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Free Radical Scavenging and Antioxidant Activity of Ascorbigen Versus Ascorbic Acid: Studies in Vitro and in Cultured Human Keratinocytes

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Ascorbigen (ABG) is formed by indole-3-carbinole and ascorbic acid in brassica vegetables. In the present study, ABG has been systematically analyzed for its free radical scavenging and antioxidant capacity. To directly measure the free scavenging activity of ABG and ascorbic acid (used as a positive control), electron spin resonance and spin trapping experiments were performed. Ascorbic acid exhibited a potent free radical scavenging activity, whereas ABG did not scavenge superoxide anion free radicals and showed only little scavenging activity toward 2,2-diphenyl-1-picrylhydrazyl free radicals. Similar data were obtained for the ferric reducing ability of plasma and trolox equivalent antioxidant capacity assays. In cultured human keratinocytes, ABG counteracted *tert*-buthylhydroperoxide-induced cytotoxicity, whereas ascorbic acid did not exhibit any protective activity. Furthermore, in ABG-treated human keratinocytes, a decrease in *tert*-buthylhydroperoxide-induced lipid peroxidation was detected, whereas an ascorbic acid pretreatment did not result in the prevention of lipid peroxidation. These data indicate that ascorbic acid seems to be a more potent free radical scavenger than ABG in vitro, while ABG prevented *tert*-buthylhydroperoxide cytotoxicity more effectively as compared to ascorbic acid in cultured cells.

KEYWORDS: Ascorbigen; ascorbic acid; glucosinolates; antioxidant capacity; HaCaT keratinocytes; lipid peroxidation

INTRODUCTION

Ascorbigen (ABG) [for the chemical structure of ABG and ascorbic acid (AA), see **Figure 1**] is a compound that naturally occurs in Brassica vegetables (*1, 2*). At first, Prochazka and co-workers (*3*) isolated ABG and Kiss and Neukom elucidated its chemical structure in 1966 (*4*). ABG is a breakdown product of the glucosinolate metabolism in plants and is formed by its precursor glucobrassicin (GB) that is enzymatically degraded by the enzyme myrosinase. The formation of ABG depends on the hydrolysis conditions including pH (*5*) and the presence of metal ions (*6*): Under neutral pH conditions, the main product formed is indole-3-carbinol (I3C) (*7*), whereas an acidic pH basically results in the formation of 3-indolyl-acteonitrile (*8*); in the presence of AA, 3-indolyl-acteonitrile and I3C form ABG (*9, 10*). Consequently, ABG formation requires two consecutive steps: first, the enzymatic hydrolysation of GB to I3C; and second, the spontaneous reaction of I3C with L-AA (*11*) to form ABG (*12*). The corresponding reaction scheme is presented in **Figure 2**. Both the chemical and the biological properties of ABG have recently been reviewed (*13*).

The human skin is constantly exposed to reactive oxygen and nitrogen species (*14*), and recent research has aimed to identify specific phytochemicals in Brassica vegetables that confer protection of the skin against oxidative stress. In fact, studies

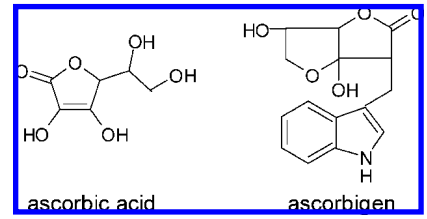

Figure 1. Chemical structures of the test compounds.

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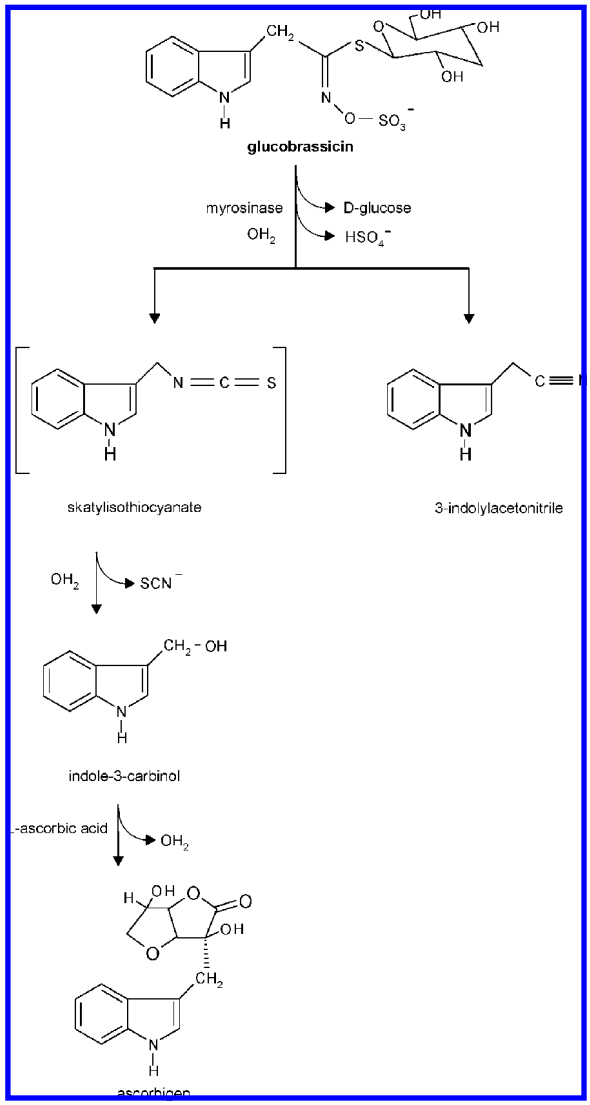

Figure 2. Mechanism of ABG formation through myrosinase-mediated hydrolysis of GB (modified according to Hrncirik et al. *2*).

in mice indicate a protection against UV light-induced skin carcinogenesis by sulforaphane-containing broccoli sprouts (*15*). However, to the best of our knowledge, there are no studies available that have systematically analyzed the free radical scavenging and antioxidant effects of ABG, although it is accepted that reactive oxygen and nitrogen species are centrally involved in skin carcinogenesis. In the present study, we determined the free radical scavenging activity of ABG by using different in vitro assays including electron spin resonance (ESR) spectroscopy and spin trapping. Additionally, cell culture studies in human keratinocytes were conducted to analyze the potential of ABG to protect against *tert*-butylhydroperoxide (tBHP)-induced cytotoxicity and lipid peroxidation.

MATERIALS AND METHODS

Chemicals. Stock solutions of 100 mmol/L AA (Carl Roth, Karlsruhe, Germany) in aqua bidest and 100 mmol/L ABG (Parish Co., Orem, UT) in 0.125% citrate/aqua bidest were freshly prepared. To measure the free radical scavenging and antioxidant capacity of AA in comparison to ABG, concentrations ranging from 0.25 to 100 mmol/L were used in the different in vitro assays. For tissue culture studies, 500 mmol/L ABG was dissolved in dimethyl sulfoxide (DMSO) and stored at −80 °C until further use. tBHP was freshly prepared in aqua bidest and diluted in phosphate-buffered saline (PBS). In the Neutral Red Assay, concentrations from 0.25 to 1000 μmol/l of both ABG and

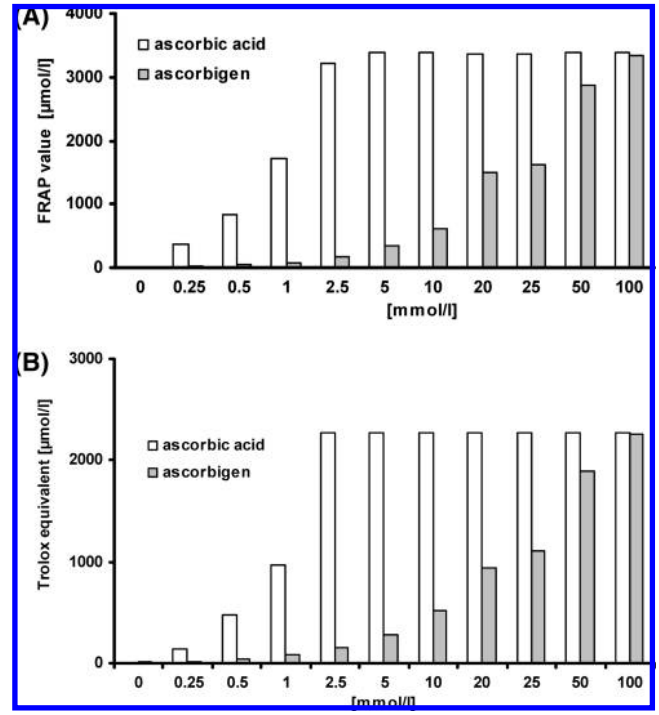

Figure 3. Antioxidative capacity of ABG vs AA. (**A**) The FRAP values of increasing concentrations of ABG vs AA showed a 40 times more effective iron reducing activity of AA as compared to ABG. Pooled $SD_{AA} = 0.0244$, pooled $variance_{AA} = 0.0006$; pooled $SD_{ABG} = 0.0768$, pooled $variance_{ABG} = 0.0059$. (**B**) In the TEAC assay, AA exhibits a 40 times higher antioxidative capacity than ABG. Analyses were performed in triplicate. Pooled $SD_{AA} = 0.0014$, pooled $variance_{AA} = 0.0001$; pooled $SD_{ABG} = 0.0038$, pooled $variance_{ABG} = 0.0001$.

tBHP were tested. AA was used as a positive control throughout the ferric reducing ability of plasma (FRAP), trolox equivalent antioxidant capacity (TEAC), and ESR and spin trapping experiments. For all in vitro and cell culture assays, vehicle controls were performed. Neither citrate nor DMSO, at the concentrations used, affected any of the parameters measured.

FRAP and TEAC Assays. The FRAP assay was conducted as described by Benzie and Strain (*16*). The antioxidant capacity of the test compounds was determined through a reduction of iron(III) to iron(II) applying Fe(II) $SO_4 \cdot 7H_2O$ ranging from 0.025 to 1 mmol/L as a reference. The samples were mixed with FRAP solution and left at room temperature for 6 min, and subsequently, the absorbance was measured at 593 nm in a spectrophotometer (DU 800, Beckman Coulter, Krefeld, Germany). The results were given as FRAP values in μmol/L referred to the Fe(II) $SO_4 \cdot 7H_2O$ standard curve.

The TEAC assay by Miller and co-workers (*17*) was based on the reduction of the 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation by antioxidants. As a reference substance, the synthetic antioxidant trolox (0.05−0.75 mmol/L) was used. The test compounds were mixed with the ABTS solution and incubated at room temperature for 6 min, and following, the absorbance was measured at 734 nm in a spectrophotometer (DU 800, Beckman Coulter).

ESR and Spin Trapping. ESR and spin trapping were applied to detect the free radical scavenging activity of DPPH and superoxide anion radicals by ABG and AA as previously described (*18*). The experiments were performed on a JEOL JES-TE 200 ESR spectrophotometer (X-Band microwave unit) and carried out in triplicate.

ABG was dissolved in citric acid (pH 6.0) and mixed with a 1:2 mixture of citrate (pH 6.0)/methanol and 40 μL of 0.5 mmol/L DPPH diluted in methanol solution. Following, the solution was transferred into a hematocrit capillary tube, and the DPPH signal was measured exactly 90 s after adding the DPPH solution by ESR spectrophotometry.

The superoxide scavenging activity of antioxidants was conducted in 300 μL of aqueous solution in a microtest tube containing 160 μL

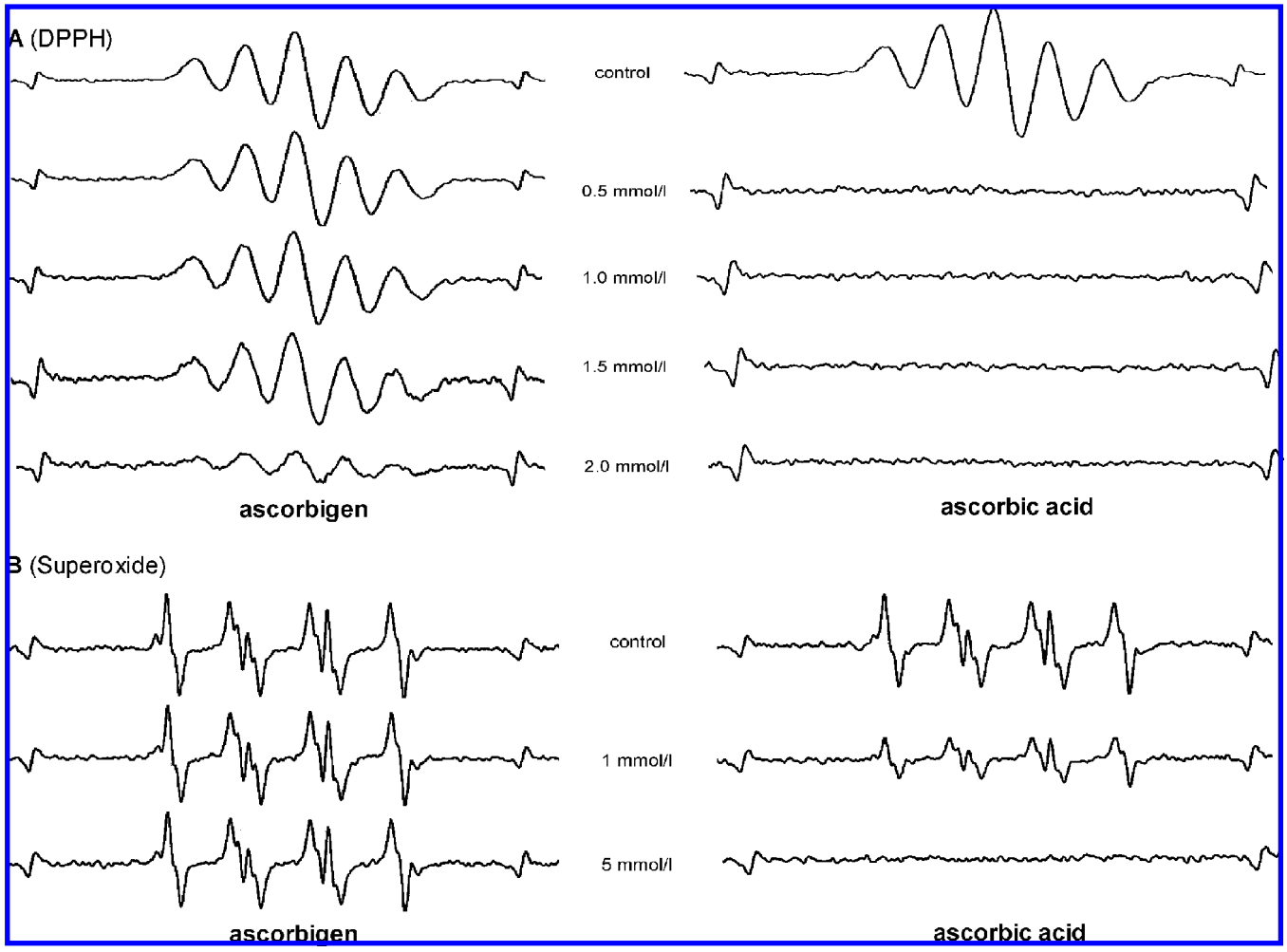

Figure 4. DPPH radical (**A**) and superoxide (**B**) scavenging activity of ABG in comparison to AA as measured by ESR spectroscopy and spin trapping. One out of three representative experiments is shown.

of 2 mmol/L hypoxanthin oxidase solution in 50 mmol/L phosphate buffer solution, 20 μL of 10 mmol/L DMPO in 50 mmol/L phosphate buffer, and 80 μL of 0.4 U/mL xanthine oxidase solution in 50 mmol/L phosphate buffer, 100 μL of 0.3−3.0 mmol/L antioxidants (final concentration, 0.1−1 mmol/L), and 60 μL of 10 mmol/L diethylenetriaminepentaacetate (DTPA) solution. The reaction was initiated by adding the xanthine oxidase solution. The reaction mixture was transferred into a 100 μL disposal capillary tube. ESR spectra were measured 60 s after the addition of xanthine oxidase solution. ESR spectrometer settings were as follows: central field, 341.0 ± 10 mT; microwave frequency, 9.43 GHz; modulation amplitude, 0.1 mT; microwave power, 8 mW; time constant, 0.03 s; gain, 5.0×10^5; sweep time, 0.5 min; and scan width, 100 G.

Cell Culture Experiments. HaCaT human keratinocytes (generated by Dr. N. Fusenig, DKFZ Heidelberg, and obtained from the Institute of Applied Cell Culture, Munich, Germany) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all PAA, Coelbe, Germany) and grown in a humidified incubator at 37 °C and 5% O_2. Cells were seeded at a density of 400000 cells/mL in a 24 well plate for 24 h, incubated with test compounds for further 24 h, and subsequently stressed with tBHP for 4 (cytotoxicity experiments) and 2 h (Bodipy measurements), respectively.

Cytotoxicity. The Neutral Red Assay (*19*) was used to determine the cell viability after incubation with different compounds. HaCaT cells were treated with 0.25−1000 μmol/L ABG and 0.25−1000 μmol/L tBHP for 24 or 4 h, respectively. tBHP was applied in PBS. To test the ability of ABG to protect the cells from tBHP toxicity, HaCaT cells were preincubated with 100 μmol/L ABG for 24 h, subsequently washed with PBS, and exposed to 500 μmol/L tBHP for 4 h. To analyze the cell viability, the Neutral Red Assay was used. In brief, the culture medium containing the test substances was replaced with fresh serum-containing medium including 60 μg/mL of Neutral Red (Carl Roth, Karlsruhe, Germany). After incubation for 3 h, the medium was removed, and the cells were extracted using a solution comprising 50:49:1 (v/v) ethanol, water, and glacial acetic acid. The absorbance was measured in a plate reader (Labsystems, Helsinki, Finland) at 540 nm.

Bodipy Measurements. Membranous lipid peroxidation was detected by fluorescence of C11-Bodipy$^{581/591}$ (Molecular Probes, Invitrogen; Karlsruhe, Germany), a lipophilic compound that is incorporated into cellular membranes (*20, 21*). HaCaT keratinocytes were treated with either 100 and 250 μmol/L of ABG and AA for 24 h, respectively. Following, the cells were incubated with 10 μmol/L C11-Bodipy$^{581/591}$ for 30 min. As the oxidation of C11-Bodipy$^{581/591}$ is comparable to the extent of lipid peroxidation of polyunsaturated fatty acids in the membrane, it serves as an index for lipid peroxidation of the cell membrane. Because the fluorescence of the oxidized (green) and the reduced (red) form of C11-Bodipy$^{581/591}$ was detected at different wavelengths, the ratio of oxidized to total (ox + red) fluorescence was calculated. C11-Bodipy$^{581/591}$ oxidation was detected at basal and after oxidative stimulation with tBHP for 2 h. The fluorescence was measured at 485/535 and 535/590 nm in a multimodus reader (Infinite F200, Tecan, Crailsheim, Germany).

Statistical Analysis. The statistical calculations were conducted using SPSS software Version 15.0 (Munich, Germany). One-way analysis of variance (ANOVA) with a Tukey's posthoc test was performed for comparison of the outcomes of the cytotoxicity assays. A Games−Howell test (heterogeneous variances) was conducted to test significant differences between oxidation rates.

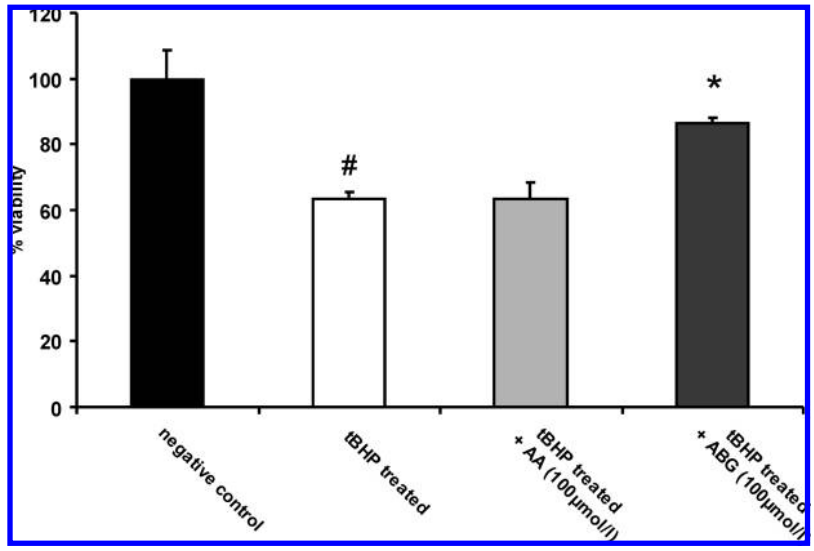

Figure 5. Viability of HaCaT cells after 24 h of preincubation with either 100 μmol/L ABG or 100 μmol/L AA and subsequent cellular oxidative stress for 4 h induced by 500 μmol/L tBHP. Results represent one out of three independent experiments performed in triplicate and are expressed as mean values ($\pm$SD). # indicates significant differences ($p < 0.05$) between tBHP-treated cells as compared to untreated control cells. * indicates significant differences ($p < 0.05$) as compared to tBHP-treated cells.

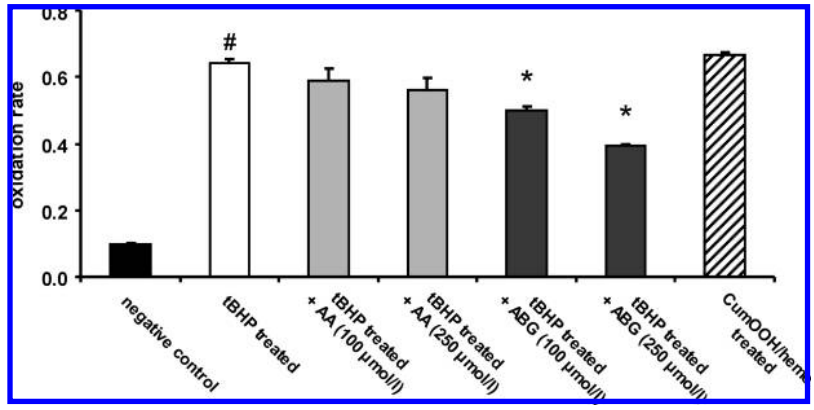

Figure 6. Oxidation rates of C11-Bodipy$^{581/591}$ in HaCaT keratinocytes following treatment with 600 μmol/L tBHP for 2 h and/or preincubation with 100 and 250 μmol/L ABG and AA, respectively. CumOOH (80 μmol/L) and heme (80 nmol/L) served as a positive control. Results represent one out of three independent experiments performed in triplicate and are expressed as mean values ($\pm$SD). # indicates significant differences ($p < 0.05$) between tBHP-treated cells as compared to untreated control cells. * indicates significant differences ($p < 0.05$) as compared to tBHP-treated cells.

RESULTS AND DISCUSSION

In the present study, the free radical scavenging and antioxidant activity of AA vs ABG has been systematically investigated. In the FRAP assay, AA exhibited a dose-dependent increase in the iron reducing ability reaching a plateau at 5 mmol/L AA. ABG also exhibited a dose-dependent iron reducing activity, however, at significantly lower concentrations (**Figure 3A**); similar results were obtained for the TEAC assay. The TEAC assay determines the capability of a test compound to reduce the ABTS free radical. In this assay, both AA and ABG showed a dose-dependent increase of trolox equivalents. Whereas 2.5 mmol/L AA already scavenged 100% of the generated ABTS radicals, 100 mmol/L ABG was necessary to achieve the same effect (**Figure 3B**). Because both the FRAP and the TEAC assay have their methodological limitations and are not highly specific (*22*), additional ESR and spin trapping studies were conducted to directly assess the free radical scavenging activity of the test compounds. ESR and spin trapping confirmed that AA has a higher potency in scavenging free superoxide and DPPH radicals. AA exhibited a potent free radical scavenging activity towards superoxide and DPPH free radicals. However, under the conditions investigated, ABG did not scavenge superoxide anion free radicals and showed only little scavenging activity towards DPPH free radicals (**Figure 4**). In this context, it needs to be taken into account that the DPPH radical does not occur under in vivo conditions and rather presents a model system. However, the cytotoxicity assays in keratinocytes indicated a significant protection of HaCaT cells against tBHP-induced cellular oxidative stress after preincubation with ABG for 24 h, whereas AA exhibited no protection. It is well-known that AA is an important water-soluble antioxidant (*23*) that scavenges reactive oxygen and nitrogen species (*24*). AA belongs to the hydrophilic scavengers and is found in cytosolic, mitochondrial, and nuclear aqueous compartments of the cell reaching concentrations between 1 and 10 mmol/L (*25*). In the in vitro assays, concentrations of AA between 0.25 and 100 mmol/L were used; thus, the concentrations administered in the present in vitro assays were partly within the physiological level. At physiological pH, AA exists only in the form of its conjugated base AH^-, while scavenging free radicals the conjugated base AH^- is transformed into an ascorbyl radical $AH^\bullet$ that in turn deprotonates to $A^{\bullet -}$, which is stabilized via electron delocalization (*25*). Wardman and colleagues described a midpoint reduction potential Em7 (AH^-/

$A^{\bullet}$) of 0.3 V for AA (*26*). However, for ABG, no such information is currently available. As the hydroxyl group is one of the main functional groups involved in the scavenging of free radicals, AA with four hydroxyl groups is, due to its chemical structure, a more potent free radical scavenger than ABG that only possesses two hydroxyl groups. This hypothesis was confirmed by our ESR data. Because tBHP mediates its cytotoxicity mainly through lipid peroxidation in the cellular membrane (*27*), AA may protect the cell membranes to a certain extent through regeneration of the tocopheroxyl radical (*28*), thereby restoring the free radical scavenging activity of vitamin E (*29*). The cytotoxicity to HaCaT cells in the presence of tBHP and AA as well as ABG was measured by using the Neutral Red Assay. While ABG alone did not mediate any cytotoxic effects up to 1 mmol/L, tBHP treatment resulted in a dose-dependent decrease of the viability of HaCaT cells (data not shown). When the cells were preincubated for 24 h with 100 μmol/L ABG and were subsequently stressed with 500 μmol/L tBHP for 4 h, a significant protection against tBHP-induced cytotoxicity was observed (**Figure 5**). In contrast to ABG, a preincubation of HaCaT cells with AA did not show a significant prevention against tBHP-induced cell death (**Figure 5**). Under baseline conditions, the lipid oxidation rate (oxidized/total fluorescence of C11-Bodipy$^{581/591}$), measured as a biomarker of lipid peroxidation, in HaCaT cells was 0.10. Following stimulation with 600 μmol/L tBHP, the oxidation rate in HaCaT cells increased 6.5-fold to 0.64. A preincubation of HaCaT cells with 100 and 250 μmol/L ABG for 24 h resulted in a dose-dependent significant decrease of the tBHP-induced oxidation rate to 0.50 and 0.40, respectively. However, a preincubation with 100 and 250 μmol/L AA for 24 h showed only little effects on the tBHP-induced oxidation rate in HaCaT keratinocytes. Cumene hydroperoxide and heme (CumOOH/heme) was used as a positive control for the induction of lipid peroxidation (**Figure 6**). The C11-Bodipy$^{581/591}$ data indicate a better protection against tBHP-induced lipid peroxidation by ABG as compared to AA. C11-Bodipy$^{581/591}$gets incorporated into cellular membranes, and the oxidized/reduced C11-Bodipy$^{581/591}$ rate is indicative for the oxidation of the membrane. The better protection against tBHP-induced lipid peroxidation by ABG in cultured keratinocytes may be related to the higher lipophilicity of ABG vs AA. Furthermore, cellular protective effects of ABG may also be mediated by other mechanisms than its free radical scavenging per se. It has been shown in rats receiving an indole-enriched diet that different phase II enzymes were significantly induced (*30, 31*). Furthermore, it has been demonstrated that broccoli seeds, containing high amounts of glucosinolates, elevated the protein levels of several antioxidant enzymes via Nrf2-dependent signal transduction pathways (*32*). Such an induction is not possible in a cell-free in vitro system and may, at least partly, explain the difference between our in vitro and our cell culture data. Further studies are required to test the hypothesis whether ABG affects cellular enzymatic and nonenzymatic antioxidants (including glutathione) in keratinocytes. In addition, the effect of ABG against UV-induced oxidative stress needs to be systematically studied both in cultured cells and in vivo.

ABBREVIATIONS USED

ABG, ascorbigen; AA, ascorbic acid; CumOOH/heme, cumene hydroperoxide and heme; DTPA, diethylenetriaminepentaacetate; ESR, electron spin resonance; FRAP, ferric reducing ability of plasma; I3C, indole-3-carbinol; tBHP, *tert*-buthylhydroperoxide; TEAC, trolox equivalent antioxidant capacity.

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