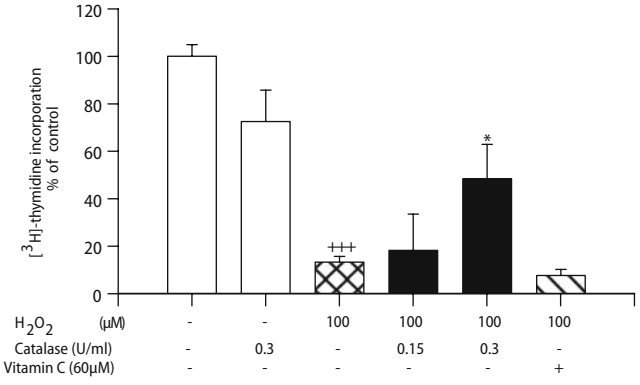

Fig. 3 Effect of H_2O_2, catalase (CAT) and vitamin C on DNA-synthesis. Subconfluent quiescent HUVECs were incubated for 20 h with H_2O_2 alone or following a preincubation (10 min) with CAT or vitamin C. H_2O_2 significantly decreased DNA-synthesis. This decrease was dose-dependently inhibitable by CAT, but not by vitamin C. Three independent experiments were performed, each in triplicates. +: vs. control; *: vs. H_2O_2. +++: $P < 0.001$, *: $P < 0.05$

ma-concentrations were reported for patients with peripheral arterial disease (median: 27.8 µM) [24] and especially during MI (mean: 19.6 µM) [25]. Both studies indicate that local lesions require high amounts of vitamin C leading to a decrease of plasma vitamin C. These high amounts of vitamin C are assumed to be required for counteracting the massive oxidative stress and thereby endothelial injury during MI. A selective proliferative stimuli for EC in addition or as part of the antioxidant protection would further support a fast regeneration of the tissue. Vitamin C possesses both properties—it acts antioxidative and we can show here that it is a mitogen for EC. In addition vitamin C can inhibit vascular smooth muscle cell growth [12], another important property to counteract atherosclerosis. Vitamin C's essentiality for the synthesis of collagen, the main protein of blood vessels [26], by stimulating type IV collagen synthesis for the basement membrane [23] further

supports the hypothesis of looking at vitamin C as mitogen and promotor of local wound healing.

The mitogenic effect could also be caused by H_2O_2 formed during the oxidation of vitamin C by metal ions (Fe^{3+}, Cu^{2+}) present in the culture medium or in the plasma. It was shown earlier that bolus injections of vitamin C during hemodialysis can lead to pro-oxidant effects like an increased generation of superoxides in iron-overloaded uremic patients [2]. Also vitamin E and C supplementation triggered oxidative stress in aortic tissue of rats [27]. Our findings that vitamin C (60 μM) induced DNA-synthesis in the presence of CAT in concentrations high enough to counteract 100 μM of H_2O_2 clearly speaks for vitamin C as mitogen for ECs in vitro. However, also CAT itself activated ERK1/2, but without inducing DNA-synthesis. ERK1/2 is known to play a dual role. The ultimate result of activation—growth promotion or inhibition—depends on the cell type, the simultaneous activation of other signal transduction kinases [13] and apparently the stimulator concentration and duration of activation. For example high concentrations of H_2O_2 activate ERK1/2 permanently, leading to apoptosis [18] whereas free radical maintained under strict cell regulation lead to a transient activation. They have been proposed to act as second messengers in a manner similar to growth factors [18, 28]. Both, vitamin C and CAT, influence the free radical balance and may thereby activate ERK1/2. The ultimate growth promotion will depend on additional factors like other MAPK-activation or e.g. the ability to promote collagen synthesis. Vitamin C did not activate the stress related MAP-kinases p38 or c-Jun, demonstrating a different activation pattern of vitamin C compared to H_2O_2. That vitamin C, compared to CAT, did not counteract the inhibitory effect of H_2O_2 (100 μM) on DNA-synthesis demonstrates that 60 μM of vitamin C are insufficient for this high amount of H_2O_2, and indicates the specifity of CAT for this task. Indeed, an overexpression of CAT due to a gene transfer into human arterial EC led to an increase in cell viability and a reduced apoptosis under exposure of oxLDL [29].

In contrast to our results with 60 μM, high (mM) concentration of vitamin C have been reported to directly activate p38 in HUVECs stimulated by tumor necrosis factor-α thereby inhibiting NF-κB activation [30]. Thus, the effect of vitamin C on p38 may be concentration dependent or may relate to specific isoforms of p38 (α, ß, γ, δ) [31] not differentiated here. Also the role of p38 itself is known to be cell-type- and stimulus specific [31].

ERK5 was activated by vitamin C during proliferation and thus may be related to growth promotion in HUVECs. This is supported by recent findings in ERK5 null mice. Their ECs which line the developing myocardium and embryonic blood vessels were disorganized and failed to mature [32]. Further, ERK5 deficiency impeded angiogenic remodelling via the vascular endothelial growth factor [33]. Also ERK5 activation by steady laminar flow is proposed to be atheroprotective by inhibiting EC apoptosis [34]. Our findings of a simultaneous upregulation of ERK5- and ERK1/2-phosphorylation in proliferating ECs support the assumption that ERK5 plays a role in organized blood vessel development and argues for a supportive role of vitamin C in these processes.

The MEK-inhibitor U0126 inhibited the vitamin C induced proliferation and the ERK1/2 activation and further supports a role of ERK1/2 for the vitamin C induced proliferation in EC. ERK-inhibitors are presently investigated as potential therapeutic anti-atherosclerotic agents since they are activated during inflammation and during VSMC-proliferation. But the pharmacological blockade of the ERK-cascade during ischemia/reperfusion by PD98059, another MEK-inhibitor, increased the induced injury in isolated rat hearts [35] and both PD98059 and U0126 augmented the infarct size in pig myocardium during sustained ischemia [36]. Our results show, that the MEK-inhibitor UO126 inhibits proliferations of ECs. An increased injury or an augmented infarct size in the presence of MEK-inhibitors may indicate that ERK1/2-activation and proliferation of ECs are essential for an optimal regeneration of vessels after injury.

In summary we demonstrate that physiological concentrations of vitamin C promote DNA-synthesis and proliferation in subconfluent ECs via targeting ERK1/2 and ERK5 without activating the stress-related MAP-kinases p38 or c-Jun. This ability may improve, in addition to antioxidant mechanisms, endothelial dysfunction by promoting a fast regeneration of the endothelium after tissue injury, particularly required during secondary prevention, early development or after balloon angioplasty which commonly goes along with an arterial injury and a delayed reendothelialization.

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