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Concentration-dependent effects of resveratrol and metabolites on the redox status of human erythrocytes in single-dose studies

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Abstract

Dietary *trans*-resveratrol (RES) is rapidly metabolized into sulfated and glucuronated conjugates in humans. This study focused on the *in vitro* determination of the antioxidant capacity of RES and its main physiological metabolites and on its relevance *in vivo*. *In vitro*, RES, RES-3-*O*-sulfate (R3S) and 3-*O*-glucuronide (R3G) showed antioxidant activities at a concentration of 1 mM when compared to Trolox using an assay in which the antioxidant inhibits iron-induced linoleic acid oxidation: 0.87±0.08 mM Trolox equivalents (TE) for RES, 0.52±0.01 mM TE for R3S and 0.36±0.02 mM TE for R3G. At a concentration of 1 μM, compounds promoted linoleic acid peroxidation (RES −0.30±0.09 mM TE, R3S −0.48±0.05 mM TE and R3G −0.57±0.07 mM TE). To elucidate whether these effects were reflected *in vivo*, total antioxidant capacity, reactive oxygen species (ROS), conjugated fatty acid dienes (CD), superoxide dismutase (SOD) and catalase (CAT) activities were determined in human plasma and erythrocytes over 24 h, after oral intake of either 0.05 g RES as piceid or 5 g RES. Oral administration of RES did not show an impact on total antioxidant capacity, ROS or CD. However, enzymatic activities of ROS scavenging SOD and CAT were significantly lower after high-dose compared to low-dose administration of RES ($P<.03$ and $P<.01$). In conclusion, in healthy subjects, neither 0.05 g nor 5 g RES changed blood oxidative state, although our *in vitro* data point to a prooxidative activity of low concentrations of RES and its metabolites, which could be important *in vivo* for individuals with compromised antioxidant defense capacity.

Keywords: Resveratrol; Antioxidant capacity; ROS; SOD; CAT; Human

1. Introduction

trans-Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, RES) is predominantly found in red grapes, peanuts or products thereof [1]. Upon ingestion, RES and its predominant form in plant-derived foods, RES-3-*O*-β-D-glycoside, termed piceid, are metabolized rapidly, forming RES-sulfated and RES-glucuronated conjugates [2]. Depending on the administered dose, plasma concentrations of RES metabolites vary from nanomolar to micromolar range [2,3]. Quantitative data from a previously conducted pharmacokinetic pilot study showed maximum plasma concentrations (c_{max}) of RES-3-*O*-sulfate (R3S), RES-disulfates (RdS) and RES-monoglucuronides and diglucuronides of 0.95 μM, 1.27 μM and 0.7 μM, respectively, after a single oral dose of 85.5 mg piceid (corresponding to 0.05 g RES) [2]. Dietary administration of a 100-fold higher bolus dose of 5 g RES revealed c_{max} values for the aforementioned metabolites of 81.3 μM, 28.2 μM and 67.6 μM, respectively [3]. RES in plasma was below the limit of detection (0.02 μM) after administration of 85.5 mg piceid and demonstrated a c_{max} value of only 0.90 μM after administration of 5 g RES [2,3]. Both conducted pharmacokinetic studies showed a low bioavailability for RES in blood, in contrast to its metabolites, leading to the hypothesis that the conjugates carry the biological activity in humans or are reconverted into RES in target organs, as reported by Patel *et al.* [4]. Hence, reconversion data are still limited, and it seems conceivable that observed health effects in humans can be attributed to RES conjugates [5]. The effects of the parent compound RES on oxidative and radical-scavenging mechanisms are controversially discussed [6]. Oxidative stress in form of reactive oxygen species (ROS) can lead to modification of proteins, lipids and carbohydrates and is hypothesized to be associated with the progression of various pathological states, e.g., cancer, age-related diseases and the development of type 2 diabetes [7,8]. In general, RES is known as a potent direct radical scavenger and has been shown to interact with redox systems *in vivo* by regulating antioxidant detoxification enzymes like superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase directly or

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via adjustment of redox-controlled gene transcription [9–11]. However, Bader *et al.* [12] investigated the degradation of RES by γ-radiation and hypothesized the formation of RES radicals in aqueous solution as a result of OH-radical attack. Accordingly, at a considerably low concentration of 8 μM, Ahmad *et al.* [13] could show that RES induced a prooxidative state in human leukemia cells *in vitro* by increasing intracellular superoxide by 25%, indicating a promoting effect on radical formation. On the other hand, RES showed a direct radical-scavenging effect in rat macrophages *in vitro* at concentrations ranging from 0.65 to 1.3 mM [14]. Furthermore, in human breast cancer cells, Bader and Getoff [15] investigated the impact of 2 μM and 50 μM RES on the effectiveness of γ-ray irradiation on cell death *in vitro*, and they could show that, at 2 μM, RES had a radical-scavenging protective effect on cancer cells, whereas cancer cell death was enhanced at 50 μM RES. Besides direct radical-scavenging activity, another mechanism of action of RES is the up-regulation of ROS detoxification enzyme activity such as that of SOD and CAT. This was shown by Mokni *et al.* [16] in an *in vivo* study of healthy rats after intraperitoneal administration of 5–25 mg/kg body weight RES for 1 week, circumventing extensive metabolism in liver and digestive tract. Considering aforementioned controversial findings, studies in healthy human subjects after dietary relevant single dosage of RES could provide useful information on the influence of this natural compound and its metabolites on the natural balance of radical-scavenging systems and are, to our knowledge, not yet available. We therefore initially assessed the redox capacity of RES and its main physiological metabolites over a wide range of concentrations (1–1000 μM) in a cell-free assay. Consecutively, the current study aimed to compare the impact of a RES-derivative piceid, present in plants, on the human blood antioxidative capacity after a single low oral dose of 85.5 mg (0.05 g RES) [2], representative for an average daily food intake from dietary sources [1], and a high oral amount of 5 g RES (10 capsules at 500 mg) [3], representing commercially available nutritional supplements. Plasma and erythrocyte samples were obtained from the pharmacokinetic studies referenced above, conducted and published earlier, by Burkon *et al.* [2] and Gualdoni *et al.* [3]. Formation of ROS, conjugated fatty acid dienes (as a marker for radical induced lipid peroxidation, CD) and ROS detoxifying enzyme activities (CAT and SOD) in human erythrocytes were compared after single, differentially dosed, oral administration of RES in healthy subjects to see if the obtained *in vitro* results were reflected in the redox status of human blood. Measurements were done in a timely manner after blood drawings, and as of now, we could show for the first time a dose-dependent impact of oral short-term administration of RES on antioxidative enzyme activities in erythrocytes of healthy subjects.

2. Materials and methods

2.1. Chemicals

The reagents and solvents used for the experiments were purchased from Sigma-Aldrich (Taufkirchen, Germany) and Merck (Darmstadt, Germany). The SOD assay kits used for the activity measurements were acquired from Dojindo (Gaithersburg, MD, USA). Benzoyl-leucomethylene blue was purchased from TCI Germany (Eschborn, Germany).

2.2. Plasma and erythrocyte samples

The plasma and erythrocyte samples were obtained from two human intervention studies, as reported by Burkon *et al.* (n=9) [2] and Gualdoni *et al.* (n=8) [3] on the pharmacokinetics of RES and metabolites, both of which were performed and published earlier [2,3]. After drawing blood samples as references, a single oral dose of either 85.5 mg piceid (corresponding to 0.05 g *trans*-RES) per 70 kg body weight (Burkon *et al.* [2]) or an absolute amount of 5 g of RES (Gualdoni *et al.* [3]) was administered. After oral administration, blood samples were drawn over 24 h (piceid) and 48 h (5 g RES), immediately separated into plasma and erythrocytes by centrifugation, washed three times with 0.9% sodium chloride solution and stored at −80°C, shortly followed by further analyses.

2.3. Total antioxidative activity

Total antioxidative activity was determined following the procedure reported by Lindenmeier *et al.* that is based on the inhibition or promotion of the iron-catalyzed peroxidation of linoleic acid by added antioxidants [17]. Initially, the total antioxidant activity of RES and the RES metabolites was measured. R3S, RdS and 3-*O*-glucuronide (R3G) were synthesized and purified as published previously [2]. Solutions of each substance (1–1000 μM in 15% aqueous ethanol) were prepared and aliquots (50 μl) of these solutions were used for testing. For the determination of the antioxidative activity of plasma samples, these were spiked with ethanol for a final concentration of 15% ethanol and aliquots (50 μl) were used for testing. The antioxidative activity was correlated with the degree of color development (conversion of benzoyl-leucomethylene blue to methylene blue), which was measured at 666 nm in disposable cuvettes on a TecanInfinite 200 (Groedig, Austria). Results were calculated using a seven-point-calibration curve with Trolox solutions as reference antioxidant (0–2 mM in 15% aqueous ethanol) and the total antioxidative activity was expressed as Trolox equivalents (TE) (mmol/L). Experiments were performed in triplicates, with two technical replicates.

2.4. Determination of total ROS in erythrocytes

Total ROS was determined by FACS measurement according to Amer *et al.* [18]. It is based on the uptake of nonfluorescent 2′,7′-dichloro-fluorescin-diacetate (DCF-DA) into cells and its conversion to 2′,7′-dichloro-fluorescin (DCFH) by endogenous esterases. ROS-containing cells convert DCFH to a fluorescent 2′,7′-dichloro-fluorescein (DCF) radical, with absorption maximum at 488 nm. A 1:20 dilution of erythrocytes in PBS was incubated for 15 min (37°C, 5% CO_2) with 0.4 mM DCF-DA, centrifuged and washed once with 1 ml of PBS. Subsequently, an aliquot was incubated with 2 mM H_2O_2 for 30 min at room temperature. Measurement of 100,000 cells was performed with a FACSCalibur (Becton-Dickinson, New Jersey, USA). The difference between fluorescent values of H_2O_2-stimulated and nonstimulated cells was normalized to hemoglobin content of the sample, and data were then calculated in percent as treated (n=8) over control (n=2).

2.5. Measurement of CD in erythrocytes

The lipid extraction from the erythrocytes was carried out by a modified method reported by Folch *et al.* [19], followed by saponification and methylation of the fatty acids according to the Association of Official Analytical Chemists method [20]. A volume of 10 μl of this fatty acid composition was injected into an HPLC column and CD were quantified using diode array detection (DAD) at a wavelength λ=234 nm. Separation was performed on a Dionex Ultimate 3000 LC system (Thermo Fisher Scientific, Vienna, Austria), using a Nucleosil 100-5 C18 analytical HPLC column (250×4.6 mm ID, 5 μm particle size; Macherey-Nagel, Düren, Germany) with a precolumn of the same material, at a flow rate of 0.8 ml/min at 25°C. Liquid chromatography was carried out using the following conditions: starting with 95% acidified H_2O bidest (0.1% formic acid) and 5% acidified acetonitrile (0.1% formic acid), the acetonitrile content was increased to 80% within 20 min then to 100% within 25 min and was maintained for 5 min. Thereafter, the column was reset to the initial conditions within 5 min and equilibrated for another 5 min. Each of the experiments was performed in triplicates. CD content was defined as the area under the curve (AUC) related to the content of total lipids extracted via the Folch extraction [19]. The total lipid content was determined by a photometric assay according to the manufacturers' protocol (Randox Laboratories, Krefeld, Germany).

2.6. Measurement of SOD activity in erythrocytes

The SOD activity in erythrocytes was determined by the SOD assay kit water-soluble tetrazolium salt, according to the manufacturers' protocol (Dojindo, Gaithersburg, MD, USA) with slight modifications. For sample preparation, 0.5 ml of erythrocytes was suspended with 2 ml of distilled water then 1 ml of ethanol and 0.6 ml of chloroform were added and the mixture was shaken vigorously with a shaker for 15 min at 4°C. Afterwards, the mixture was centrifuged at 600*g* for 10 min at 4°C, the upper water/ethanol phase was transferred into a new tube and diluted 1:8 with distilled water. To achieve the sample solution for the assay, a final dilution step was accomplished with 0.25% aqueous ethanol. In the SOD assay, a highly water soluble tetrazolium salt (WST-1) was used, which was converted to a water-soluble formazan dye through reduction by superoxide anions. The rate of reduction of the superoxide anions was linearly related to the xanthine oxidase activity and inhibited by SOD (sample solution). Thus, the SOD activity (50% inhibition) was determined colorimetrically in duplicates in 96-well microplates at a wavelength λ=450 nm. Finally, the SOD activity was related to the hemoglobin content of the sample.

2.7. Measurement of CAT activity in erythrocytes

The CAT activity in erythrocytes was determined following a photometric assay reported by Aebi [21]. For sample preparation, erythrocytes were diluted 1:5 with distilled water. A further 1:500 dilution step was performed with phosphate buffer immediately before the assay was started by adding H_2O_2. The determination was performed in duplicates and the decrease of H_2O_2 absorbance was recorded at 240 nm for 30 s (at 20°C) in disposable cuvettes. Results were calculated and demonstrated as

the rate constant of a first-order reaction (k) [21]. Finally, CAT activity was related to the hemoglobin content of the sample.

2.8. Hemoglobin content in erythrocytes

The hemoglobin content in erythrocytes was determined following a method with Drabkin's reagent according to the manufacturers' protocol (Sigma-Aldrich, Taufkirchen, Germany) with slight modifications. For sample preparation, the erythrocytes were diluted 1:2 with distilled water and aliquots (20 µl) were used in the colorimetric assay. The determination was performed in duplicates and the absorbance was recorded at a wavelength λ=540 nm. Finally, the results were calculated using a five-point-calibration curve with human hemoglobin solutions as standards (0.15–0.92 mg/ml in distilled water).

2.9. Statistical analysis

All data presented are expressed as means±S.E.M. The differences in the enzyme activities and the oxidative exposure of erythrocytes (CD) between time point 0 h and the various time points after administration of piceid and RES were calculated using two-tailed Student's t test for nonpaired samples. The same test was used for calculation of differences between high-dose and low-dose administrations of RES, using AUC values. $P \leq .05$ was considered significant.

3. Results and Discussion

3.1. Total antioxidative capacity in vitro

The antioxidative activity of RES and its main physiological metabolites (R3S, RdS and R3G) was measured following a protocol by Lindenmeier *et al.* [17]. This method is based on the Fenton-type reaction, with linoleic acid as a substrate for iron-catalyzed radical attack by H_2O_2-derived OH^- radicals. Resulting linoleic acid peroxides then catalyze the conversion of colorless benzoyl-leucomethylene blue to methylene blue, which absorbs at 666 nm. Addition of a given substance can either inhibit (antioxidative) or promote (prooxidative) linoleic acid peroxidation, resulting in differential color development [22,23]. Results were calculated using an eight-point Trolox calibration curve (0–2 mmol/L, R^2=0.995). RES, R3S, RdS and R3G at concentrations 1, 10 and 100 µM showed a promoting effect on linoleic acid peroxidation, with lowest TE values (mM TE) of −0.30±0.09 mM TE, −0.48±0.05 mM TE, −0.36±0.09 mM TE and −0.57±0.07 mM TE, respectively, at a concentration of 1 µM (Fig. 1). At 100 µM, the parent compound showed an inhibiting effect on linoleic acid peroxide formation, compared to the metabolites, which still induced peroxidation. Whereas at 1000 µM, the antioxidative activity of RES resulted in 0.87±0.08 mM TE, values for the metabolites were lower with 0.52±0.01 mM TE for R3S and 0.36±0.02 mM TE for R3G. No effect with respect to total antioxidant capacity was observed for RdS at 1000 µM. These results clearly demonstrate that the redox capacity of RES and its conjugates varied dose dependently from 1 µM to 1000 µM from prooxidant to antioxidant in our cell-free assay. On the one hand, the linoleic acid peroxidation inducing effect of low concentrations of RES corresponds to *in vitro* findings by Martins *et al.*, where RES, at concentrations of 1–50 µM, increased ROS levels in hepatic cells after a 24 h treatment [24]. On the other hand, a decrease in superoxide anion production could be shown at only 3 and 6 µM RES *in vitro*, however in a model of hyperglycemic THP-1 monocytes to mimic diabetes, with higher levels of oxidative stress to begin with [25]. In our cell-free study, at a concentration of 100 µM, RES is more antioxidatively active than its metabolites R3S, RdS and R3G (ANOVA, *post hoc* Holm-Sidak, $P \leq .007$). At 1000 µM, RES inhibits linoleic acid peroxidation more effectively than R3S and R3G (ANOVA, *post hoc* Holm-Sidak, $P \leq .003$). This could be due to the conjugation of 3-hydroxy and 5-hydroxy groups, which was shown to impair the thermodynamic stabilization of the radical-scavenging system of RES [26]. The potential of free RES to inhibit linoleic acid peroxidation at concentrations of 100 and 1000 µM, compared to RES conjugates, is further confirmed by Storniolo *et al.*, where at 50 µM, activity of RES was more profound than that of R3S or R3G [27].

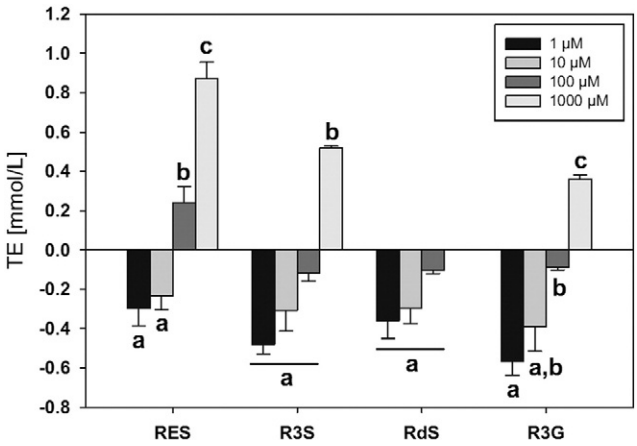

Fig. 1. *In vitro* total antioxidant activity of RES and its main physiological metabolites R3S, RdS and R3G in aqueous solution (in mM TE); concentration-dependent effects for individual compounds are indicated by lowercase letters as determined by ANOVA, followed by *post hoc* Holm-Sidak testing ($P \leq .05$); data are expressed as means±S.E.M. (n=3–6).

3.2. Oxidative status in vivo

3.2.1. Total antioxidant capacity in plasma

In a study by Olas *et al.* [28], a pronounced protective effect against the ROS species peroxynitrite could be shown in plasma of healthy volunteers for 100 µM RES. Therefore, after determining the antioxidant effect of RES and its conjugates on the iron-induced linoleic acid peroxidation *in vitro*, we analyzed the redox status in plasma of healthy subjects after oral administration of 0.05 g RES, in form of 85.5 mg piceid by means of the *in vitro* applied test (Table 1) [17]. Contrary to our *in vitro* findings at 1 µM, at c_{max} values of 0.95 µM, 1.27 µM and 0.7 µM, for R3S, RdS and RES-glucuronides, respectively, as determined earlier by Burkon *et al.* [2] for the same study population, no changes compared to basal levels (t_0, set at 100%) were observed *in vivo*. The absence of any measurable effect of RES and metabolites *in vivo* might be explained by the higher abundancies of other antioxidants present, such as ascorbic acid, tocopherol, retinol or glutathione [29].

3.2.2. Total ROS in erythrocytes

The *in vitro* observed prooxidative effect of RES and its conjugates was not reflected *in vivo* by the total antioxidant activity in plasma after administration of 85.5 mg piceid (0.05 g RES). Therefore, the effects of RES and its metabolites on nonenzymatic and more specific markers of oxidative stress (ROS and CD) were determined in erythrocytes after a high oral load of 5 g. Levels of ROS in erythrocytes were determined by the intracellular conversion of DCFH to a DCF radical and were calculated as treated (n=8) over control (n=2), with time point t_0 set at 100% as baseline value, as depicted in Fig. 2. No

Table 1
Total antioxidant activity in human plasma after the administration of a single dose of 85.5 mg piceid per 70 kg body weight (n=9); time point 0 h (t_0) was set as 100%, and data represent means±S.E.M.

Time points (t_x) (h)	Total antioxidant activity (t_x/t_0) (%)
0	100±6.38
1	106±5.00
2	105±6.06
4	105±5.05
6	109±4.90
8	106±6.00
10	109±4.90
24	103±3.09

differences to baseline level could be observed, suggesting no impact of metabolized RES on ROS formation in erythrocytes. After an amount of 5 g RES, c_{max} values in plasma were 0.09 μM, 81.3 μM, 28.2 μM and 67.6 μM, at time points (t_{max}) 2.5 h, 5 h, 10 h and 2.5–5 h, for RES, R3S, RdS and RES-glucuronides, respectively, as reported by Gualdoni *et al.* [3]. According to our here-presented results on ROS formation in human erythrocytes, those concentrations are apparently not relevant for an antioxidant effect. On the other hand, we could not observe a prooxidant effect either, as suggested by our *in vitro* data (Fig. 1), although ROS values show an increase from 5 to 10 h (Fig. 2). This interval corresponds approximately to c_{max} for metabolites, namely R3S and the RES-4′-*O*-glucuronide with t_{max} at 5 h and RdS with t_{max} at 10 h after oral administration, as previously determined by Gualdoni *et al.* [3]. However, it should be mentioned that, *in vitro*, at a concentration of 100 μM, we could observe a radical inducing effect for RES conjugates, with values for predominating metabolites R3S and R3G of −0.12±0.04 and −0.09±0.01 mM TE, whereas in an *in vitro* study by Storniolo *et al.* [27], 50 μM of the respective compounds showed a radial-scavenging effect of 0.11±0.02 and 0.12±0.03 mM TE. In the referenced study, results were also compared to TE but achieved by means of a commercially available kit, based on the inhibition of the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) to $ABTS^+$ by present compounds [30]. As evaluated by Tabart *et al.* [31], total antioxidant values can vastly differ for individual compounds, depending on the applied test. Measuring the scavenging activity of the 2,2-diphenyl-1-picrylhydrazil radical and measuring the oxygen radical-scavenging capacity, for example, showed distinct differences in antioxidant capacities for selected flavonoids due to different reaction mechanisms [31]. We therefore estimate the impact of predominant RES conjugates on ROS levels in concentrations up to approximately 100 μM in human erythrocytes to be negligible, especially, as other antioxidants very likely cover their effect due to higher molar abundances [29].

3.2.3. CD in erythrocytes

To examine whether the metabolites formed *in vivo* after oral intake of 0.05 g or 5 g RES affected the oxidative exposure of erythrocytes, CD were quantified in erythrocytes by HPLC DAD (Fig. 3). As CD are more stable than ROS, these oxidized fatty acids can be used to determine changes in erythrocyte redox state that would be overseen due to short half-life of ROS, in combination with the time periods between blood drawings. In accordance with our data

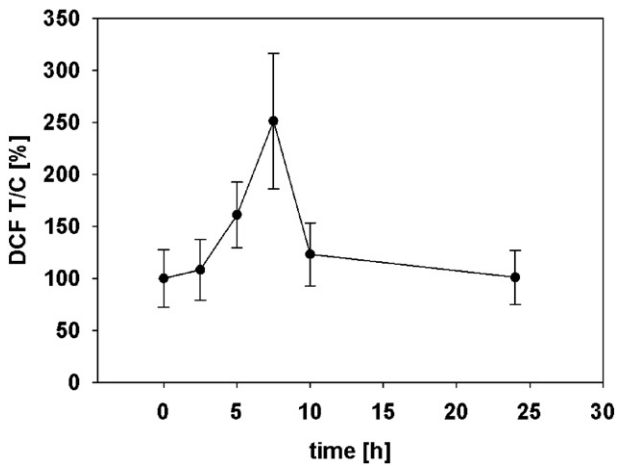

Fig. 2. ROS levels in erythrocytes, as determined by DCF fluorescence; values are normalized to hemoglobin content and calculated as treated (n=8) over control group (n=2) with baseline at time point t_0 set at 100%; data represent means±S.E.M.; a two-tailed Student's t test for nonpaired samples was applied to calculate differences between time points, and P≤.05 was considered significant.

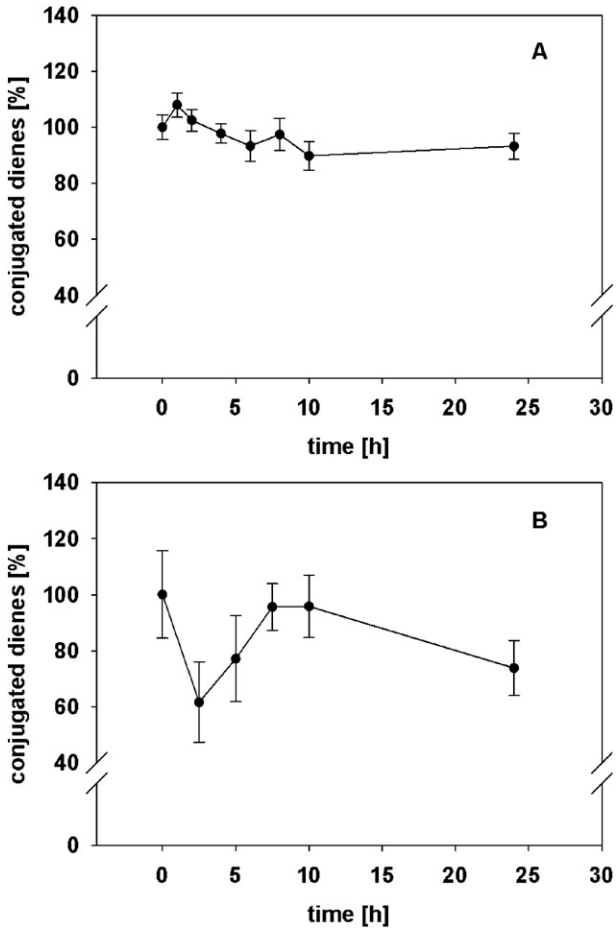

Fig. 3. CD in human erythrocytes after the administration of (A) single-dose piceid (0.05 g RES, n=9) and (B) single-amount RES (5 g, n=8); results are normalized to total lipid content and t_0 was set as 100%; data represent means±S.E.M.; a two-tailed Student's t test for nonpaired samples was applied to calculate differences between time points, and P≤.05 was considered significant.

on antioxidant capacity and ROS, no changes to baseline values (100%) could be determined at any measured time point. Comparison of AUCs (in arbitrary units, a.u.) between dietary low RES ($AUC_{0.05\ g}$=2260± 76.8 a.u.) and high RES, after a 5 g supplement ($AUC_{5\ g}$=2018± 156 a.u.) showed no significant difference for CD in erythrocytes, although Fig. 3B showed a decrease in CD levels in the first 5 h, which would indicate an antioxidant effect. This potential antioxidant effect is in agreement with other studies where incubation of erythrocytes with 0.1–100 μM RES attenuated oxidative-injured erythrocyte membrane *in vitro* [32,33]. A dose-dependent decrease of *tert*-butylhydroperoxide-induced formation of malondialdehyde was obtained after treatment of the erythrocytes with RES. However, in the current study, no changes in CD levels were determined after a dose of only 0.05 g RES (Fig. 3A).

3.2.4. Antioxidant enzyme activities in erythrocytes

The function of antioxidant enzymes (SOD, CAT, glutathione peroxidase) in blood and tissues is to protect from ROS-mediated damage [34]. Down-regulation of gene transcripts and the activity of those enzymes have been linked with increased amounts of ROS and cause increased susceptibility to oxidative damage [35,36]. As erythrocytes are not able to transcribe and translate genes, antioxidant enzyme activities were measured in red blood cells of participants, as a marker for oxidative stress.

3.2.4.1. SOD in erythrocytes. To elucidate the influence of RES on ROS detoxifying enzymes, SOD and CAT activity in erythrocytes was determined after oral administration of different amounts of RES over 24 h (Figs. 4 and 5). No differences compared to basal levels could be observed after administration of either 0.05 g or 5 g RES. However, by comparison of AUC values of the SOD activity in both studies, SOD activity was reduced after intake of 5 g RES compared to the low-dose intake of 0.05 g RES in form of piceid ($AUC_{0.05\ g}$=2365±43.0 a.u. and $AUC_{5\ g}$=1829±216 a.u., P=.03). This effect of the higher oral amount of RES might be at least partially explained by lower values at 2.5 h after administration of 5 g RES (Fig. 4B), corresponding to the time points for c_{max} of glucuronated metabolites (total of 67.6 μM), ranging from 2.5 to 5 h, as documented by Gualdoni *et al.* [3], and could indicate changes in erythrocyte oxidative state, which might not occur with low-dose RES, where c_{max} for glucuronides was only 0.7 μM with t_{max} at 6 h after ingestion [2].

3.2.4.2. CAT in erythrocytes. As for SOD, no differences to basal levels could be observed for CAT activity in either group, but again, a lower enzyme activity could be demonstrated for the high amount RES group by AUC comparison to the low-dose RES group ($AUC_{0.05\ g}$=2373±119 a.u. and $AUC_{5\ g}$=1411±230 a.u., P=.01, Fig. 5). For the parent compound RES, an enzyme inhibiting effect on CAT has been observed by Martins *et al.* [24], at 50 μM *in vitro* in liver cells. As the parent compound is not present in plasma in such high concentrations

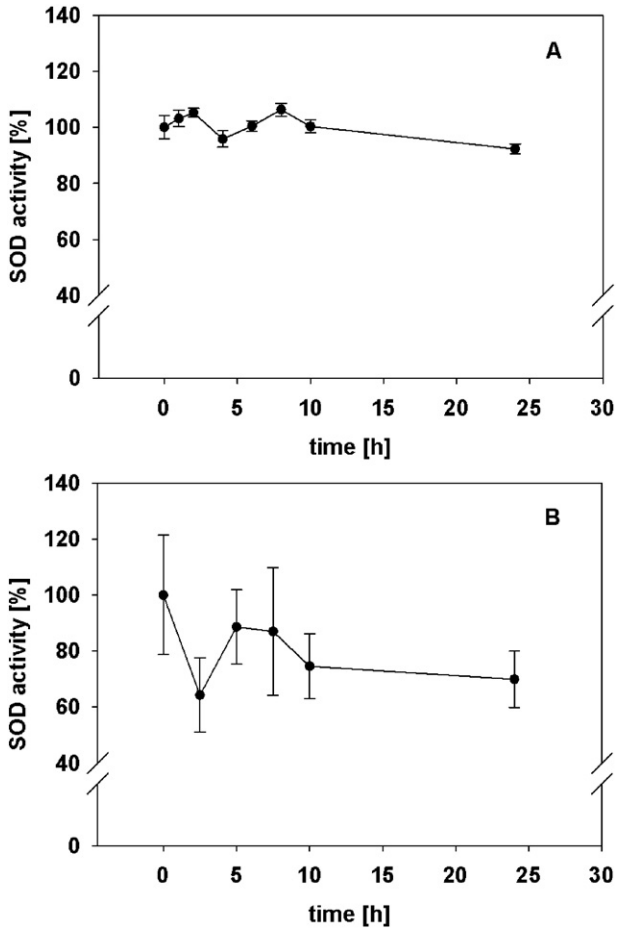

Fig. 4. SOD activity in human erythrocytes, normalized to hemoglobin content after the administration of (A) single-dose piceid (0.05 g RES, n=9) and (B) single-amount RES (5 g, n=8); t_0 was set as 100%; data represent means±S.E.M.; a two-tailed Student's t test for nonpaired samples was applied to calculate differences between time points, and P≤.05 was considered significant.

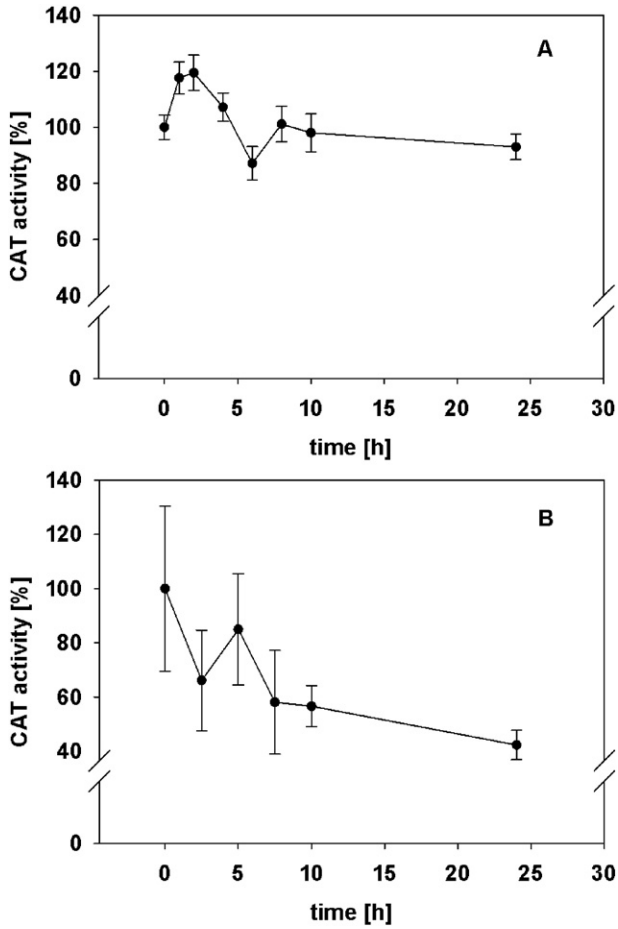

Fig. 5. CAT activity in human erythrocytes normalized to hemoglobin content after the administration of (A) single-dose piceid (0.05 g RES, n=9) and (B) single-amount RES (5 g, n=8); t_0 was set as 100%; data represent means±S.E.M.; a two-tailed Student's t test for nonpaired samples was applied to calculate differences between time points, and P≤.05 was considered significant.

(c_{max}=0.09 μM) after oral administration of 5 g RES, those changes in enzyme activity could be attributed to the main conjugates. Fig. 5B shows a trend for lower enzyme activities, albeit not reaching the level of significance (P=.063), CAT activity at 2.5 h (t_{max} of glucuronide conjugates) and additionally from 5 h to 24 h, corresponding to t_{max} after oral administration for R3S at 5 h (c_{max}=81.3 μM) and for RdS at 10 h (c_{max}=28.2 μM), as determined by Gualdoni *et al.* [3].

4. Conclusions

Oral administration of RES did not affect the iron-catalyzed linoleic acid peroxidation and ROS formation in human erythrocytes, presumably due to the presence of more potent antioxidants [29] and the comparably low concentrations of RES metabolites (c_{max} from 0.27 to 1.27 μM after oral intake of 0.05 g RES and from 28.2 to 81.3 μM after 5 g RES) [2,3]. Moreover, the high-plasma-protein binding capacity of RES and its metabolites of over 93% for RES, 66% for R3S and 56% for RdS might also impede their effect on ROS formation [2]. However, our data suggest a prooxidative effect of orally ingested RES as demonstrated by decreased SOD and CAT activity in erythrocytes, after administration of a high bolus dose of 5 g RES, compared to low-dose administration as 85.5 mg piceid, especially at time points corresponding to maximum plasma concentrations of individual metabolites (c_{max} for glucuronides being 67.6 μM with t_{max} at 2.5 h and 81.3 μM for R3S with t_{max} at 5 h after 5 g RES intake). Down-

regulation of SOD and CAT activity has been associated with an increased ROS formation [35,36], which was most likely compensated by the presence of other antioxidants in our *in vivo* studies. In our *in vitro* assay, antioxidant activities were demonstrated for concentrations of 100 μM, which were not reached even after the bolus dose of 5 g RES, resulting in a mean maximum plasma concentration for the 3-O-sulfated metabolite of 81.3 μM, followed by RES glucuronides with 67.6 μM [3]. Additionally, plasma half lives in humans for RES conjugates were determined to be only 5–8 h, after an oral dose of 5 g, resulting in rapid elimination from the body [37]. In accordance with our *in vitro* data, at concentrations of 1 and 10 μM, Ahmad *et al.* [13] demonstrated that treatment with 4–8 μM RES for 24 h profoundly increased intracellular radical concentrations (up to 25%) in human leukemia cells and even showed an interference with chemotherapeutic-induced apoptosis. Our *in vitro* data indicate that the major RES metabolites have a similar potential on radical formation. Considering the antioxidant effect of higher concentrations of RES and its metabolites, blood or tissue accumulation resulting in higher steady-state concentrations of RES could have not been reported in healthy human subjects so far, as demonstrated by Almeida *et al.* [38]. Here, a mean c_{max} value in plasma was only 0.28 μM and half-life of RES was determined to be 2–5 h after intake of 4× 150 mg RES over 24 h. Accordingly, after oral intake of 1 g RES administered twice a day for a week to healthy human subjects, a mean c_{max} value in plasma was 5.58 μM, and half-life was 2.4 h [39]. In one of our conducted pharmacokinetic studies, intake of 85.5 mg piceid led to plasma metabolite concentrations below 1.27 μM (RdS), and in line with their short elimination half-lives and our herein obtained data, we conclude that dietary ingested amounts of RES or piceid do not significantly influence the redox balance in blood samples of healthy subjects [2]. Taken together, even with single oral administration of RES in a dose corresponding to a high-dose nutritional supplementation, it seems unlikely to achieve plasma concentrations high enough for antioxidative effects in healthy subjects as observed in our *in vitro* experiments. However, Brasnyó *et al.* [40] showed a decrease of oxidative stress in urine of type 2 diabetic patients, after only 5 mg oral RES twice daily for 4 weeks. It has to be considered, though, that type 2 diabetics have higher basal systemic oxidative stress levels than metabolically healthy subjects and endogenous cellular stress response is attenuated, possibly featuring the effects of administered xenobiotics [41,42]. In conclusion, further investigations on the short-term and long-term dose-dependent effects of RES on blood and tissue redox mechanisms and enzymes especially in patients with compromised antioxidant defense capacity are needed to determine its antioxidant potential in disease.

Disclosure statement

The authors declare no conflict of interest.

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