

ORIGINAL ARTICLE

The red-vine-leaf extract AS195 increases nitric oxide synthase–dependent nitric oxide generation and decreases oxidative stress in endothelial and red blood cells

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Abstract

The red-vine-leaf extract AS195 improves cutaneous oxygen supply and the microcirculation in patients suffering from chronic venous insufficiency. Regulation of blood flow was associated to nitric oxide synthase (NOS)-dependent NO (nitric oxide) production, and endothelial and red blood cells (RBC) have been shown to possess respective NOS isoforms. It was hypothesized that AS195 positively affects NOS activation in human umbilical vein endothelial cells (HUVECs) and RBC. Because patients with microvascular disorders show increased oxidative stress which limits NO bioavailability, it was further hypothesized that AS195 increases NO bioavailability by decreasing the content of reactive oxygen species (ROS) and increasing antioxidant capacity. Cultured HUVECs and RBCs from healthy volunteers were incubated with AS195 (100 μ mol/L), tert-butylhydroperoxide (TBHP, 1 mmol/L) to induce oxidative stress and with both AS195 and TBHP. Endothelial and red blood cell–nitric oxide synthase (RBC-NOS) activation significantly increased after AS195 incubation. Nitrite concentration, a marker for NO production, increased in HUVEC but decreased in RBC after AS195 application possibly due to nitrite scavenging potential of flavonoids. S-nitrosylation of RBC cytoskeletal spectrins and RBC deformability were increased after AS195 incubation. TBHP-induced ROS were decreased by AS195, and antioxidative capacity was significantly increased in AS195-treated cells. TBHP also reduced RBC deformability, but reduction was attenuated by parallel incubation with AS195. Adhesion of HUVEC was also reduced after AS195 treatment. Red-vine-leaf extract AS195 increases NOS activation and decreases oxidative stress. Both mechanisms increase NO bioavailability, improve cell function, and may thus account for enhanced microcirculation in both health and disease.

Abbreviations

AU, arbitrary units; AS195, red-vine-leaf extract (Antistax[®]); Du, densitometric units; EI max, maximum deformability; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cell; NO, nitric oxide; NOS, nitric oxide synthase; RBC, red blood cell; RBC-NOS, red blood cell–nitric oxide synthase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SS 1/2, shear stress required for one half of maximum deformability; TBHP, tert-butylhydroperoxide.

Introduction

The red-vine-leaf extract AS195 (Antistax[®]; Boehringer Ingelheim Pharma GmbH & Co, Ingelheim am Rhein, Germany) of *Vitis Vinifera Folium* contains a variety of natural nutrients including the flavonoids quercetin-3-O- β -glucuronide (63%), isoquercitrin (quercetin-3-O- β -glucoside; 32%), and kaempferol-3-O- β -D-glucosid (5%). AS195 has been shown to reduce lower leg edema and circumference of patients with chronic venous insufficiency (Kiesewetter et al. 2000). AS195 further improves the cutaneous microcirculation and oxygen supply in humans with chronic venous insufficiency (Kalus et al. 2004).

Microcirculation represents the blood circulation through the microvessels (Levy et al. 2001) and facilitates the gas exchange between the blood and the surrounding tissue. The blood flow is influenced by the tonus of the arterioles and the rheological properties of the blood cells. These involve plasma viscosity, red blood cell (RBC) aggregation, and RBC deformability (Baskurt and Meiselman 2003) with the latter being positively influenced by nitric oxide (NO) produced within RBC (Bor-Kucukatay et al. 2003; Grau et al. 2013; Bizjak et al. 2015). NO produced by endothelial NOS (eNOS) readily diffuses across the cell membrane to smooth muscle cells and interacts with the enzyme soluble guanylyl cyclase finally leading to vasorelaxation (see for review Bruckdorfer 2005). In RBC, NO is produced by red blood cell-nitric oxide synthase (RBC-NOS) (Kleinbongard et al. 2006) and among others binds to the cytoskeletal α - and β -spectrin, thus modulating RBC deformability (Grau et al. 2013).

Microcirculatory complications, described for a variety of cardiovascular diseases such as venous insufficiency (Huisman et al. 2013), diabetes (Fowler 2008) or sickle cell disease (Cheung et al. 2002), are due to limited NO production and/or bioavailability (Tessari et al. 2010; Bizjak et al. 2015; Grau et al. 2015), paralleled by increased amounts of reactive oxygen and nitrogen species (reactive oxygen species/ reactive nitrogen species[ROS/RNS]) (Dalle-Donne et al. 2006) and reduced antioxidant capacity (Adelekan et al. 1989; Marra et al. 2002) finally leading to oxidative/nitrosative stress (Obrosova et al. 2005).

Some flavonoids, including the flavonols quercetin and kaempferol, were shown to increase eNOS activity (Olszanecki et al. 2002) and may thus represent potent active agents to enhance NO bioavailability. AS195 is an effective antiedema drug improving the microcirculation, but the cellular mechanisms responsible for this action are unknown. We here showed that the active substance of Antistax[®] positively influences NO bioavailability by improving NO synthesis and antioxidative capacity and by reducing free radicals in both RBC and endothelial cells.

Materials and Methods

Ethical approval

The protocols involving human subjects were approved by the Ethics Committee of the German Sport University Cologne (#26/2015). The applied protocols align with the Declaration of Helsinki, and all participants gave written informed consent to participate in this study.

Part I: Influence of AS195 on NOS-dependent NO production in HUVEC and RBC

Treatment of HUVEC

Primary human umbilical vein endothelial cells (HUVECs; Life Technologies, Darmstadt, Germany) were cultured at 37°C in a humidified atmosphere containing 5% CO₂ (Gimbrone et al. 1978; Maciag et al. 1981). HUVECs were seeded either into 250-mm² cell culture flasks with 6.2×10^4 cells/flask for the measurement of nitrite concentration as marker for NO production (Ignarro 1990; Lauer et al. 2001) or onto 0.1% gelatin precoated cover slips in 24-multiwell plates with 5×10^4 cells/well for immunohistochemical staining of eNOS. Treatment was started when cells reached a confluent monolayer. HUVECs were incubated with AS195 (medium = 0 μ mol/L as control and 100 μ mol/L) for up to 24 h at standard cell culture conditions. At seven time points (0, 0.5, 1, 2, 4, 6, and 24 h), medium was transferred into clean tubes and stored at -80°C until measurement. For the immunohistochemical staining of eNOS, AS195 incubation was stopped by aspirating the cell culture medium. HUVECs were then incubated with 4% paraformaldehyde (PFA) for 25 min at room temperature (RT). PFA-treated cells were washed with 0.1 mol/L phosphate-buffered saline (PBS) and permeabilized with 0.25 mol/L Triton-X 100 and 0.5 mol/L NH₄Cl in 0.05 mol/L Tris-buffered saline (TBS) for 10 min. The cells were washed with 0.05 mol/L TBS, and unspecific binding sites were blocked with 5% bovine serum albumin (BSA) in 0.05 mol/L TBS for 1 h at RT. Then, the primary antibody diluted in 0.8% BSA (total eNOS: rabbit anti-eNOS; BD Transduction Laboratories, Heidelberg, Germany, dilution 1:500; eNOS^{Ser1177}: rabbit anti-eNOS [phospho S1177], dilution 1:200; Abcam, Cambridge, UK) was applied and incubated over night at 4°C. The cells were washed with TBS and incubated with the corresponding biotinylated secondary antibody for chromogen visualization (goat anti-rabbit IgG; Vector Laboratories, Burlingame USA; dilution 1:500 in 0.05 mol/L TBS) for 1 h at RT, followed by an incubation step with a streptavidin-horseradish complex (HRP; GE Healthcare, Solin-

gen, Germany; dilution 1:150 in 0.05 mol/L TBS) for 1 h. Finally, staining was conducted with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.1 mol/L PBS.

For staining intensity detection, pictures were taken using a Leitz microscope (Wetzlar, Germany) coupled to a Leica camera (DCF 280, Wetzlar, Germany). Magnification for all images was 500-fold. The analysis was conducted using the software "Image J" (National Institutes of Health, Bethesda, Maryland, USA). For each condition, a total of 50 HUVECs were counted from at least four visual fields. The intensity of immunostaining is reported as the mean of measured cell gray value minus background gray value, which was detected at a cell-free area of the slide (Korkmaz et al. 2007).

Treatment of RBC

Blood from 10 healthy male subjects (age [years]: 27.0 ± 4.95 ; height [m]: 1.77 ± 0.68 ; weight [kg]: 73.75 ± 14.80) was taken from the *vena mediana cubiti* into sodium-heparin vacutainer (BD Vacutainer, Franklin Lakes, USA). RBCs were separated (800g, 4°C and 10 min) and resuspended in autologous plasma to yield a hematocrit of 40%. RBCs were then treated with AS195 (0.1 mol PBS, pH 7.4 = 0 μ mol/L as control and 100 μ mol/L) for up to 60 min. After each time point, RBCs were fixed with 4% PFA and processed as previously described (Suhr et al. 2012; Grau et al. 2014) for immunohistochemical staining of RBC-NOS. A second aliquot was separated by centrifugation (5000g, 1 min, 4°C), and the RBC pellet was mixed with a ferricyanide-based preservation solution in a 1:5 ratio (v/v; preservation solution/RBCs) (Hendgen-Cotta et al. 2008), snap-frozen, and stored at -80°C until nitrite measurement.

Red blood cell-nitric oxide synthase staining of fixed RBC was conducted according to the protocols of Suhr et al. (2012) and Grau et al. (2014). Briefly, fixed cells were dispersed on a slide and heat fixed. A control and a test area were marked and washed with 0.05 mol/L TBS. The following steps include incubation with 0.1% trypsin for 30 min at 37°C, 80% methanol/5% hydrogen peroxide/15% $\text{H}_2\text{O}_{\text{dest}}$ for 30 min at RT, and 3% milk powder solution in 1 \times TBS for 30 min at RT. The test area was incubated with the primary antibody (total RBC-NOS: rabbit anti-eNOS; BD Transduction Laboratories, Heidelberg, Germany, dilution 1:500; RBC-NOS^{Ser1177}: rabbit anti-eNOS (phospho S1177), dilution 1:200; Abcam) for 1 h at RT. Final incubation steps included 3% Normal Goat Serum (Dako, Glostrup, Denmark) for 30 min at RT, secondary goat anti-rabbit antibody (Dako; dilution 1:400 in 0.05 mol/L TBS) for 1 h at RT, HRP (Sigma-

Aldrich, St. Louis, USA) (dilution 1:400 in 0.05 mol/L TBS) for 30 min at RT, and development of staining with 3,3'-diaminobenzidine-tetrahydrochloride solution (Sigma-Aldrich).

For staining intensity detection, a Leica microscope coupled to a CCD camera (DXC-1850P; Sony, Berlin, Germany) was used, and the analysis was conducted using the software "Image J" (National Institutes of Health, Bethesda, Maryland, USA). Magnification by all images was 400-fold. For each condition/subject, gray values of a total of 100 RBCs were determined from at least four visual fields (test area) and 50 RBCs from at least two visual fields (control area). The actual RBC gray value was calculated considering background gray value and gray value of RBC from the control area.

Nitrite measurement

HUVEC samples were thawed on ice and nitrite concentration was directly measured without further preparation of the samples. For nitrite measurement in RBCs, methanol (VWR International, Darmstadt, Germany) was added to the frozen samples in a 1:2 ratio and centrifuged at 21,000g, 4°C for 15 min. Nitrite levels of the RBC supernatant and of the HUVEC medium were determined by injecting 100 μ L into an acidified tri-iodide solution that reduces nitrite to NO gas. Along with a helium gas stream, NO was purged into an ozone-based chemiluminescence NO detector (CLD 88 NO; Ecophysics, Munich, Germany) (Pelletier et al. 2006; Hendgen-Cotta et al. 2008). The Power Chrome software (Ecophysics) was used to integrate the area under the curve. All samples were measured in triplicate. Using aqueous calibration solutions with known nitrite concentration allowed calculation of sample nitrite content.

Part II: Impact of AS195 on oxidative stress and antioxidant capacity in HUVEC and RBC

The influence of AS195 on oxidative stress and antioxidant capacity of HUVEC and RBC was tested by adding tert-butylhydroperoxide (TBHP, 1 mmol/L) (Sigma-Aldrich, Darmstadt, Germany), to the cells. Cultured HUVEC and RBC (hematocrit 40%) were treated with PBS/medium as control, AS195 (100 μ mol/L), TBHP (1 mmol/L), or AS195 (100 μ mol/L) + TBHP (1 mmol/L) for 30 min at 37°C. The incubation time of 30 min was chosen because this was the first time point showing significant differences between control and AS195 treatment. A parallel incubation of TBHP and AS195 was chosen to observe whether AS195 is capable to reduce TBHP-dependent modifications.

Detection of ROS/RNS using the OxiSelect assay kit

The OxiSelect In Vitro ROS/RNS Assay Kit (Cell Biolabs, Inc., San Diego, USA) employs a ROS/RNS-specific probe. Dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ) was first processed to receive a highly reactive DCFH form which reacts with ROS/RNS to the highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF) form. HUVEC and RBC were thawed on ice and lysed via ultrasound and vortex. Samples as well as standards were pipetted into a 96-multiwell fluorescence plate and incubated with a catalyst for 5 min at RT that helps accelerate the oxidative reaction. The prepared DCFH probe was then added to the samples and standards, and the reaction was allowed to proceed for 45 min at RT. Fluorescence was read with a fluorescence plate reader (Fluoroskan Ascent Microplate Fluorometer; Thermo Fisher Scientific, Schwerte, Germany) at 480nm excitation (ex)/530 nm emission (em). Samples were then measured against a DCF standard, and concentration was calculated by linear regression. The influence of AS195, TBHP, and AS195 + TBHP on free ROS/RNS was indicated as percent changes compared to the control value (PBS or cell culture medium).

Detection of ROS/RNS by cellular ROS/RNS detection assay kit

The Cellular ROS/RNS Detection Assay Kit (Abcam) was used to measure NO, superoxide, and ROS/RNS in vivo in living HUVEC using fluorescence microscopy (Laser Scanning Microscope 510; Carl Zeiss, Jena, Germany). The assay was only applied in HUVEC because the kit was not suitable for RBC. HUVECs were seeded onto glass plates covered with 0.1% gelatin with 30,000 cells per plate. The HUVECs were cultured under standard cell culture conditions until confluence.

Human umbilical vein endothelial cells were loaded with a ROS/RNS 3-Plex Detection Mix for NO detection, nitrosative stress detection, and superoxide detection and incubated under normal tissue culture conditions for 2 h at 37°C with periodic shaking. The 3-Plex Detection Mix was aspirated, and the cells were washed to remove redundant mix. HUVECs were treated with PBS, AS195, TBHP, or AS195+TBHP for 30 min at 37°C. The supernatant was removed and replaced by regular cell culture medium. The staining was analyzed with filter set to ex/em 490/525 nm (nitrosative stress detection), 550/620 nm (superoxide detection), and 650/679 nm (NO detection). Pictures were taken at 400-fold magnification, and a line scan was performed to

quantify the fluorescence signal (DU, densitometric units). A total of 50 HUVECs were analyzed for each condition.

Antioxidant capacity

Reduced Cu²⁺ was measured as marker for the total antioxidant capacity (Total Antioxidant Capacity Assay Kit [Abcam]) in the cell culture medium of HUVEC (diluted 1:4 with medium), in lysed HUVEC, in plasma (diluted 1:100 with PBS), and in lysed RBC (1:10,000). Cell lysis was achieved via ultrasound and vortex. Both, Trolox standard (0, 4, 8, 12, 16, 20 nmol) and samples were incubated with a Cu²⁺ reagent for 90 min at RT on an orbital shaker and the absorbance was read at 570 nm using the Multiskan FC Microplate Photometer (Thermo Fisher Scientific). The absorbance was plot as a function of Trolox concentration, and the sample antioxidant Trolox equivalent concentrations were calculated by linear regression.

S-nitrosylation of RBC α - and β -spectrin

S-nitrosylation of RBC cytoskeletal proteins was determined using S-Nitrosylated Protein Detection Kit (Cayman Chemicals, Ann Arbor, USA) which employs the Biotin-Switch Assay after Jaffrey and Snyder (2001). The protocol has been described in detail elsewhere (Grau *et al.* 2013). Briefly, RBCs were lysed, free thiol groups were blocked, and S-nitrosothiols were reduced. The newly formed thiols were covalently labeled with maleimide biotin. Subsequent detection by avidin-coupled reagents was used to localize the biotinylated proteins. The protein concentration of the samples was determined using the DC-Protein Assay Kit (BioRad, Munich, Germany), and 60 μ g of total protein was loaded into each lane of a 4–12% Bis-Tris gel (BioRad). Proteins were separated for 1 h under constant 90 mA in a 1 \times MOPS running buffer (BioRad). The separated proteins were transferred to a polyvinylidene fluoride membrane (0.45 mm pore size). The background of the membrane was blocked in 2% bovine serum albumin (in 1 \times TBS with 0.1% Tween 20) overnight at 4°C. A horseradish peroxidase (dilution 1:2000) was applied which binds to the biotin-avidin complex, and the reaction was developed using an enhanced chemiluminescence kit containing peroxidase substrate (Thermo Fisher Scientific). S-nitrosylated protein bands at 240 kDa and 220 kDa, previously identified as α -spectrin and β -spectrin, respectively (Grau *et al.* 2013), were examined for different “Integrated densities” using the (National Institutes of Health, Bethesda, Maryland, USA) software (Grau *et al.* 2013).

RBC deformability

RBC deformability was determined for nine shear stresses between 0.3 and 50 Pa using the laser-assisted optical rotational cell analyzer (LORCA; RR Mechatronics, Hoorn, The Netherlands) (Hardeman et al. 2001). RBCs were mixed with 0.14 mmol/L polyvinylpyrrolidone (LORCA) in a 1:250 ratio and immediately sheared in a glass Couette system with a laser beam directed through the sheared sample. The diffraction pattern produced by the deformed RBC was analyzed and an elongation index (EI) was calculated by the LORCA software. Maximum deformability (EI max), the theoretical maximum deformability at infinite shear stress, and SS 1/2, the shear rate required for half of maximum deformability, were calculated as previously described (Baskurt et al. 2009).

Cell adhesion assay

The wells of a 96-multiwell plate were precoated with adhesive proteins (laminin: 0 μ g, 1.25 μ g, 2.5 μ g, 5 μ g, 10 μ g or 20 μ g/well) overnight at 37°C, followed by a subsequent incubation with 1% BSA for 4 h. After the AS195/TBHP treatment, HUVECs were seeded into 96-multiwell plates with a density of 5×10^4 cells in a final volume of 100 μ L cell culture medium per well. MnCl₂ (100 mmol/L in PBS) and MgCl₂ (200 mmol/L in PBS) were added to the wells to activate the integrins and incubated at 37°C for 30 min. Medium and nonadhered cells were removed by inverting the plates (without tapping the multiwell plates). The wells were washed with PBS, and then, remaining cells were fixed with 1% glutaraldehyde in PBS for 30 min at RT. Adherent HUVECs were stained with 0.1% crystal violet in water for 25 min. The multiwells were washed under running water and dried by tapping out the remaining water. 0.2% Triton X-100 was added to each well and incubated overnight at 4°C with gentle shaking. The absorbance was then measured at 570 nm using the Multiskan FC Microplate Photometer (Bald et al. 2014).

Statistical analysis

Statistical software packages Origin 8.5 Pro (Northampton, USA) and GraphPadPrism 6 (La Jolla, USA) were used for statistical analyses of the data and graphical representation of data. Data were tested for normal distribution using the D'Agostino and Pearson omnibus normality test. Data of Part I were analyzed by two-tailed *t*-test (Gaussian distribution) to test for differences within one time point between the two tested concentrations. Data of Part II were calculated by one-way ANOVA with multicomparison test to test for significant differences in the means. The data

are presented as mean \pm standard error of means (SEM) unless described otherwise. Statistical differences were considered to be significant for values of $P < 0.05$.

Results

AS195-dependent NOS generation and activation

In HUVEC, total NOS content increased in the control condition (0 μ mol/L) but increase was even more pronounced in AS195-treated cells with highest values measured after 24 h ($P < 0.001$ compared to 0 μ mol/L). Phosphorylation of eNOS^{Ser1177} decreased in the control condition (0 μ mol/L) with lowest values measured after 24 h ($P < 0.001$ compared to 0 h). Application of 100 μ mol/L AS195 increased eNOS^{Ser1177} with highest levels measured after 24 h of incubation ($P < 0.001$ compared to 0 μ mol/L AS195) (Fig. 1A and C). In RBC, total NOS was not affected by the intervention. RBC-NOS^{Ser1177} staining was significantly increased after 30 min of AS195 incubation ($P < 0.01$ compared to 0 μ mol/L AS195) but did not further increase after 60 min of AS195 incubation (Fig. 1B and D).

AS195 changed nitrite concentration

Nitrite concentration remained unaltered in the control conditions of RBC and HUVEC. In HUVEC, medium nitrite concentration increased with increasing incubation time. Highest levels were measured after 24 h of incubation ($P < 0.01$ compared to 0 μ mol/L AS195) (Fig. 1E). In RBC, nitrite concentration significantly decreased during AS195 incubation (30 min; $P < 0.05$; 60 min; $P < 0.05$) (Fig. 1F).

AS195 decreased TBHP mediated oxidative stress

Free ROS/RNS in HUVEC and RBC

In HUVEC, free ROS/RNS content was significantly decreased in AS195-treated samples ($P < 0.001$ compared with control) and in cells treated with both AS195 and TBHP ($P < 0.001$ compared with control; $P < 0.01$ vs. TBHP). Single incubation with TBHP significantly increased ROS/RNS in HUVEC ($P < 0.01$; Fig. 2A). In RBC, free ROS/RNS content significantly decreased after AS195 incubation ($P < 0.05$ compared with control) and increased after TBHP application ($P < 0.01$ compared with control). Parallel incubation of AS195 and TBHP decreased free ROS/RNS compared with single incubation of TBHP ($P < 0.01$; Fig. 2B).

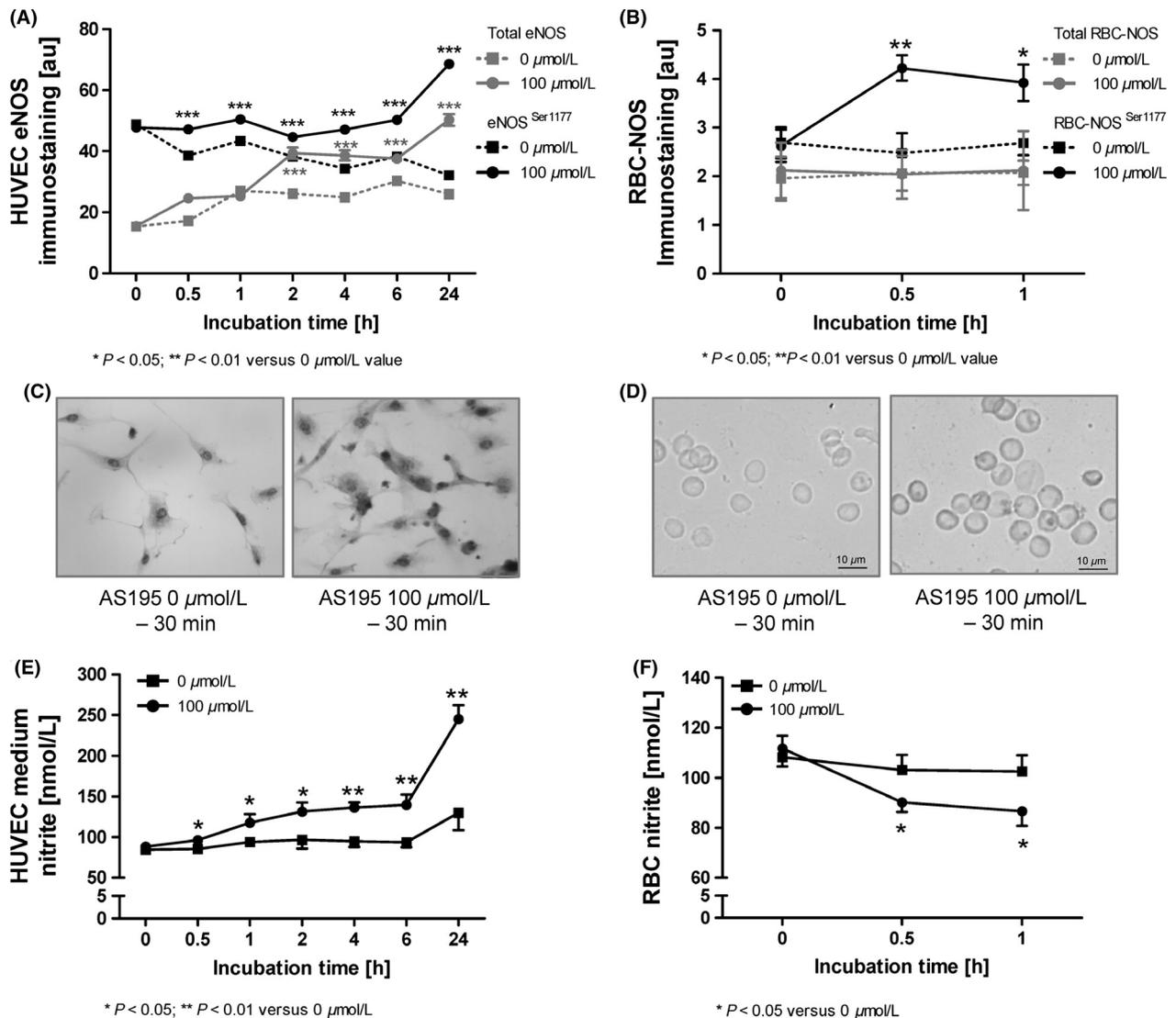


Figure 1. NOS (nitric oxide synthase) content, NOS activation, and nitrite generation of HUVECs (human umbilical vein endothelial cells) and RBC after AS195 (red-vine-leaf extract) treatment. (A) AS195 (100 μmol/L) significantly increased total eNOS (endothelial nitric oxide synthase) signal and eNOS serine 1177 phosphorylation in HUVEC starting 0.5 h after application ($P < 0.001$). Highest NOS levels and phosphorylation signals were measured 24 h after AS195 application. (B) In RBC, total NOS signal was not affected by the treatment but NOS phosphorylation at serine 1177 residue significantly increased after 0.5 h ($P < 0.01$) and 1 h ($P < 0.05$) of AS195 treatment. Representative photographs of (C) HUVEC and (D) RBC-NOS (red blood cell–nitric oxide synthase) serine 1177 staining induced by AS195. Magnification of images was 400-fold (RBC) and 500-fold (HUVEC). (E). Nitrite concentration of HUVEC medium significantly increased during AS195 (100 μmol/L) incubation, starting 0.5 h after application ($P < 0.05$) with highest concentration measured after 24 h ($P < 0.01$). (F) RBC nitrite concentration significantly decreased during AS195 incubation ($P < 0.05$ after 0.5 h and $P < 0.05$ after 1 h). Data were analyzed using *t*-test to test for differences between the two AS195 concentrations within one time point. Data of (A) and (B) represent mean \pm SEM (standard error of means) of $n = 5$ each. Data of (E) and (F) represent mean \pm SEM of $n = 5$ (HUVEC) and $n = 10$ (RBC).

NO, ROS/RNS, and superoxide in HUVEC

NO signal was significantly increased in HUVEC after AS195 incubation ($P < 0.05$ compared with control) (Fig. 3A). ROS/RNS significantly increased after TBHP incubation ($P < 0.001$). Parallel incubation of

AS195 + TBHP reduced ROS/RNS ($P < 0.001$) compared with single TBHP incubation ($P < 0.001$) (Fig. 3B). Superoxide signal was significantly decreased after AS195 incubation ($P < 0.001$), while TBHP increased superoxide signal ($P < 0.001$) and parallel incubation of TBHP + AS195 again decreased superoxide ($P < 0.001$

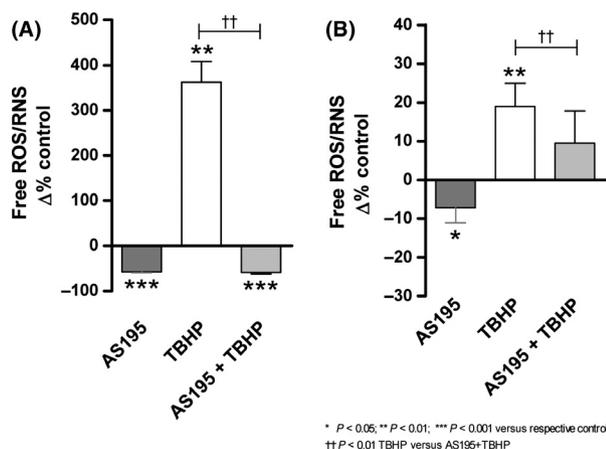


Figure 2. Reduction of free (reactive oxygen species) ROS/RNS after AS195 treatment of HUVECs (human umbilical vein endothelial cells) and RBC. (A). In HUVEC, free ROS/RNS was significantly decreased in AS195-treated samples ($P < 0.001$), while TBHP (tert-butylhydroperoxide) significantly increased ROS/RNS content ($P < 0.01$). (B). In RBC, AS195 significantly reduced free ROS/RNS ($P < 0.05$). TBHP in contrast increased free ROS/RNS ($P < 0.01$), and parallel incubation of AS195 and TBHP attenuated this increase. Data are analyzed by one-way ANOVA. Data represent mean \pm SEM (standard error of means) of $n = 3$ (HUVEC) and $n = 5$ (RBC).

compared with TBHP) (Fig. 3C). Representative photographs of treated HUVEC with the corresponding fluorescence signal of NO (blue), ROS/RNS (green), and superoxide (red) and line scan signals are shown in Figure 4 A and D (magnification 400-fold).

AS195 improved total antioxidant capacity

Cu^{2+} reduction was significantly increased after AS195 incubation in the HUVEC cell culture medium ($P < 0.001$ compared with control), in lysed HUVEC ($P < 0.01$ compared with control), in the plasma fraction ($P < 0.001$ compared with control), and lysed RBC ($P < 0.05$ compared with control). Cu^{2+} reduction was also increased in samples incubated with both AS195 + TBHP (medium: $P < 0.001$; lysed HUVEC: $P < 0.01$; plasma: $P < 0.001$) (Fig. 5A–D).

AS195 increased S-Nitrosylation of RBC spectrins

Quotient of S-nitrosylation calculated for α -spectrin (240 kDa) and β -spectrin (220 kDa) was significantly increased ($P < 0.05$) after AS195 incubation. TBHP incu-

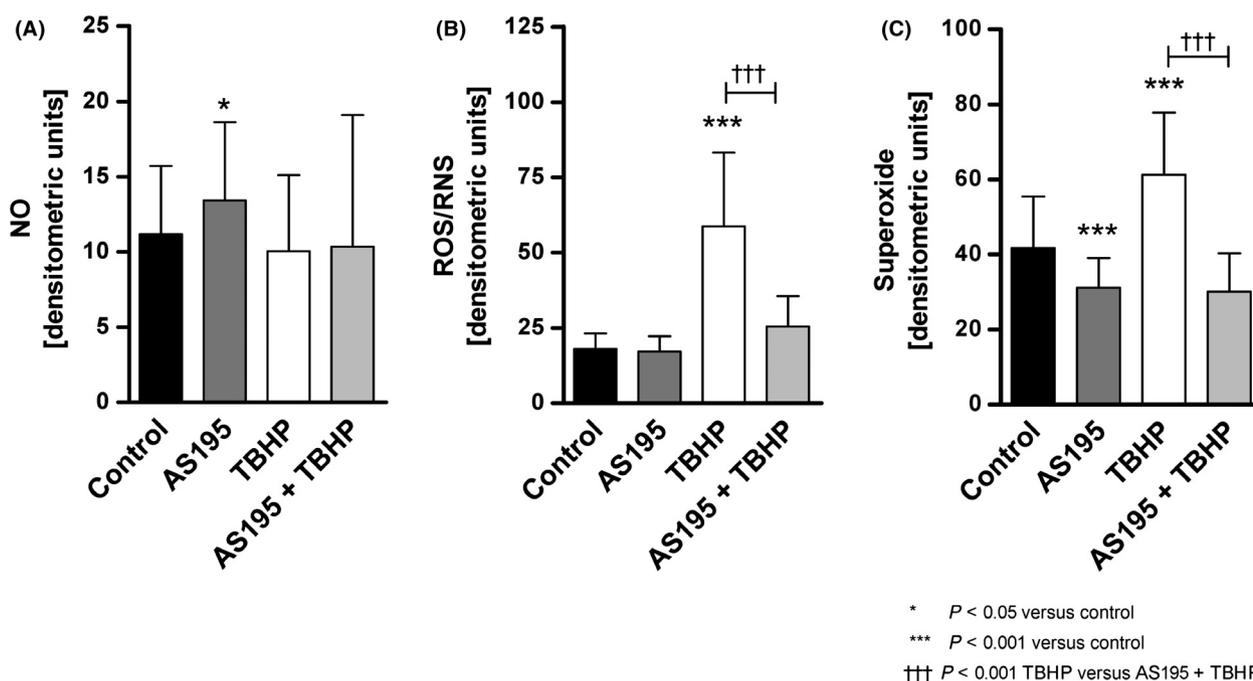


Figure 3. AS195 (red-vine-leaf extract) mediated increase of NO and reduction of (reactive oxygen species) ROS/RNS (reactive nitrogen species) and superoxide in HUVECs (human umbilical vein endothelial cells). Cell imaging of HUVEC revealed (A) significantly increased NO (nitric oxide) signal after 30 min of AS195 (100 $\mu\text{mol/L}$) incubation ($P < 0.05$). (B) TBHP (tert-butylhydroperoxide) (1 mmol/L) significantly increased ROS/RNS signal ($P < 0.001$) which was not observed when TBHP was incubated in parallel with AS195. (C) Superoxide signal was significantly decreased in AS195-treated cells ($P < 0.001$) and significantly increased in TBHP-treated cells ($P < 0.001$). This increase was not observed in cell treated with both TBHP and AS195. Data are analyzed by one-way ANOVA. Data of (A–C) represent mean \pm SEM (standard error of means) of $n = 3$ each.

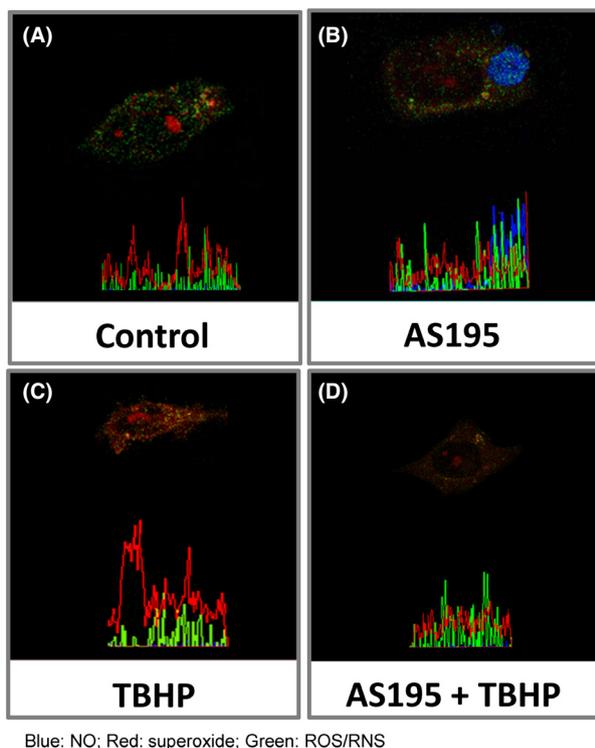


Figure 4. Representative photographs of HUVEC (human umbilical vein endothelial cells) with corresponding line scans. (A) Control cell showed no prevalent signal. (B) AS195 increased NO signal (blue), while (C) TBHP (tert-butylhydroperoxide) increased superoxide signal (red). (D) Parallel incubation of AS195 and TBHP showed no prevalent signal. Magnification of all pictures was 400-fold.

bation significantly decreased S-nitrosylation of β -spectrin ($P < 0.05$) but not of α -spectrin. Parallel incubation of AS195 + TBHP increased S-nitrosylation of β -spectrin ($P < 0.05$) compared with single incubation of TBHP (Fig. 6A and B).

AS195 increased RBC deformability and decreased HUVEC adhesion

Compared with control, EI max significantly increased in AS195-treated RBC ($P < 0.01$) and significantly decreased after TBHP treatment ($P < 0.05$). EI max in AS195 + TBHP-treated samples was significantly higher compared with single TBHP incubation ($P < 0.01$) (Fig. 7A). SS 1/2 was not affected by the different conditions (Fig. 7B).

Adhesion of HUVEC decreased with decreasing laminin concentration. Adhesion was significantly lower in HUVEC treated with AS195. Adhesion further decreased in HUVEC incubated with TBHP. Lowest adhesion was observed in cells treated with both AS195 and TBHP (Fig. 7C).

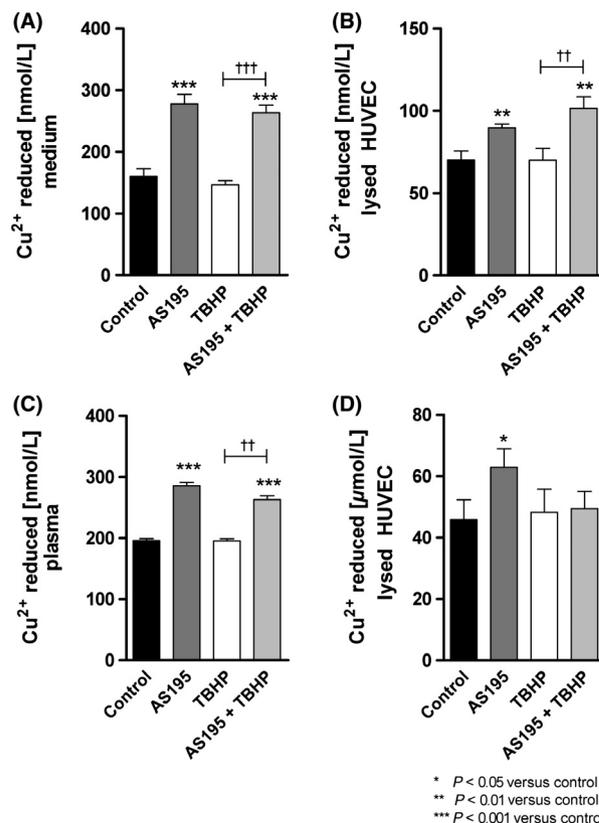


Figure 5. Improvement of total antioxidant capacity in HUVECs (human umbilical vein endothelial cells) and RBC after AS195 treatment. AS195 increased Cu^{2+} reduction as representative of total antioxidant capacity in (A) HUVEC cell culture medium ($P < 0.001$), (B) lysed HUVEC ($P < 0.01$), (C) human plasma ($P < 0.001$), and (D) lysed human RBC ($P < 0.05$). This increase was also observed in samples treated with both TBHP and AS195. Data are analyzed by one-way ANOVA. Data represent mean \pm SEM (standard error of means) of $n = 6$ each.

Discussion

Red-vine-leaf extract AS195 increased NOS activation in HUVEC and RBC. This increased nitrite concentration in the cell culture medium of HUVEC, which is a valid marker of externalized NO. In contrast, AS195 significantly decreased nitrite concentration in RBC. Additional NO reaction pathways were tested in RBC, and S-nitrosylation of the cytoskeletal proteins was enhanced by AS195. As a consequence, RBC deformability was improved. Further, AS195 decreased ROS/RNS, including superoxide, and increased total antioxidant capacity in RBC and endothelial cells. In HUVEC, AS195-dependent NO production was accompanied by reduced cell adhesion.

Plant extracts, also known as phytopharmaca, are widely accepted in the medical treatment of patients or in self-medication. Flavonoids, a group of secondary plant

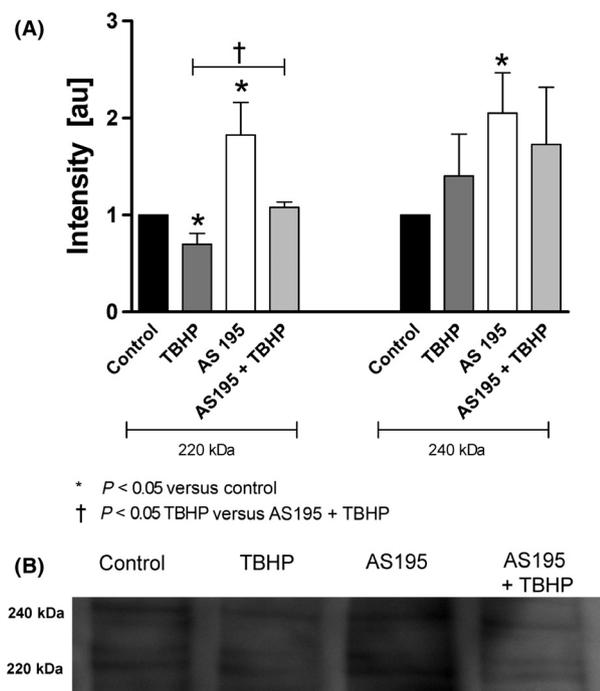
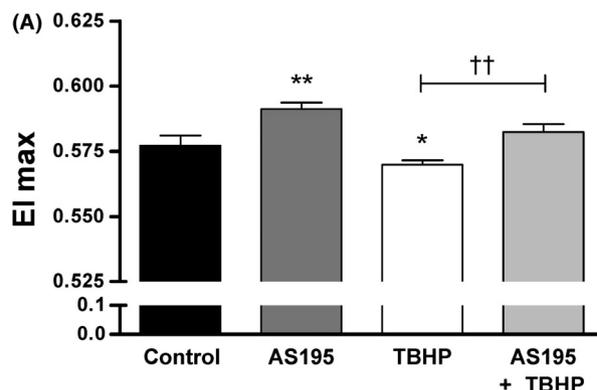


Figure 6. AS195 (red-vine-leaf extract) increased S-nitrosylation of α - and β -spectrin. (A). AS195 increased S-nitrosylation of β -spectrin (220 kDa) ($P < 0.05$), while TBHP (tert-butylhydroperoxide) significantly decreased S-nitrosylation of β -spectrin (220 kDa) ($P < 0.05$). S-nitrosylation was significantly increased in samples incubated with both AS195 and TBHP ($P < 0.05$ compared to single TBHP incubation). S-nitrosylation of α -spectrin (240 kDa) was significantly increased after AS195 incubation ($P < 0.05$). Data are analyzed by one-way ANOVA. Data represent mean \pm SEM (standard error of means) of $n = 5$. (B). Representative image of adjacent lanes of one western blot displaying α -spectrin (240 kDa; upper lane) and β -spectrin (220 kDa) (lower lane).

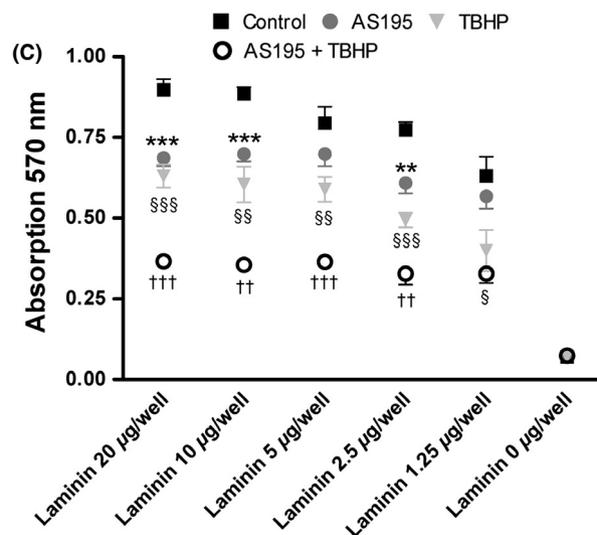
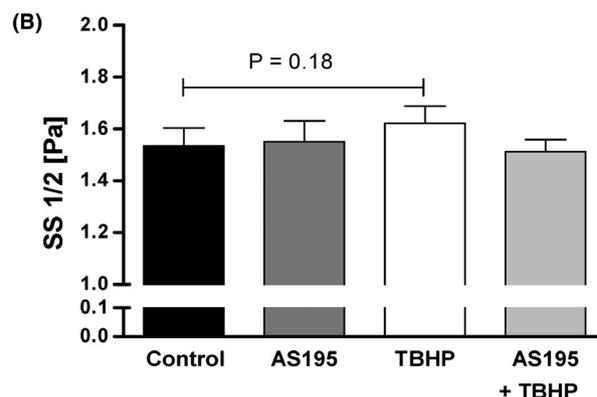
products, include flavanols, anthocyanidins, flavonols, isoflavones, flavones, or flavanones. Flavonoids are shown to have antioxidative capacity, free-radical scavenging capacity, coronary heart disease prevention, and

Figure 7. AS195 (red-vine-leaf extract) improves RBC deformability and reduces adhesion of endothelial cells. (A) Maximum deformability (EI max) was significantly increased in AS195-treated RBC ($P < 0.01$) and TBHP (tert-butylhydroperoxide) significantly decreased EI max ($P < 0.05$). Parallel incubation of AS195 and TBHP again increased EI max ($P < 0.01$ compared to single TBHP incubation). (B) SS $\frac{1}{2}$ (shear stress required for one half of maximum deformability) was not affected by the different conditions. (C) Adhesion of HUVECs (human umbilical vein endothelial cells) decreased with decreasing laminin concentration. Adhesion of HUVEC was significantly reduced in AS195-treated cells. Least adhesion was observed in cells treated with AS195 + TBHP. Data are analyzed by one-way ANOVA. Data of (A) + (B) represent mean \pm SEM (standard error of means) of $n = 10$ and data of (C) represent mean \pm SEM (standard error of means) of $n = 3$.

anticancer activity (Yao et al. 2004). The main flavonols within the red-vine-leaf extract AS195 are quercetin glucuronide, isoquercitrin, and kaempferol glucoside (Boucheny and Brum-Bousquet 1990). Clinical studies



* $P < 0.05$; ** $P < 0.01$ versus control
†† $P < 0.01$ TBHP versus AS195 + TBHP



** $P < 0.01$; *** $P < 0.001$ control versus AS195
§ $P < 0.05$; §§ $P < 0.01$; §§§ $P < 0.001$ control versus TBHP
†† $P < 0.01$; ††† $P < 0.001$ TBHP versus AS195 + TBHP

already indicated AS195 to be a potent and well-tolerated anti-edema drug (Kiesewetter *et al.* 2000; Kalus *et al.* 2004; Rabe *et al.* 2011), and it was hypothesized herein that the positive effects of AS195 are associated with improved NOS-dependent NO bioavailability.

Total NOS content was significantly increased in AS195-treated HUVEC. Flavonoids extracted from Artichoke have been shown to increase eNOS protein expression in HUVEC (Li *et al.* 2004), and the recent data suggest a positive role of red-vine-leaf flavonols on the expression of eNOS. Total RBC-NOS was not affected by the intervention because RBCs are incapable of protein neoformation. NOS activation, reflected by phosphorylation of serine 1177 (Dimmeler *et al.* 1999; Kleinbongard *et al.* 2006), significantly increased in both AS195-treated HUVEC and RBC while eNOS phosphorylation decreased in HUVEC incubated under control conditions. It is speculated that this might be related to a reduction in NOS cofactors during 24h incubation possibly related to nutrient consumption from the medium. But this hypothesis requires further investigation. Several (phyto)pharmacological stimuli for NOS^{Ser1177} phosphorylation have been identified so far, including insulin (Kleinbongard *et al.* 2006; Grau *et al.* 2013), bradykinin (Arnal *et al.* 1999), or red wine polyphenols (Leikert *et al.* 2002). The present results indicate that AS195 is an additional stimulant that effectively activates NOS through phosphorylation of serine 1177, possibly through phosphatidylinositol 3-kinase-, PKA-, and Akt-dependent pathways as shown for other flavonoids (Lorenz *et al.* 2004). NOS activation increased NO production but direct measurement of NO is challenging. Thus, nitrite was established as marker for NO (Lauer *et al.* 2001). AS195 increased extracellular nitrite concentration in HUVEC cell culture medium but decreased nitrite concentration within RBC. In endothelial cells, eNOS produced NO is released; it diffuses locally and relaxes the underlying smooth muscle cells thus controlling vascular diameter and blood pressure. Inhibition of NO production increases blood pressure and produces severe hypertension (Arnal *et al.* 1999). Pharmacological compounds that induce the release of NO are useful therapeutic agents in the treatment of cardiovascular diseases (Ignarro *et al.* 2002). From the recent data, it is concluded that AS195 can be added to the list of phytopharmaca that are capable to stimulate NOS-dependent NO generation and release. Administration of AS195 may improve NO content in vascular disorders (hypertension, diabetes, atherosclerosis) associated with reduced NO availability (Cooke and Dzau 1997).

Reduction of RBC nitrite concentration observed after AS195 application was rather surprising. Recent studies support a positive relation between increasing nitrite content and increased RBC-NOS activation (Bizjak *et al.*

2015; Grau *et al.* 2013; Suhr *et al.* 2012). Another study also suggests that RBC-produced NO is exported from the RBC and contributes to the regulation of blood pressure and nitrite homeostasis (Ulker *et al.* 2011). According to these findings, plasma nitrite would have been expected to increase after AS195 treatment, but it remained unaffected by AS195 (data not shown). Flavonoids were found to be potent nitrite scavengers (Choi *et al.* 1989), suggesting that NO oxidation to nitrite is not reduced by AS195 but instead, AS195 may additionally scavenge nitrite thus decreasing its concentration. In HUVEC, nitrite scavenging might be superimposed by increased NO/nitrite production due to increased protein expression which was not the case in RBC.

The oxidation of NO to nitrite is not the only reaction route within RBC (Özüyaman *et al.* 2008; Grau *et al.* 2013). NO can also bind to reactive protein cysteine thiols to form a S-nitrosothiol. This reaction, termed S-nitrosylation, is a reversible and posttranslational modification that regulates the activity of a large number of targets, including structural, cytoskeletal, and signaling proteins (Stamler *et al.* 1992). S-nitrosylation of RBC proteins was examined after AS195 and TBHP treatment. The results evidenced increased S-nitrosylation of α - and β -spectrin, the most abundant cytoskeletal proteins within RBC and major determinants of RBC deformability (Grau *et al.* 2013) after AS195 incubation. Indeed, RBC deformability was also improved after AS195 incubation, suggesting that this might also positively affect blood flow in the microcirculation. AS195 treatment could thus be beneficial for the health situation of patients suffering from hypertension, diabetes, or atherosclerosis because these are also associated to impaired deformability (Schwartz *et al.* 1991; Yedgar *et al.* 2002; Subasinghe and Spence 2008).

Tert-butylhydroperoxide decreased deformability which may be related to altered S-nitrosylation of the spectrins. TBHP decreased S-nitrosylation of β -spectrin compared with control but did not affect S-nitrosylation of α -spectrin. The spectrins form a network at the cytoplasmic side of the RBC lipid bilayer incorporating several other proteins into this network. Oxidative damage induced by TBHP may affect spectrin sulfhydryl groups. These spectrin units may become cross-linked by disulfide bonds, thus altering membrane mechanical properties (Hale *et al.* 2011). S-nitrosylation induced by AS195-produced NO may prevent this damage as previously proposed (Grau *et al.* 2013). Susceptibility to oxidative damage differs between RBC proteins (Celedón *et al.* 2001), and thus, further investigations are needed to investigate the different effects of free radicals on spectrins.

AS195 was further shown to decrease free-radical content and increase total antioxidant capacity in both

HUVEC and RBC. Oxidative/nitrosative stress represents an imbalance between ROS/RNS and the antioxidant capacity of a system. This imbalance can be manifested in acute or chronic overproduction of ROS/RNS or reduced antioxidants, such as glutathione, superoxide dismutase, or catalase. Sources of ROS/RNS may be xanthine oxidase, mitochondria, or uncoupling of NOS (Young and Woodside 2001; Madamanchi et al. 2005). High oxidative stress has been found responsible for diabetic vascular complications (Giugliano et al., 1996), sickle cell disease (Amer et al. 2006), or heart failure (Singh et al. 1995), causing damage to cell structures including lipids, membranes, proteins, and DNA (see for review Valko et al. 2007). The results of this study indicated that AS195 reduced free radicals and increased total antioxidant capacity. The data further indicated that the free-radical buffering capacity of HUVEC is lower compared with RBC leading to higher percental increase in measureable free radicals in HUVEC after TBHP incubation. Given the finding that overall antioxidant capacity was higher in RBC, free radicals seem to be faster trapped by antioxidants in RBC decreasing the amount of measureable free radicals. A study by Kučera et al. (2014) showed that TBHP-induced oxidative stress reduces mitochondrial function by reducing the activity of complex I. This reduces the electron transport which augments the production of superoxide anion (Kushnareva et al. 2002). This explains increased superoxide content observed in HUVEC treated with TBHP. Quercetrin and kaempferol isolated from red bushwillows have strong antioxidant activity (Aderogba et al. 2012). The recent results showed a reduction in TBHP induced free-radical content and increased total antioxidant capacity of cells treated with AS195 concluding that the flavonols present in AS195 also act as effective antioxidants. Administration of AS195 may thus be advantageous for patients exhibiting oxidative/nitrosative stress to improve their medical situation.

Oxidative stress caused by impaired NO production has been also shown to increase endothelial cell adhesion (Singh et al. 1995). But also human RBCs were shown to adhere to subendothelial matrix laminin via the basal cell adhesion molecule and Lutheran protein (BCAM/Lu) with higher blood cell–endothelium interactions in various diseases such as diabetes or sickle cell anemia which contributes to the vasoocclusive crises reported for sickle cell patients (El Nemer et al. 2008). AS195 decreased the adhesion of endothelial cells and may thus represent a useful addition to conventional therapeutic approaches in the treatment of these vascular diseases.

In conclusion, the administration of AS195 combines two key elements to increase NO bioavailability. First, NOS activation is increased which improves NO production. Further, the amount of ROS/RNS is reduced, and

the total antioxidant capacity is increased. Both effects improve cell function of both endothelial and red blood cells. These findings may thus help to explain improved microcirculation observed after AS195 treatment.

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Conflict of Interest

None declared.

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