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Original article

'Antioxidant' berries, anthocyanins, resveratrol and rosmarinic acid oxidize hydrogen sulfide to polysulfides and thiosulfate: A novel mechanism underlying their biological actions

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ABSTRACT

Nutraceutical polyphenol catechins in green tea oxidize H_2S to polysulfides (PS) in buffer and in cells thereby conveying their cytoprotective effects. Here we measure H_2S oxidation in buffer and HEK293 cells by over-the-counter nutraceuticals, blueberry, bilberry and cranberry, and by polyphenols, cyanadin (Cya), quercetin (Que), rosmarinic acid (RA) and resveratrol (Res). H_2S and PS were measured with specific fluorophores, AzMc and SSP4 respectively, and thiosulfate (TS) production was measured in buffer using silver nanoparticles (AgNPs). All compounds increased polysulfide production from H_2S in buffer and increased polysufides in cells. Decreasing oxygen from 100% to 21% and 0% progressively decreased PS production by Que and RA in buffer and Que decreased PS production in cells incubated in 5% O_2 compared to 21% O_2. Que, RA and Res, but not Cya, increased TS production from H_2S in 21% O_2 but not in 0% O_2. Superoxide dismutase did not affect PS production from H_2S by Que or TS production from H_2S by Que, RA or Res, whereas catalase inhibited TS production by all three polyphenols. Conversely, these polyphenols only slightly reduce a mixed polysulfide (K_2S_n) or thiosulfate to H_2S in 0% O_2. Collectively, our results suggest that polyphenols are autoxidized to a semiquinone radical and that this, in turn, oxidizes H_2S to a thiyl radical from which polysulfides and thiosulfate derived. They also suggest that this is catalyzed by a semiquinone radical and it is independent of either superoxide or hydrogen peroxide concomitantly produced during polyphenol autoxidation. The polysulfides produced in these reactions are potent antioxidants and also initiate a variety of downstream cytoprotective effector mechanisms. It is also possible that H_2S can be regenerated from the thiosulfate produced in these reactions by other cellular reductants and reused in subsequent reactions.

1. Introduction

There is increasing interest in the potential health benefits of certain colored fruits, berries and spices. Blueberries, cranberries, bilberries and grapes are especially notable in this regard as they have high concentrations of polyphenols that presumably convey the antioxidant properties from which the various health benefits are derived. Their purported effects include an anticancer activity and protection from a variety of cognitive and neurological disorders, metabolic syndrome and cardiovascular disease, enhance exercise performance, and modification of the microbiome [1–7].

Polyphenol-containing spices, such as rosmarinic acid, have received similar attention. Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, both subunits containing a phenolic ring. RA is prevalent in herbal plants [8,9] and it is used as a spice and for medicinal purposes where its purported beneficial effects include anticancer and protection of most organ systems against a variety of stressors, especially those related to oxidative stress [10–17].

The antioxidant effects of these bioactive polyphenols are generally thought to be directed against reactive oxygen species (ROS). However,

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it is also possible that their effects may be mediated through metabolism of reactive sulfur species (RSS), namely hydrogen sulfide (H_2S) and polysulfides (H_2S_n). RSS are chemically and biologically similar to ROS and they exert identical biological actions via common cysteine thiols on regulatory proteins [18]. The numerous health benefits attributed to polyphenols can also be explained by RSS as polysulfides are potent reductants [19], scavengers of ROS, especially superoxide [20], and through persulfidation of Keap1 they free it from Nrf2 leading to activation of the genomic antioxidant response elements [21–26].

We recently showed that green tea and tea catechin polyphenols oxidize H_2S to produce polysulfides in buffer and in cells [27] and we proposed that this could account for some of the purported health benefits of these nutraceuticals. As catechin polyphenols are similar in structure to berry and spice polyphenols, we hypothesize that the latter would also affect sulfur metabolism. The present studies were conducted to determine if a variety of health food 'antioxidants' available as over-the-counter supplements shared the ability to oxidize H_2S. We chose blueberries, cranberries, bilberries and resveratrol as these are commonly available and extensively promoted in advertising campaigns. We also examined a number of their purportedly bioactive polyphenols, cyanadin and quercetin as well as the spice polyphenol, rosmarinic acid. We show that all of these compounds concentration-dependently increase oxidation of H_2S to polysulfides and thiosulfate in buffer. In HEK293 cells they increase cellular polysulfides and this appears to be at the expense of intracellular H_2S. Collectively, these results suggest that many of the potentially beneficial effects of berries can be explained by their effects on sulfur metabolism and this is likely a common feature of nutraceutical polyphenols.

2. Methods

2.1. H_2S and polysulfide measurements in buffer

Compounds were aliquoted into 96-well plates in a darkened room and tape was placed over the plates to reduce H_2S volatilization. Fluorescence was measured with a SpectraMax M5e plate reader (Molecular Devices, Sunnyvale, CA). Excitation/emission wavelengths for 3′,6′-Di (O-thiosalicyl)fluorescein (SSP4), and 7-azido-4-methylcoumarin (AzMC) were 482/515 and 365/450 nm, respectively per manufacturer's recommendations. These fluorophores have been shown to have sufficient specificity relative to other sulfur compounds and reactive oxygen and nitrogen species (ROS and RNS, respectively) to be effectively employed for their respective analysis [28–30]. All experiments were performed in phosphate buffer (PBS) at room temperature.

2.2. Thiosulfate measurement in buffer

Thiosulfate was measured with silver nanoparticles (AgNPs) as previously described [31]. The particles were prepared by vigorously mixing 1 mL of 20 mM $AgNO_3$ with 200 μL of 0.5 mM $HAuCl_4$ in 98 mL of Milli-Q water at room temperature followed by addition of 1 mL of 5.0 mM tannic acid with continuous mixing. The mixture turned yellow within 30 min indicative of formation of the AgNPs. The solution was then titrated to pH 6.0 and stored at 4 °C until use. Thiosulfate standards were made in PBS and added to the AgNPs in 96-well plates at a ratio of 30 μL PBS to 200 μL AgNPs and absorbance measured at 419 nM. All subsequent experiments were analyzed with this 30 μL PBS: 200 μL AgNP ratio. Initial studies indicated that 60 min was sufficient for maximum changes in absorbance.

Although Dong et al. [31] reported that 10 μM H_2S did not interfere with the silver nanoparticle thiosulfate assay, we noticed in preliminary experiments that 100 and 300 μM H_2S produced absorbance responses similar to those of 100 and 300 μM thiosulfate. However, if the well-plates were uncovered, the H_2S effect progressively decreased over time indicating that indeed this was due to H_2S reacting directly with the AgNP (Supplemental Fig. S1). These studies also showed that by 90 min most of the H_2S effect had disappeared and they suggest that the residual effect on absorbance was largely due to thiosulfate that was present either as a contaminant in the Na_2S salt, or due to H_2S oxidation during the experiment. In order to examine the effects of polyphenols on thiosulfate production in subsequent experiments, the wells were covered with tape for an initial 120 min to minimize volatilization of H_2S and allow the reaction to proceed and then the wells were uncovered for an additional 90 min to allow the H_2S to dissipate prior to adding the samples to AgNP. The thiosulfate that was present as a contaminant or produced by H_2S oxidation was measured in polyphenol-free experiments and it was subtracted from the total thiosulfate produced by polyphenols in these experiments.

2.3. Oxygen dependency of polyphenol reaction with H_2S in buffer

In order to determine if polyphenols were autoxidized prior to reacting with H_2S we bubbled 5 mL of PBS with either 100% N_2 or 100% O_2, in glass vials, or used PBS equilbrated with room air (21% O_2) and then added quercetin or rosmarinic acid, capped the vial, and incubated the mixture for 60 min. Aliquots were assayed for polysulfides and thiosulfate as described above.

2.4. Polyphenols as reductants of oxidized sulfur

To determine if reduced polyphenols could produce H_2S from oxidized sulfur, we incubated the polyphenols with either the mixed polysulfide, K_2S_n, or thiosulfate and measured H_2S with either the H_2S-specific fluorophore, AzMC in 96-well plates, or with a home-made amperometric H_2S sensor in a 1 ml reaction chamber. For K_2S_n experiments the buffer was first sparged with 100% N_2 and placed in the hypoxia chamber at 100% N_2. K_2S_n was dissolved in this buffer and left uncovered for 90 min to reduce the small amount of H_2S that is present as a contaminant. The K_2S_n, polyphenols and AzMC were then added to the 96-well plates while in the hypoxia chamber, the wells were covered with tape and the plates removed for fluorescence measurements. As there is minimal H_2S in thiosulfate, the thiosulfate was not left uncovered prior to addition of compounds. Samples for amperometric measurements were treated similarly except the polyphenols were added to deoxygenated buffer in the reaction chamber after the K_2S_2 and thiosulfate samples.

2.5. Cells

Human embryonic kidney (HEK293) cells were cultured in T-25 tissue culture flasks and maintained at 37 °C in a 5% CO_2 humidified incubator with 21% O_2 supplemented with DMEM (low glucose) containing 10% FBS and 1% Pen/Strep. They were then transferred to 96-well plates and experiments were conducted when cells were 80–95% confluent. AzMC and SSP4 fluorescence was measured on a SpectraMax M5e plate reader as described above.

For experiments conducted at different O_2 tensions, the cells were transferred to 96-well plates with gas-permeable bottoms (Coy Laboratory Products, Inc. Grass Lake, MI) and grown to 80–95% confluency. AzMC or SSP4 and compounds of interest were added and after an initial baseline reading one plate was returned to the tissue incubator while the other plate was placed in a model 856-HYPO hypoxia chamber (Plas Labs, Inc. Lansing, MI) and the cells were incubated in 5%O_2/5% CO_2 (balance N_2) at 37 °C. The plates were removed at timed intervals, fluorescence measured, and the plates were returned to their respective environments.

2.6. Chemicals

Berry extracts and resveratrol were purchased 'off the shelf' from the health foods section of local supermarkets. SSP4 (3′, 6′-di(O-thiosalicyl) fluorescein) was purchased from Dojindo molecular Technologies Inc.

(Rockville, MD). All other chemicals were purchased from either ThermoFisher Scientific (Grand Island, NY) or Sigma-Aldrich (St. Louis, MO).

Bilberry capsules contained 300 mg of bilberry *Vaccinium myrtillus* fruit extract (equivalent to 1200 mg of bilberry), blueberry softgels contained 800 mg whole blueberry, cranberry tablets contained 400 mg cranberry concentrate and 30 mg vitamin C concentrate, and resveratrol softgels contained 250 mg resveratrol extract from *Polygonun cuspidatum* root and 10 mg grape skin extract from *Vilis vinifera*. The contents of the capsules and softgels were removed and the tablets crushed with a mortar and pestle. They were then suspended in distilled water and vortexed for a minimum of 2 min. The solids were allowed to settle out and the supernatant was extracted. The actual amount of extract used in individual experiments was an approximation of the dissolved extract. Stock solutions of resveratrol, roamarinic acid, cyanidin and quercetin were made in DMSO. Control samples were run in buffer or the appropriate concentration of DMSO.

H_2S is used to denote the total sulfide (sum of H_2S + HS^-) derived from Na_2S; S^{2-} does not exist under these conditions [32]. Phosphate buffer (PBS; in mM): 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 2 NaH_2PO_4, pH was adjusted with 10 mM HCl or NaOH to pH 7.4.

2.7. Statistical analysis

Data was analyzed and graphed using QuatroPro (Corel Corporation, Ottawa Ont, Canada) and SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA). Statistical significance was determined using one-way ANOVA and the Holm-Sidak test for multiple comparisons as appropriate using SigmaStat (Systat Software, San Jose, CA). Results are given as mean ± SE; significance was assumed when $p \leq 0.05$. Statistical comparisons of thiosulfate production were based on absorbance values as thiosulfate concentrations were derived from average absorbance values.

3. Results

3.1. Oxidation of H_2S to polysulfides by berry extracts and polyphenols in buffer

In order to determine if the berry extracts oxidized H_2S, we added berry extracts to H_2S (prepared as Na_2S) and monitored polysulfide formation with SSP4. Because the active antioxidant components of many berries and fruits are attributed to anthocyanins, flavonoids and related polyphenols, we repeated the above experiments with some of the important polyphenol components of the berry extracts, cyanidin, quercetin and resveratrol. All berry extracts and polyphenols produced a concentration-dependent increase in SSP4 fluorescence indicative of polysulfide production from H_2S (Fig. 1). Addition of 100 μM cyanidin to SSP4 in the absence of H_2S did not affect fluorescence. Similarly, SSP4 fluorescence was not affected by either 300 μM quercetin or 300 mg/L resveratrol; these are not shown in Fig. 1 for the sake of clarity. SSP4 fluorescence was inhibited by the highest concentrations of quercetin and resveratrol suggesting an optical inhibitory effect which was most notable for these two compounds and subsequently examined in greater detail (see section 3.5). These results indicate that berry extracts directly oxidize H_2S to polysulfides.

3.2. Effects of berry extracts and polyphenols on H_2S and polysulfides in HEK293 cells

We then examined if the berry extracts and polyphenols affected sulfur metabolism in HEK293 cells (Fig. 2). All three berry extracts decreased AzMC fluorescence (H_2S) at 1000 mg/L. This appeared to be partially attributable to H_2S oxidation to polysulfides and partially due to interference with light transmission (section 3.5). Extracts of all three berry products time- and concentration-dependently increased SSP4 fluorescence in HEK293 cells indicative of increased polysulfides. There was an initial rapid increase in fluorescence within the first 4 h followed by a sustained, slower increase in SSP4 fluorescence that appeared to last for at least 70 h.

As shown in Fig. 3, cyanidin, quercetin and resveratrol produced essentially the same concentration-dependent effects in cells as did the

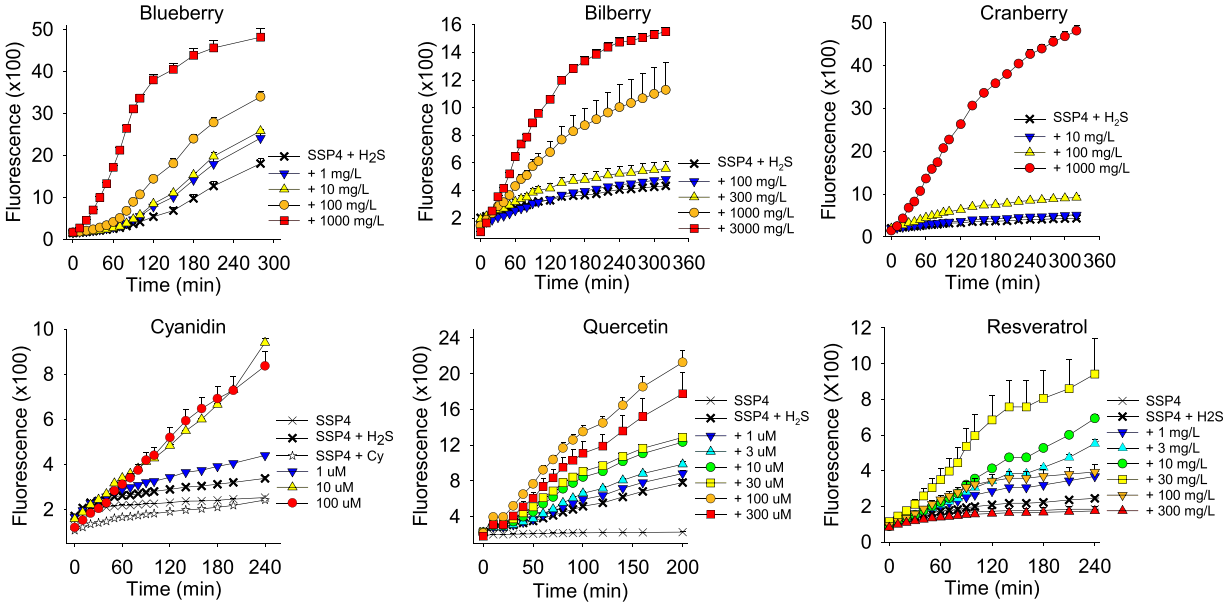

Fig. 1. Effects of blueberry, bilberry and cranberry extract and cyanidin, quercetin and resveratrol on polysulfide formation (SSP4 fluorescence) in the presence of 1 mM H_2S (as Na_2S) in buffer. All compounds concentration-dependently increased polysulfides over time. Mean + SE, n = 4 wells per treatment.

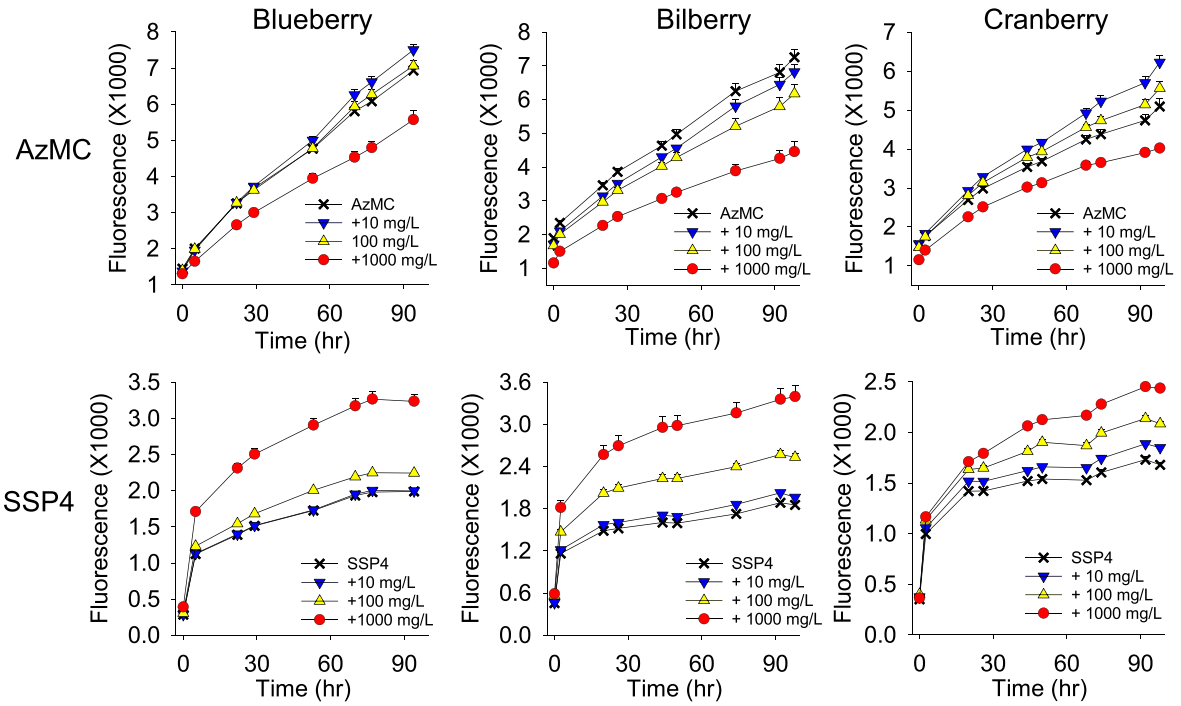

Fig. 2. Effects of blueberry, bilberry and cranberry extract on H_2S (AzMC fluorescence) and polysulfide (SSP4 fluorescence) in HEK293 cells. By 2.5 h all three compounds at 1000 mg/L significantly ($p < 0.001$) decreased AzMC fluorescence. All three also increased SSP4 fluorescence at 1000 mg/L ($p < 0.001$) and 100 mg/L (blueberry, $p < 0.01$; bilberry and cranberry, $p < 0.001$) and 10 mg/L cranberry increased SSP4 fluorescence ($p < 0.01$). Mean + SE, n = 8 wells per treatment.

berry extracts, although the highest concentrations of quercetin and resveratrol completely inhibited AzMC fluorescence and depressed SSP4 fluorescence. While some of the effects of these compounds on AzMC fluorescence could be due to optical interference, they clearly cannot account for the increase in SSP4 fluorescence at lower concentrations of the compounds. Collectively, these results suggest that the effect of berry extracts on HEK293 cells can be attributed, at least in part, to their polyphenols, cyanidin, quercetin and resveratrol.

3.3. Short-term effects of berry extracts and polyphenols on SSP4 fluorescence in HEK293 cells

The relatively rapid increase in polysulfide (SSP4 fluorescence) produced by berry extracts and polyphenols in HEK293 cells in the initial 4 h after treatment (Figs. 2 and 3), prompted a re-examination of their effects over these initial 4 h. Within the first 30 min, the highest concentrations of blueberry and bilberry extract (1000 mg/L) and polyphenols, cyanidin and quercetin (100 μM) significantly increased polysulfides as did lower doses of bilberry and quercetin (100 mg/L or 10 μM, respectively; Supplemental Fig. S2). These results suggest that berry extracts and their polyphenols have relatively rapid effects on cell sulfur metabolism.

3.4. Effects of rosmarinic acid on RSS metabolism in buffer and cells

The effects of rosmarinic acid on sulfur metabolism in buffer and HEK293 cells are shown in Fig. 4. Rosmarinic acid concentration-dependently increased polysulfide production from H_2S in buffer. While rosmarinic acid did not appreciably affect cellular H_2S, it concentration-dependently increased polysulfide production in HEK293 cells.

3.5. Berry extracts and polyphenols affect light transmission at high concentrations

Because the berries are colored it is possible that this might affect light transmission of the reporting fluorophores. To examine this possibility we added 30 μM H_2S to AzMC or 10 μM of mixed polysulfides (K_2S_n, n = 1–8) to SSP4 and allowed the fluorophores to fully react for 120 min before adding the berry extracts or polyphenols. Supplemental Figs. S3 and S4 show the effects of increasing concentrations of polyphenols and berry extracts on activated AzMC and SSP4 fluorescence, respectively. At 100 μM, quercetin, resveratrol and cyanidin inhibited AzMC fluorescence by approximately 60, 25 and 20%, respectively and SSP4 fluorescence by approximately 15, 20 and 10%, respectively. Considerably more inhibition, especially for AzMC was observed at higher concentrations of polyphenols. Blueberries at 1000 mg/L inhibited AzMC and SSP4 fluorescence by approximately 15% and 10%, respectively, bilberries inhibited AzMC and SSP4 fluorescence by approximately 30% and 20%, respectively, whereas cranberries were the least inhibitory at 6% and 10%. These results show that there is optical inhibition of fluorophore fluorescence by these compounds and they limited the concentrations of extracts and polyphenols used in subsequent studies.

3.6. Effects of oxygen on H_2S metabolism by quercetin and rosmarinic acid in buffer

The sulfur in H_2S is in its most reduced form and must be oxidized to produce polysulfides. This requires prior oxidation of reduced polyphenols. In order to determine if oxygen contributed to, or was directly involved in autoxidation of the polyphenol, we incubated quercetin or rosmarinic acid for 1 h in PBS that was previously bubbled with 100% N_2, 100% O_2 or open to room air (21% O_2) prior to adding H_2S and SSP4. As shown in Fig. 5A, polysulfide production by both polyphenols

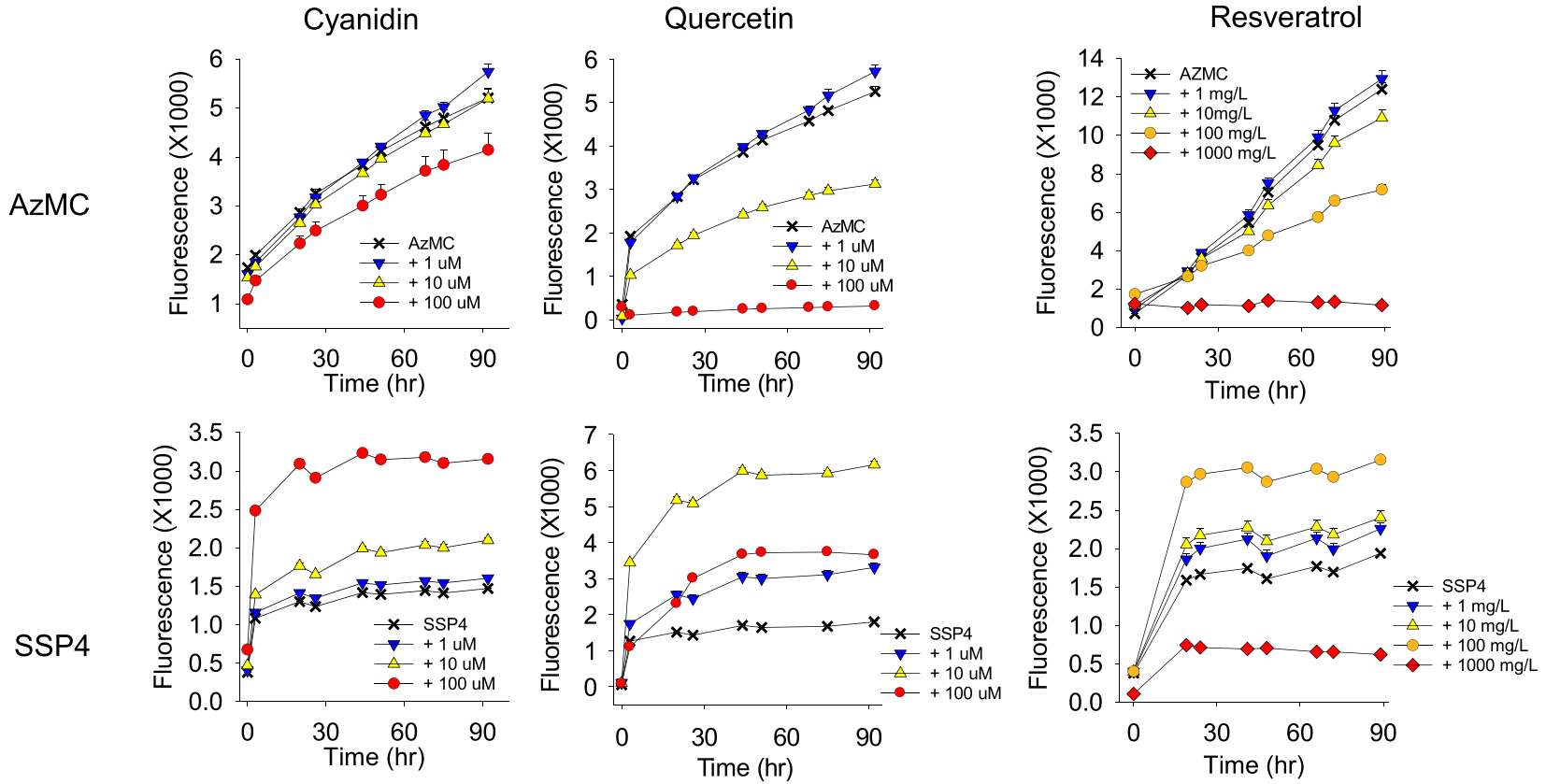

Fig. 3. Effects of cyanidin, quercetin and resveratrol on H_2S (AzMC fluorescence) and polysulfide (SSP4 fluorescence) in HEK293 cells. AzMC fluorescence was significantly decreased by 100 μM cyanidin ($p < 0.001$), 10 and 100 μM quercetin ($p < 0.001$) and by 10 mg/L resveratrol after 48 h ($p < 0.05$), 100 mg/L resveratrol by 24 h ($p < 0.001$) and completely inhibited by 1000 mg/L resveratrol ($p < 0.001$) at all times. SSP4 fluorescence was increased by 1 ($p < 0.05$), 10 ($p < 0.001$) and 100 ($p < 0.001$) μM cyanidin, 1, 10 and 100 μM quercetin (all $p < 0.001$). SSP4 fluorescence was increased ($p < 0.001$) by 1–100 mg/L resveratrol and decreased ($p < 0.001$) by 1000 mg/L resveratrol. Mean + SE, n = 8 wells per treatment.

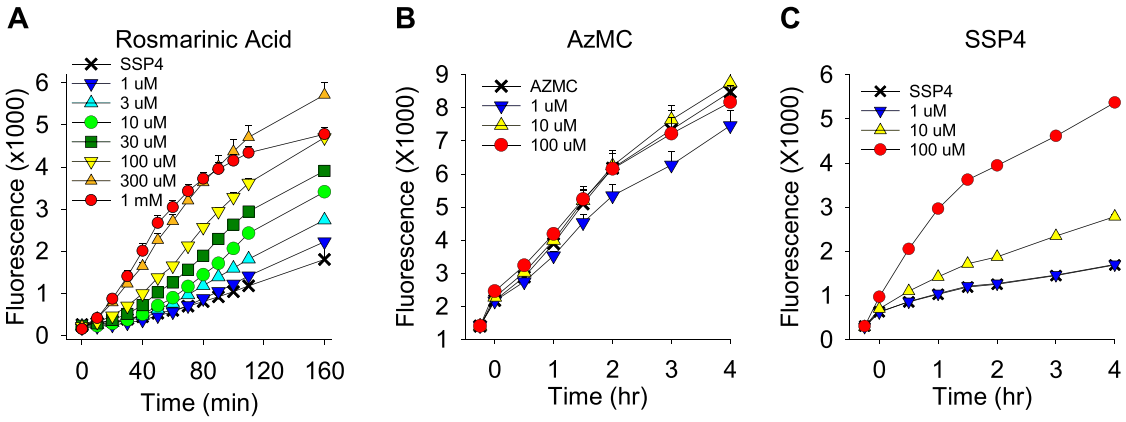

Fig. 4. Effects of rosmarinic acid (RA) on sulfur metabolism in buffer (**A**) and HEK293 cells (**B-C**). RA concentration-dependently increased polysulfide (SSP4 fluorescence) production from 300 μM H_2S in buffer (**A**). RA did not appreciably affect cellular H_2S (AzMC fluorescence, **B**), whereas it concentration-dependently increased polysulfides (SSP4 fluorescence, **C**). Mean + SE, n = 8 wells per treatment.

increased as the O_2 tension increased indicating that O_2 is required for H_2S oxidation.

3.7. Effects of quercetin at different O_2 tension on RSS metabolism in HEK293 cells

The above experiments suggest that an increase in O_2 tension in cells might also increase polysulfide production in cells treated with polyphenols. However, we have previously shown that a decrease in O_2 tension increases H_2S in HEK293 cells [33] and this would, conversely, promote polysulfide production in the presence of polyphenols. To resolve this issue we added quercetin to HEK293 cells incubated in either 21% O_2 or 5% O_2. As shown in Fig. 5B and C, 5% O_2 increased H_2S production, as we have shown previously, whereas quercetin appeared to decrease cellular H_2S at both O_2 tensions. Conversely, while 5% O_2 alone did not affect cellular polysulfide production the stimulatory effect of quercetin was significantly decreased in 5% O_2. This suggests that either the cellular redox state, which would be more oxidative at 21% O_2, has a greater effect on overall H_2S/polysulfide balance in the presence of quercetin, or that O_2 is involved in H_2S oxidation by quercetin in cells. Our experiments in buffer (section 3.6.) favor the latter hypothesis.

3.8. Effects of superoxide dismutase on quercetin oxidation of H_2S

Oxygen-mediated autoxidization of polyphenols produces superoxide and it is possible superoxide is responsible for oxidation of H_2S to polysulfides. To examine this possibility, we incubated quercetin with H_2S in the presence of superoxide dismutase (SOD) which should inhibit polysulfide production if superoxide was the H_2S oxidant. As shown in Fig. 6, SOD produced polysulfides from H_2S in the absence of quercetin, which we have shown previously [34]. SOD plus quercetin produced a further increase in polysulfide production, but this appeared to be solely a quantitatively additive effect of SOD and quercetin. These results suggest that superoxide is not involved in H_2S oxidation by quercetin. We could not examine the effects of catalase because of interference with SSP4.

3.9. Oxidation of H_2S to thiosulfate by polyphenols in buffer

We have previously shown that both polysulfides and thiosulfate are products of H_2S oxidation by catechin polyphenols in green tea [27]. In order to determine if thiosulfate production was a general property of polyphenols, we then examined thiosulfate production by cyanidin, quercetin, resveratrol and rosemarinic acid. H_2S alone and in the presence of cyanidin, quercetin, rosemarinic acid and resveratrol all decreased absorbance compared to AgNP alone indicative of thiosulfate production, however, there was no difference between H_2S alone and cyanidin and the effect of quercetion was only slightly, but significantly ($p < 0.05$) greater than that of H_2S. The effect of both rosmarinic acid and resveratrol was considerably greater (Fig. 7A). Fig. 7B shows the concentration of thiosulfate produced in these reactions and the percent H_2S converted to thiosulfate (assuming 2 mol of H_2S per 1 mol of thiosulfate) and the percent converted by the polyphenols corrected for H_2S alone are shown in Fig. 7C. Approximately 17% of the H_2S is autoxidized to thiosulfate and 0, 5, 18, and 35% of the H_2S is oxidized to thiosulfate by cyanidin, quercetion, rosmarinic acid and resveratrol, respectively.

To determine if thiosulfate production by polyphenols is oxygen dependent we then measured thiosulfate production by quercetin, rosmarinic acid and resveratrol in buffer bubbled with 100% O_2 or 100% N_2 (Fig. 7C and D, respectively). Thiosulfate production in 100% O_2 was similar to that in 21% O_2, whereas it was nearly completely inhibited in the absence of O_2. These results show that polyphenol oxidation of H_2S to thiosulfate requires O_2.

One-electron autoxidation of a polyphenol hydroquinone will produce a semiquinone radical and superoxide and a two-electron oxidation will produce a quinone and hydrogen peroxide. Any of these can potentially oxidize H_2S to thiosulfate. To determine if either superoxide or peroxide was involved in oxidation of H_2S, we measured thiosulfate production by H_2S and resveratrol in the presence of Cu/Zn superoxide dismutase (SOD) and catalase (Cat). As shown in Fig. 7E–G, SOD alone increased thiosulfate production, as we have shown previously [34], and although SOD further increased thiosulfate production in the presence of resveratrol, this appeared to be solely an additive effect. Catalase essentially prevented thiosulfate production from H_2S alone and from H_2S plus SOD but it did not substantially affect thiosulfate production by H_2S and resveratrol and only moderately (~30%) decreased the effect of SOD plus resveratrol.

We then examined the possibility that resveratrol, SOD or Cat indirectly affected the thiosulfate detection by AgNP (Fig. 7H). Resveratrol alone did not substantially affect AgNP absorbance nor did SOD affect the reaction of AgNP with thiosulfate. Cat partially inhibited the reaction between thiosulfate and AgNP with or without SOD. These results suggest that neither superoxide nor peroxide produced by autoxidation of resveratrol substantially contribute to thiosulfate production by resveratrol oxidation of H_2S. Furthermore, the ability of catalase to essentially completely inhibit thiosulfate formation from H_2S (Fig. 7E-G) with only minimal effects on the AgNP reaction with thiosulfate (Fig. 7H) suggests that a substantial fraction of thiosulfate produced by H_2S occurs in the reaction buffer during the experiment.

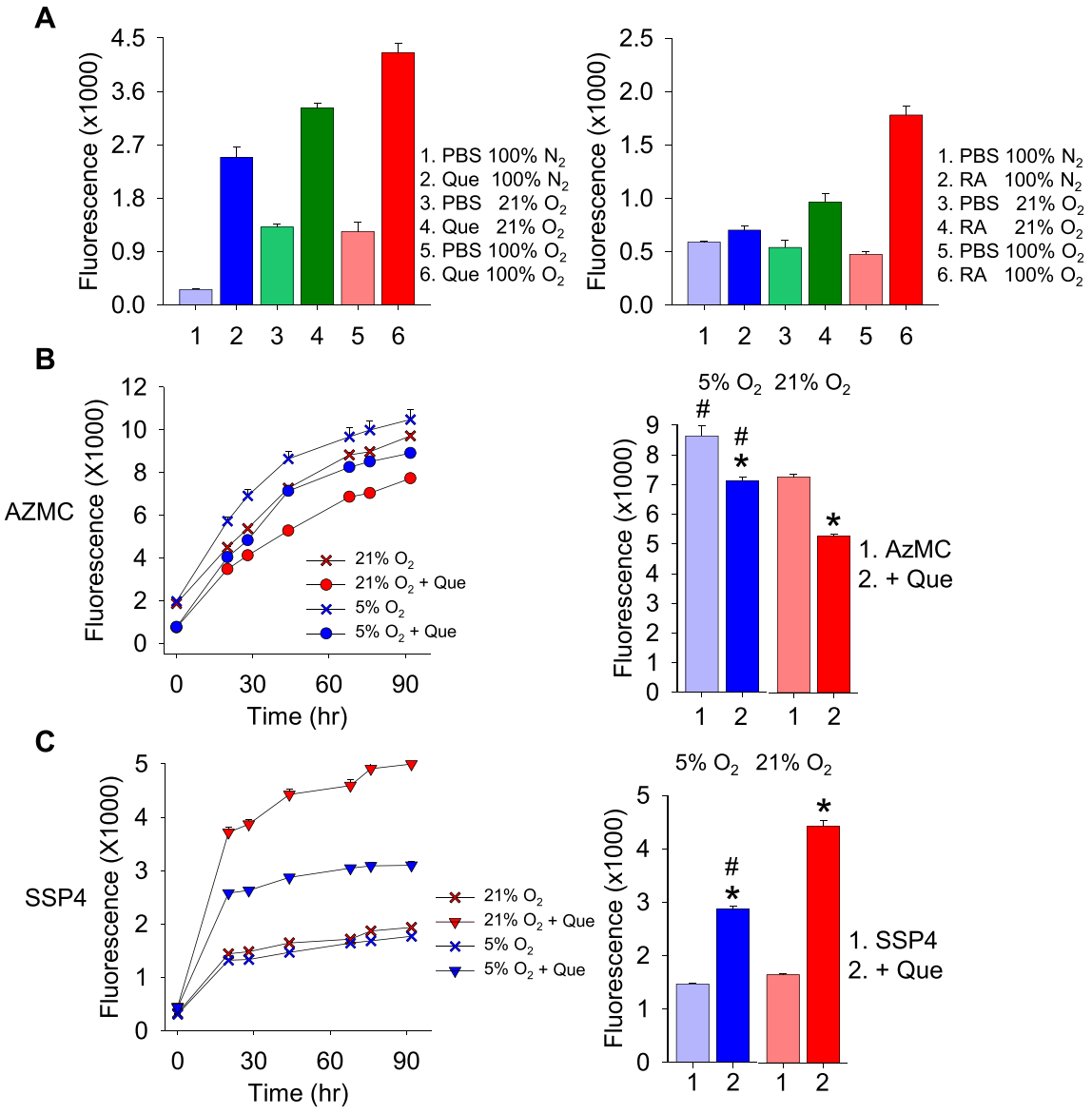

Fig. 5. Effects of oxygen on sulfur metabolism by polyphenols in buffer (**A**) or HEK293 cells (**B, C**). (**A**) 100 μM quercetin or 100 μM rosmarinic acid were incubated for 60 min in buffer previously bubbled with 100% N_2 or 100% O_2 or air equilibrated (21% O_2) PBS prior to addition of 300 μM H_2S and SSP4. Polysulfide production was increased by increasing O_2. Mean + SE, n = 4 wells per treatment; in each experiment the production at different O_2 tensions are significantly different from each other ($p < 0.002$). (**B, C**) Effects of 100 μM quercetin on H_2S (AzMC fluorescence) and polysulfides (SSP4 fluorescence) in HEK293 cells in 21% and 5% O_2. Line figures (left panels) show time course of fluorescence changes; right panels show fluorescence at 44 h. AzMC fluorescence was increased in untreated cells in 5% O_2. Quercetin decreased AzMC fluorescence at 44 h by 27% in cells in 21% O_2 and 17% in 5% O_2 cells. Oxygen tension did not affect SSP4 fluorescence in untreated cells. Quercetin increased SSP4 fluorescence at both O_2 tensions and the effects were greater in cells in 21% O_2. Mean + SE, n = 8 wells per treatment; *, different from respective control ($p < 0.001$); #, different from respective treatment at 21% O_2 ($p < 0.001$).

3.10. Polyphenols as reductants of oxidized sulfur

In order to determine if the reduced polyphenols could reduce oxidized forms of sulfur we measured H_2S production in the absence of oxygen with either the H_2S-sensitive fluorophore, AzMC, or an amperometric H_2S sensor. When incubated with K_2S_n, quercetin slightly decreased and resveratrol slightly increased AzMC fluorescence, but there was little evidence of a general production of H_2S (Supplemental Fig. S5A), whereas fluorescence was decreased by all polyphenols when incubated with thiosulfate (Supplemental Fig. 5B). When H_2S production was measured with the amperometric sensor, the polyphenols produced less than 2 μM H_2S from either 50 μM K_2S_n or 50 μM thiosulfate (Supplemental Figs. 5C and D). These results suggest that polyphenols do not readily reduce oxidized inorganic sulfur.

4. Discussion

4.1. Support for nutraceutical actions via polysulfide signaling

The vast majority of the health benefits of dietary phytochemicals have been attributed to their antioxidant, free radical-scavenging properties, although there is a general agreement that the mechanism (s) are yet to be fully elucidated. Foreman et al. [35] provided a compelling argument that the free radical scavenging hypothesis is

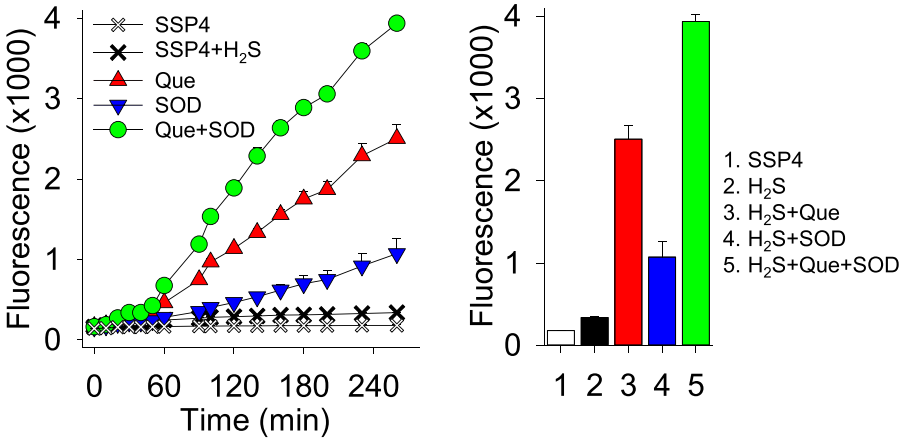

Fig. 6. Effects of 1 μM Cu/Zn superoxide dismutase (SOD) on polysulfide production (SSP4 fluorescence) by 30 μM quercetin (Que) oxidation of 300 μM H_2S. SOD directly forms polysulfides from H_2S and this is additive to the effect of Que. Bar graph shows results at 260 min. Mean + SE, n = 4 wells per treatment.

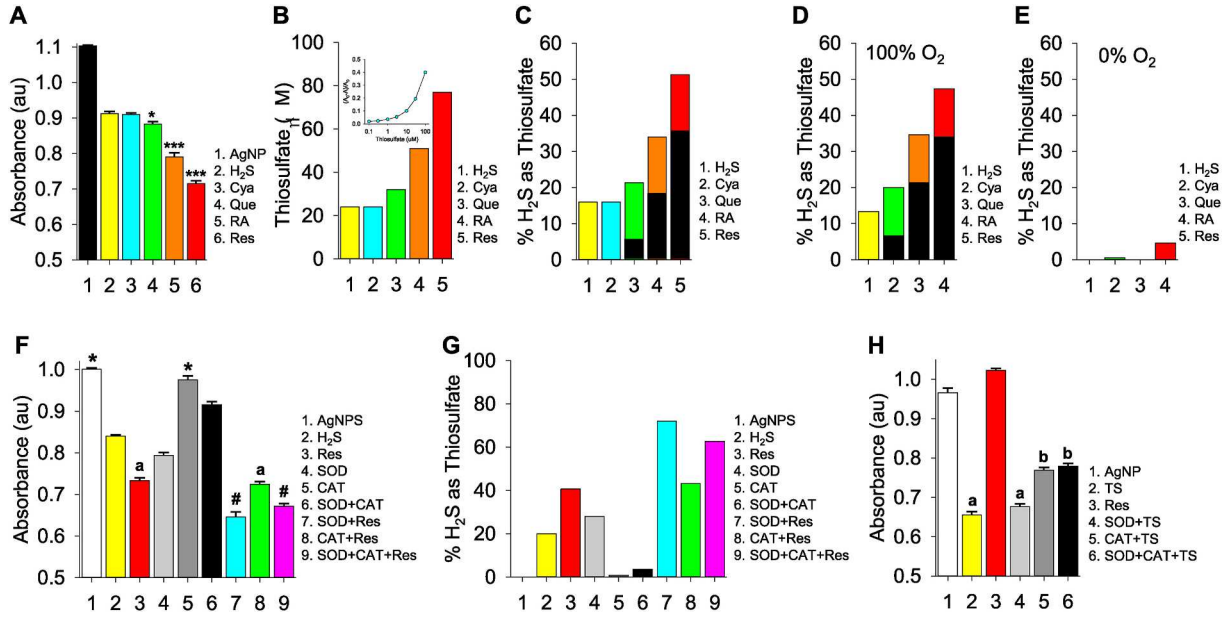

Fig. 7. Effects of polyphenols on oxidation of H_2S to thiosulfate. (**A**) Absorbance of AgNPs alone, AgNPs plus 300 μM H_2S (H_2S) and H_2S with 100 μM cyanadin (Cya), 100 μM quercetin (Que), 100 μM rosmarinic acid (RA) and 100 mg/L resveratrol (Res) in 21% O_2. All compounds significantly decreased absorbance compared to AgNPs alone ($p < 0.001$) and all but cyanidin decreased absorbance compared to H_2S (mean + SE, n = 8 wells per treatment; *, $p < 0.05$; ***, $p < 0.001$ vs H_2S). (**B**) Concentration of thiosulfate produced in these reactions; values derived from standard curve (inset). (**C**) Percent H_2S oxidized to thiosulfate assuming 2 moles of H_2S per mole thiosulfate, colored bars show total percent oxidzed, black bars are corrected for thiosulfate in H_2S. (**D**) 100 %O_2 does not affect thiosulfate production by Que, RA and Res, whereas it is inhibited in 0% O_2 (**E**), black bars are corrected for thiosulfate in H_2S. (**F, G**) Effects of 0.1 μM superoxide dismutase (SOD) and 0.1 μM catalase (Cat) on thiosulfate production from 300 μM H_2S and 100 mg/L resveratrol (Res). Neither enzyme substantially affected resveratrol oxidation of H_2S, whereas Cat inhibited autoxidation of H_2S with or without SOD. Mean + SE, n = 8 wells per treatment; **a**, no difference between like letters; * and #, $p < 0.05$ between like symbols; all other comparisons $p < 0.001$). (**H**) Effects of 100 mg/L resverstrol (Res) on AgNP and effects of 0.1 μM SOD and Cat on AgNP reaction with 100 μM thiosulfate. Res did not react with AgNP, SOD did not affect reaction between AgNP and thiosulfate and Cat partially decreased reaction between AgNP and thiosulfate with or without SOD. Mean + SE, n = 8 wells per treatment; **a**, **b**, no difference between like letters; all other comparisons $p < 0.001$).

kinetically unrealistic and that the cellular polyphenol concentration is not sufficient to react with all the free radicals being produced. Instead, they propose that the phytochemicals help maintain 'nucleophilic tone' by inducing protective phase II enzymes and increasing levels of nucleophilic substrates such as glutathione, thioredoxin and NADPH. They argue that the polyphenol is initially oxidized to the semiquinone or quinone by the free radicals and the semiquinone then directly conjugates Keap1, thereby freeing Nrf2. In fact, tea catechins, which are structurally similar to berry anthocyanins and flavonoids, have been shown to form covalent bonds with thiols via the 2′ carbon in the B ring [36].

Results from our experiments show that polyphenols oxidize H_2S and produce polysulfides in both cell-free buffer and in cells. Polysulfides are effective antioxidants [19] and, perhaps more relative to the hypothesis

of Forman et al. [37] these polysulfides will directly persulfidate cysteine in Keap1, causing it to dissociate from Nrf2. Nrf2 then activates the nuclear antioxidant response elements (ARE) which produces antioxidant responses identical to those initiated by peroxide and oxidative stress [21–26].

We have recently shown that tea catechin polyphenols also produce polysulfides from H_2S and proposed that this would ultimately activate Nrf2. We also showed that this was a catalytic reaction that relied on an initial autoxidation of the catechin. The present studies suggest that the berry extracts and associated polyphenols also metabolize H_2S and that this is a common property of biologically active polyphenols. Collectively, our results provide an alternate metabolic mechanism to explain the biological effects of these nutraceuticals.

4.2. Mechanism of H_2S oxidation

The chemical structures of polyphenols used in this study and those of select green tea catechins are shown in Fig. 8. Although an exhaustive

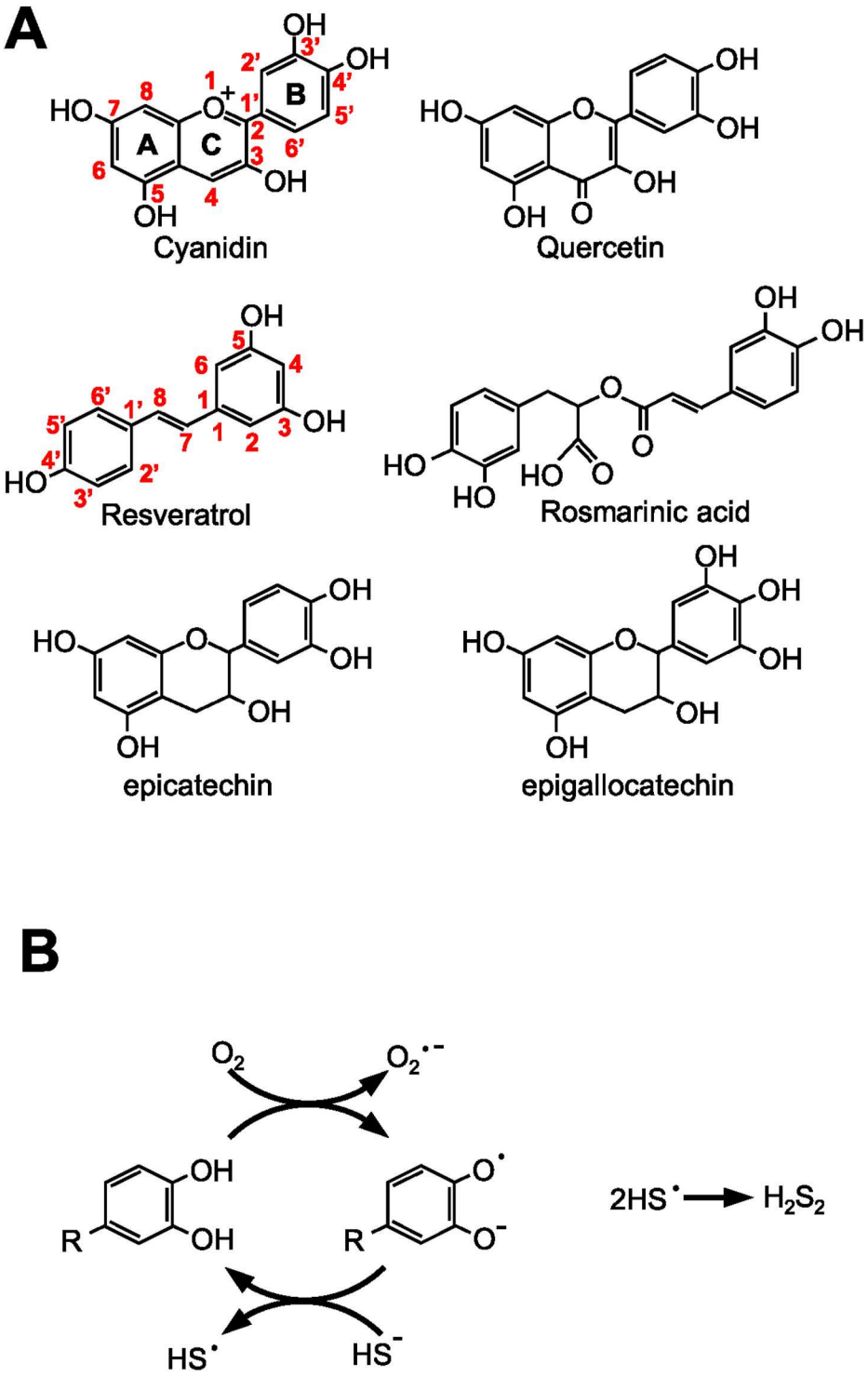

Fig. 8. (**A**) Structures of phenolic compounds used in this study and two green tea catechins. (**B**) Proposed scheme for H_2S oxidation by polyphenols. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

examination of the reactions involved in polyphenol-catalyzed oxidation of H_2S to polysulfides and thiosulfate was beyond the focus of this study, our experiments with varying oxygen tensions, SOD and catalase suggest a few general possibilities.

We propose that H_2S oxidation requires an initial autoxidation of the polyphenol dihydroquinine to a semiquinone radical concomitantly reducing oxygen to superoxide ($O_2^{\bullet-}$; eq. (1)).

$$BOH + O_2 \rightarrow BO^{\bullet} + O_2^{\bullet-} + H^+ \quad \text{(eq. 1)}$$

Both superoxide and hydrogen peroxide could potentially oxidize H_2S to polysulfides and thiosulfate [20,38]. However, the lack of effect of either SOD or catalase suggests that neither superoxide nor hydrogen peroxide are involved in H_2S oxidation by the polyphenols used in our study. Instead, we propose that the semiquinone radicals produced by oxidation of the hydroxypolyphenols are the H_2S oxidants and that they oxidize a hydrosulfide anion to a thiyl radical (eq. (2)); and two thiyl radicals can combine to form a persulfide (eq. (3)); or a thiyl radical can combine with a hydrosulfide anion to form a persulfide radical (eq. (4));

$$BO^{\bullet} + HS^- \rightarrow HS^{\bullet} + BOH \quad \text{(eq. 2)}$$

$$2HS^{\bullet} \rightarrow H_2S_2 \quad \text{(eq. 3)}$$

$$HS^{\bullet} + HS^- \rightarrow HS_2^{\bullet-} + H^+ \quad \text{(eq. 4)}$$

If the starting compound is a quinone (BO), then it will oxidize HS^- producing a thiyl radical and the semiquinone radical, the latter can then react with a second HS^- to form a second thiyl radical and the fully reduced hydroquinone (eqs. 5, 6);

$$HS^- + BO \rightarrow HS^{\bullet} + BO^{\bullet} \quad \text{(eq. 5)}$$

$$HS^- + BO^{\bullet} + H^+ \rightarrow HS^{\bullet} + BOH \quad \text{(eq. 6)}$$

Our experiments also suggest that either the thiyl or persulfide radical then reacts with O_2 to produce thiosulfate through an as yet unidentified reaction as discussed below.

Autoxidation of H_2S to thiosulfate is oxygen dependent but different from polyphenol-catalyzed reactions. The initial reaction (eq. (7)) is well known [39,40];

$$2HS^- + 2O_2 \rightarrow S_2O_3^{2-} + H_2O \quad \text{(eq. 7)}$$

As catalase appears to inhibit this reaction, it suggests that H_2S is actually reacting with peroxide (H_2O_2) which would be an initial two-electron reduction of O_2 [38], presumably catalyzed by a transition metal impurity such as Fe (eqs. 8, 9);

$$HS^- + H_2O_2 \rightarrow HSOH + OH^- \quad \text{(eq. 8)}$$

where HSOH is the reactive and unstable sulfenic acid formed from the reaction of atomic sulfur and water [41–44]. The sulfenic acid is then oxidized to sulfurous acid (eq. (9));

$$HSOH + O_2 \rightarrow H_2SO_3 \quad \text{(eq.9)}$$

followed by reaction with a second sulfenic acid to form thiosulfate (eq. (10)),

$$HSOH + H_2SO_3 \rightarrow H_2S_2O_3 + H_2O \quad \text{(eq.10)}$$

We have previously shown that sulfurous acid (sulfite), is produced by green tea oxidation of H_2S but it rapidly disappears [27]. Whether this results in formation of thiosulfate as in eq. (9), or sulfur dioxide (SO_2), which is another biologically relevant signaling molecule, is not known.

The initial peroxide formation would be derived from the two-electron oxidation of hydrogen sulfide (eq. (11));

$$2H_2S + O_2 \rightarrow H_2S_2 + H_2O_2 \quad \text{(eq. 11)}$$

The reaction of hydrogen sulfide with molecular oxygen is thermodynamically favorable but a spin-forbidden reaction [45] and the reaction in absence of any catalyst is very slow. However, in presence of redox active metals the reaction is greatly accelerated even though in some reactions the metal ion need not undergo oxidation-reduction cycles [46]. Thus, if a transition metal is present in our reaction solutions, direct formation of peroxide from the reaction of eq. (11) might occur. An alternate pathway to formation of peroxide is the more conventional reaction of hydrogen sulfide with oxygen in the presence of redox active metals ions such as ferric or cupric ions (eq. 12, 13); and peroxide is generated by the spontaneous disproportionation of superoxide.

$$HS^- + M^n \rightarrow HS^{\bullet} + M^{n-1} \quad \text{(eq. 12)}$$

$$M^{n-1} + O_2 \rightarrow M^n + O_2^{\bullet-} \quad \text{(eq. 13)}$$

The subsequent reactions beginning with eq. (8) proceed to make thiosulfate as we observed in our reactions of hydrogen sulfide with oxygen without polyphenols. However, whether with or without polyphenols, polysulfides are generated on the reaction pathway to thiosulfate, and our data indicate that peroxide is a required intermediate only for the reaction in absence of polyphenols. In the presence of polyphenols hydrogen peroxide is not needed as seen in eq. (2) and eq. (6) where the direct oxidation of the polyphenols reacting with hydrogen sulfide results in redox cycling of the polyphenol and the production of the thiyl radical and superoxide radical anion. Once the thiyl radical is formed then it can react with the hydrosulfide anion to make a radical disulfide anion (see eq. (4)) which in turn reacts with molecular oxygen to make a polysulfide and another superoxide anion (eq. (14));

$$H_2S_2^{\bullet-} + O_2 \rightarrow H_2S_2 + O_2^{\bullet-} \quad \text{(eq. 14)}$$

In the presence of H_2S, polysulfides form an equilibrium distribution of polysulfides, sulfide and elemental sulfur [47] and, as in eq. (15);

$$H_2S_2 + H_2O \rightarrow H_2S + HOSH \quad \text{(eq. 15)}$$

where HOSH is the reactive sulfenic acid as a result of the formation of sulfur in aqueous solution. Thiosulfate would then be made as in eq. 9 and 10.

4.3. Autoxidation of polyphenols

Although we did not attempt to quantitatively compare efficacy of polysulfide production by the polyphenols used in this study, it can be seen from Figs. 1 and 4 that at 160 min and with 10 μM polyphenol the relative potency is; quercetin > cyanidin > resveratrol > rosmarinic acid. This is different from the potency for thiosulfate production; resveratrol > rosmarinic acid > quercetin > cyanidin (Fig. 7). Cyanidin, quercetin and rosmarinic acid are ortho-dihydroxybenzenes, whereas resveratrol is a 3,5,4′-trihydroxy molecule with only one meta-hydroxyl group.

Autoxidization of ortho-hydroxybenzenes will produce semiquinone radicals [48,49], whereas meta-hydroxybenzenes are stable and do not autoxidize [48]. In fact, Shingai et al. [50] reported that less than 50% of resveratrol was autoxidized after 20 days in 21% O_2 and 50 μM $FeCl_3$. However, Prysyazhna et al. [51] found that in phosphate buffer *trans*-resveratrol readily isomerized to *cis*-resveratrol and this was then autoxidized to a semiquinone radical and then to the quinone at the 4′ carbon. Resveratrol oxidation of H_2S is oxygen dependent (Fig. 7) as are the other polyphenols used in this study and it is likely that the semiquinone in the 3 position is involved [52]. Not surprisingly, resveratrol also activates Nrf2 in pulmonary endothelial cells [53] and in primary epidermal keratinocytes in which Nrf2 is activated and increased ROS production is seen especially in bicarbonate containing solutions [54]. Clearly the chemistry and biological actions of resveratrol are complicated but this molecule does react with hydrogen sulfide to make

polysulfides and this pathway may be responsible for part or even the majority of its biological effects as an antioxidant.

4.4. Polyphenols as sulfur reductants

It is well known that even though the sulfur in H_2S_2 is oxidized, H_2S_2 is a stronger reductant than H_2S [19]. However, H_2S can be regenerated from polysulfides or thiosulfate by dithiothreitol or other reductants [55,56]. We were unable to substantially reduce the mixed polysulfide, K_2S_n, with any polyphenol and regenerate more than a few percent of the sulfur as H_2S (Supplemental Fig. 5A, C). This could be that either the polyphenols aren't sufficiently strong reductants, or that they do not catalyze this reaction. To further evaluate this, we next reacted the polyphenols with thiosulfate, but, again, we failed to seen any evidence for substantial H_2S production (Supplemental Fig. 5B, D). Collectively, our results suggest that the polyphenols used in this study do not significantly reduce oxidized inorganic sulfur.

4.5. Conclusions

Our experiements show that nutraceutical polyphenols present in berries and spices are autoxidized enabling them to subsequently oxidize H_2S to polysulfides, thiosulfate and possibly other, as yet unidentified bioactive sulfur molecules. We also show that these polyphenols increase polysulfide production in cells. As it is well known that polysulfides effectively scavenge ROS and initiate downstream antioxidant responses, it seems likely that many of the health benefits attributed these compounds are mediated through their effects on sulfur metabolism.

A number of questions remain regarding the specific intermediates involved in the above reactions as well as the identity of other products that might also be produced both in buffer and in different cells. It is also important to evaluate these reactions in different cellular compartments where oxygen tensions vary. These, as well as details on the effector pathways activated by H_2S metabolites, will provide a better picture of the cytoprotective effects of nutraceuticals and provide the framework for a directed approach to more effacious synthetic analogs. Supplementing nutraceuticals with H_2S donors may further enhance their health benefits.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2021.01.035.

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