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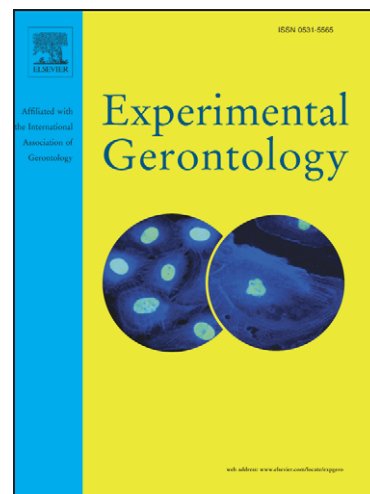
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4 **DCFH<sub>2</sub> interactions with hydroxyl radicals and other oxidants -**  
5 **influence of organic solvents.**  
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8 **Hans-Jürgen Brömme<sup>a</sup>, Leoni Zühlke<sup>b</sup>, Rolf-Edgar Silber<sup>b</sup>, Andreas Simm<sup>b</sup>**  
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**Abstract**

The oxidation of 2',7'-dichlorofluorescein (DCFH<sub>2</sub>) is widely used as a measure to detect the generation of reactive oxygen species (ROS) and to analyze oxidative stress. Other factors beside commonly known radicals may influence the results of such measurements. Therefore, the effects of H<sub>2</sub>O<sub>2</sub>, KMnO<sub>4</sub>, decomposition products of AAPH, ethanol and DMSO, antioxidants like ascorbic acid, different ferrous ion chelates, and heme-containing proteins like cytochrome c, myoglobin, hemoglobin, and horseradish-peroxidase were comparatively analyzed with respect to their impact on DCFH<sub>2</sub> oxidation. The study evaluates the effects of various oxidants with different oxidative potentials regarding their ability to induce DCF-fluorescence. Furthermore, we analyzed the inhibitory effect of organic solvents like ethanol or DMSO on the oxidation of DCFH<sub>2</sub> by hydroxyl radicals. The results of our study indicate that the potential of an oxidant does not always correlate with its efficiency to generate DCF-fluorescence.

## 1. Introduction

Free radical formation and oxidative stress play an important role in the etiology of several degenerative diseases (Halliwell and Cross 1994). Harman proposed 50 years ago that aging is the result of the accumulation of oxidative damage from radicals generated endogenously in cells during normal metabolism (Harman, 1956). Trying to find a partner for its lone electron, the free radical captures an electron from another molecule, which in turn becomes unstable and reacts with further molecules (Simm and Brömme 2005). Harmful molecules can arise which damage proteins, membranes, and nucleic acids, particularly DNA. There is a linkage between the age-dependent accumulation of oxidized enzymes and the loss of physiological function (Stadtman et al., 1992). By using models like transgenic mice and fruit-flies it has been shown that modification of the antioxidant defence mechanisms like the superoxide dismutase (SOD) can influence the ageing process and thereby modify the life span (Landis and Tower 2005). In contrast, radicals are an important physiological integral part of signaling pathways as well as of defense mechanisms of neutrophils and macrophages (Epling et al., 1992; Simm and Brömme 2005). Therefore, in many laboratories, including those, which investigate the impact of radical species on processes involved in the pathogenesis of degenerative diseases and in aging, the measurement of the formation of reactive oxygen species (ROS) is of increasing importance. The methods used should be easy to perform and valid regarding the determined reactive species. A frequently used method is the oxidation of the non-fluorescent probe 2',7'-dichlorodihydrofluorescein (DCFH<sub>2</sub>). DCFH<sub>2</sub> in its acetylated form (DCFH<sub>2</sub>-DA) can easily pass lipid membranes and after deacetylation by cellular esterases can be oxidized to form the fluorochrome 2',7'-dichlorofluorescein (DCF), which upon excitation at 480 nm emits light at 520 nm. This method has been widely used in flow cytometry, fluorescence microplate assays, or fluorescence microscopy (Silveira et al., 2003; Tada et al., 2004; Watanabe 1998). Originally, it has been assumed that DCF-fluorescence appears as a consequence of the formation of superoxide anion radicals or hydrogen peroxide

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4 and, therefore, has been regarded as a measure for the generation of this reactive oxygen  
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6 species (Al-Mehdi et al., 1998; Hempel et al., 1999; Kurose et al., 1997). Recently it became  
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8 obvious that oxidation of different fluorescent probes (including DCFH<sub>2</sub>) is less a result of  
9  
10 particular oxidizing species but rather of a generalized oxidative stress (Halliwell and  
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12 Whiteman 2004). The present investigation analyzes the influence as well as the individual  
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14 impact of different compounds on DCFH<sub>2</sub> oxidation and DCF fluorescence, examines the  
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16 influence of ethanol and DMSO as commonly used solvents for DCFH<sub>2</sub> regarding their effects  
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18 on generation of fluorescence induced by •OH, and interprets data concerning their influence  
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20 on cellular systems.  
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## 24 **2. Materials and methods**

### 25 *2.1. Material*

26  
27 2',7'-dichlorodihydrofluorescein diacetate (DCFH<sub>2</sub>-DA), ascorbic acid (AA), cytochrome c  
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29 (Cyt c) from equine heart, dimethylformamide (DMF), dimethylsulfoxide (DMSO),  
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31 ethylenediaminetetraacetic acid (EDTA), ferrous sulphate heptahydrate (FeSO<sub>4</sub> x 7 H<sub>2</sub>O),  
32  
33 ferric chloride hexahydrate (FeCl<sub>3</sub> x 6 H<sub>2</sub>O), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), myoglobin (Mb)  
34  
35 from equine heart, nitrilotriacetic acid (NTA), and potassium permanganate (KMnO<sub>4</sub>) were  
36  
37 purchased from Sigma (Taufkirchen, Germany), 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO)  
38  
39 was obtained from Alexis (Grünberg, Germany), Citric acid and Water (HPLC grade) were  
40  
41 from Merck (Darmstadt, Germany), hemoglobin (Hb), and peroxidase from horseradish (hr-  
42  
43 POD) were purchased from ICN Biomedicals GmbH (Eschwege, Germany). 2',2'-azobis-2-  
44  
45 methyl-propanimidamide, dihydrochloride (AAPH) was obtained from Polysciences Inc.  
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47 (Warrington, PA, USA). All other chemicals were obtained from Fluka (Taufkirchen,  
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49 Germany).  
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### 56 *2.2. In vitro deacetylation of DCFH<sub>2</sub>-DA*

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58 5 µL of DCFH<sub>2</sub>-DA (10 mmol/L dissolved in DMSO or DMF) and 45 µL of ethanol (96 %  
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60 v/v) were hydrolyzed in the presence of 200 µL of NaOH (10 mmol/L stock) and thereafter  
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4 neutralized with 1000  $\mu\text{L}$  of phosphate buffer (25 mmol/L stock). After neutralization, the  
5  
6 final concentration of DCFH<sub>2</sub> (total 1250  $\mu\text{L}$ ) amounted to 40  $\mu\text{mol/L}$ . The concentration of  
7  
8 DMSO and ethanol in the mixture was 56 and 616 mmol/L respectively.  
9

### 10 *2.3. Sample composition for fluorescence measurements*

11  
12 To 145  $\mu\text{L}$  of phosphate buffer according to Soerensen (27.4 parts KH<sub>2</sub>PO<sub>4</sub> 25 mmol/L and  
13  
14 72.6 parts Na<sub>2</sub>HPO<sub>4</sub> x 2 H<sub>2</sub>O, 25 mmol/L, pH 7.2) 40  $\mu\text{L}$  of DCFH<sub>2</sub> (40  $\mu\text{mol/L}$  stock), 5  $\mu\text{L}$   
15  
16 of H<sub>2</sub>O<sub>2</sub> (20 mmol/L stock) and 10  $\mu\text{L}$  of an oxidizing compound were added into 96 Well  
17  
18 Plates, Greiner, Omega Scientific (Venture, CA, USA). In the case of chemical oxidants, like  
19  
20 KMnO<sub>4</sub> or AAPH, hydrogen peroxide was not present in the sample. When hemoproteins  
21  
22 were used the composition of the sample was slightly different: To 150  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$   
23  
24 DCFH<sub>2</sub>, 5  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> and 5  $\mu\text{L}$  of either Cyt c, Mb, Hb or POD were pipetted. The control  
25  
26 consisted of 160  $\mu\text{L}$  of buffer and 40  $\mu\text{L}$  of neutralized DCFH<sub>2</sub>.  
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31 The fluorescence in the sample was monitored 2.5 min after starting DCFH<sub>2</sub>-oxidation in a  
32  
33 fully automated microplate based multi-detection reader FLUOstar OPTIMA from BMG  
34  
35 Labtech GmbH (Offenburg, Germany) using identical measuring conditions (excitation: 485  
36  
37 nm, emission: 520 nm, gain 1500, 25 °C).  
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### 40 *2.4. Sample composition for electron spin resonance (ESR)-spectroscopy*

41  
42 The blank (control) consisted of 80  $\mu\text{L}$  of water, 5  $\mu\text{L}$  of DMPO (1 mol/L stock), 10  $\mu\text{L}$  of  
43  
44 H<sub>2</sub>O<sub>2</sub> (20 mmol/L) and 5  $\mu\text{L}$  of Fe<sup>2+</sup> (0.5 mmol/L). When different organic solvents were  
45  
46 tested the composition of the mixture was: 67.5  $\mu\text{L}$  of buffer, 20  $\mu\text{L}$  of organic solvent  
47  
48 (DMSO, Ethanol, DMF), 2.5  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub>, 5  $\mu\text{L}$  of Fe<sup>2+</sup>, and 5  $\mu\text{L}$  of DMPO. In the presence  
49  
50 of organic solvents the mixture consisted of 60  $\mu\text{L}$  of buffer, 20  $\mu\text{L}$  of ethanol or DMSO, 5  
51  
52  $\mu\text{L}$  of DMPO, 10  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> and 5  $\mu\text{L}$  of Fe<sup>2+</sup>.  
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56 50  $\mu\text{L}$  of each mixture were immediately placed into disposable microtubes Brand GmbH  
57  
58 (Wertheim, Germany) and sealed carefully with plasticine. The tubes were then placed into  
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60 the cavity of an ESR-spectrometer Miniscope MS 100 Magnostech GmbH (Berlin, Germany).  
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4 Spectrometer settings were: modulation amplitude: 1 G, field scan: 100 G, scan time: 30 sec,  
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6 number of repeats: 5, B<sub>0</sub>-field: 3365 G, power attenuation: 12 dB.  
7

### 8 *2.5. Statistical analysis*

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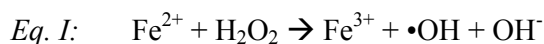
10 Data are expressed as mean ± standard deviation (SD) of at least 5 independent experiments.  
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12 For comparison of means the Student's *t*-test was applied. P-values less than 0.05 were  
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14 considered significant.  
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## 20 **3. Results**

21  
22 Several chemical oxidants, like e.g. alkylperoxyl radicals (decomposition products of AAPH  
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24 formed in the presence of oxygen) (Niki 1990) or KMnO<sub>4</sub> are able to induce a concentration-  
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26 dependent oxidation of DCFH<sub>2</sub>. Hydrogen peroxide, which is often used as a positive control  
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28 in cell culture experiments, oxidizes DCFH<sub>2</sub> in the absence of additional factors only to a very  
29  
30 small degree, if at all (Fig. 1A-C ). However, if ferrous ion chelates of NTA, EDTA or citrate  
31  
32 are present, DCFH<sub>2</sub> will be rapidly oxidized by H<sub>2</sub>O<sub>2</sub> (Fig. 2A). In the absence of H<sub>2</sub>O<sub>2</sub>, iron  
33  
34 chelates oxidize DCFH<sub>2</sub> only marginally (not shown). The oxidizing species in this type of  
35  
36 experiments appear to be hydroxyl radicals (•OH) which are formed by the FENTON reaction  
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38 according to equation I. ESR spectroscopy is the only method that allows a direct  
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40 measurement of radicals. Due to the short half-life of oxygen derived radicals their  
41  
42 monitoring needs the presence of a spin trap like DMPO. A diamagnetic spin trap can be  
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44 transformed by a given radical into a paramagnetic derivative or adduct, which can be  
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46 characterized by its individual ESR-spectrum. An ESR-spectrum corresponding to a •DMPO-  
47  
48 OH adduct indicating the formation of hydroxyl radicals can be seen in Figure 2B, trace b.  
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50 Without H<sub>2</sub>O<sub>2</sub> no •OH are generated und no •DMPO-OH adducts are formed (trace a).  
51  
52 Ethanol as well as DMSO prevent greatly the formation of such adducts (trace d, e). But, if  
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54 the spin trap (DMPO) is added after •OH has already interacted with ethanol and DMSO, the  
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56 formed carbon-centered radicals produce mixed adducts with DMPO (trace c). Figure 2C  
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4 shows the influence of ascorbate on DCFH<sub>2</sub> oxidation induced by •OH. Here ascorbate acts  
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6 predominantly as a pro-oxidant. According to equation III, ascorbate reduces the produced  
7  
8 ferric ions back into their catalytically active ferrous state. The surplus of the formed •OH  
9  
10 significantly increases the oxidation of DCFH<sub>2</sub> as seen by the increased fluorescence in the  
11  
12 presence of ascorbate.



22  
23 Heme-containing proteins like cytochrome c, myoglobin, hemoglobin, or peroxidases  
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25 produce much more DCF from DCFH<sub>2</sub> than the •OH-triggered oxidation of DCFH<sub>2</sub>. The  
26  
27 higher the concentration of the heme-containing protein the more intense will be the  
28  
29 fluorescence (Fig. 3A-D). Without H<sub>2</sub>O<sub>2</sub> no DCFH<sub>2</sub>-oxidation is observed (Fig. 3A).

#### 30 31 **4. Discussion**

32  
33 Hydroxyl radicals (•OH) belong to the most aggressive radicals known in biology (Buettner  
34  
35 1987; Halliwell and Gutteridge 1999; Mason et al., 1994). Their oxidizing potential is higher  
36  
37 than that of any other known cellular oxidant (Buettner 1993). Nevertheless, the ability of  
38  
39 •OH to oxidize DCFH<sub>2</sub> appears to be only modest when compared with the oxidizing  
40  
41 potential of KMnO<sub>4</sub>, AAPH derived alkylperoxyl radicals, and the oxidizing intermediates  
42  
43 originated from heme-containing proteins.

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47 How can this discrepancy be explained? The higher the oxidizing potential of a compound,  
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49 the higher will be its chance to react with any other electron donating compound in its  
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51 immediate vicinity. Although the composition of the reaction mixture is of low complexity,  
52  
53 nevertheless, beside DCFH<sub>2</sub> the sample contains some additional targets for •OH. The most  
54  
55 important of these supplementary targets are the organic solvents (ethanol and DMSO) in  
56  
57 which DCFH<sub>2</sub> is dissolved. From both solvents it is known that they are excellent scavengers  
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59 of •OH (Cederbaum et al., 1977; Heikkila 1977; Heikkila et al., 1974). This means that if such  
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4 additional targets are present, they compete with DCFH<sub>2</sub> for the •OH. The probability from  
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6 which target a hydrogen atom will be abstracted depends on the local concentrations of all  
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8 potential targets. The concentration ratio of ethanol, DMSO and DCFH<sub>2</sub> in the sample  
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10 amounts to 15400 : 1400 : 8 μmol/L. Therefore, DCFH<sub>2</sub> should not be the favored target for  
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12 •OH. Under the conditions applied, a maximum of 25 μmol/L •OH could be generated.  
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14 However, the trapping efficiency (the chemical yield of the diamagnetic spin trap converted to  
15  
16 the paramagnetic adduct) is normally less than 100 %. From DMPO it is known that its  
17  
18 trapping efficiency amounts to 33 % (Schwarz et al., 1997). A corresponding trapping  
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20 efficiency for DCFH<sub>2</sub> is not known. But it can be assumed that most of DCFH<sub>2</sub> present in the  
21  
22 sample should be oxidized by this strong oxidant. Assuming an approximately similar  
23  
24 electron donating potential for these three competing compounds, it can be expected that both  
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26 organic solvents must be the preferred targets for •OH. This is indeed substantiated by the  
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28 ESR experiments. As a result, the intensity of the •DMPO-OH adduct signal formed in the  
29  
30 presence of DMSO or ethanol is remarkably lower than the signal intensity in the absence of  
31  
32 organic solvents (see Fig. 2B). Regarding the preferred interaction of ethanol and DMSO with  
33  
34 •OH the low efficiency of •OH to oxidize DCFH<sub>2</sub> can therefore be understood. The low  
35  
36 efficiency of •OH to oxidize DCFH<sub>2</sub> was also reported by others (LeBel et al., 1992).  
37  
38 However, until now it remains unclear whether the carbon-centered radicals derived from the  
39  
40 •OH driven oxidation of organic solvents are able to participate also in the oxidation of  
41  
42 DCFH<sub>2</sub>.

43  
44 Under certain conditions the presence of ascorbate can intensify the formation of •OH from  
45  
46 H<sub>2</sub>O<sub>2</sub> and iron chelates (Udenfriend et al., 1954). Under these conditions ascorbate probably  
47  
48 acts as a pro-oxidant by reducing ferric ions back into their catalytically active state (redox-  
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50 cycling of iron ions at the expense of ascorbate, see equations I-III).  
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4 On the other hand, oxidants less potent than  $\bullet\text{OH}$ , like e.g.  $\text{KMnO}_4$  or alkylperoxyl radicals,  
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6 seem to be nearly ineffective in oxidizing DMSO or ethanol. Therefore they oxidize mainly  
7  
8 DCFH<sub>2</sub> and produce significantly more DCF-fluorescence.  
9

10 Heme-proteins, like cytochrome c (Burkitt et al., 2004; Burkitt and Wardman 2001; Lawrence  
11  
12 et al., 2003), myoglobin or hemoglobin, can act as pseudoperoxidases. This heme-containing  
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14 proteins, in general, lack substrate specificity. Hydrogen peroxide, organic peroxides, and  
15  
16 molecular oxygen all can serve as oxidizing agents, while the list of reducing agents is even  
17  
18 longer. The oxidizing entity of pseudoperoxidases appears to be *compound I*, a transiently  
19  
20 formed heme intermediate (Tarpey and Fridovich 2001). It is believed that *compound I*  
21  
22 consists of a ferryl ( $\text{Fe}^{\text{IV}}$ ) species and a porphyrin radical state of heme which abstracts  
23  
24 electrons from the reductant, thereby regenerating the catalytic activity of the  
25  
26 pseudoperoxidase (Cartoni et al., 2004; Goldman et al., 1998; Kawano et al., 2002). Under the  
27  
28 *in vitro* conditions applied, the only available reductant seems to be DCFH<sub>2</sub>. Therefore,  
29  
30 DCFH<sub>2</sub> is oxidized continuously by the pseudoperoxidases. The higher the amount of  
31  
32 pseudoperoxidases, the more DCFH<sub>2</sub> is oxidized per unit of time. Thus DCFH<sub>2</sub>-oxidation is  
33  
34 limited mainly by the availability of  $\text{H}_2\text{O}_2$ . Since in our batch natural substrates (cellular  
35  
36 reductants) for peroxidases were omitted, horse radish peroxidase (hr-POD) utilizes only  
37  
38 DCFH<sub>2</sub> as an artificial reductant. It might be expected that in the presence of e.g. GSH or  
39  
40 other reductive antioxidants, the rate of oxidation of DCFH<sub>2</sub> would decline significantly as  
41  
42 has been shown recently (Lawrence et al., 2003).  
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49 Which scenario can be expected in cells? Which oxidants could be able to oxidize DCFH<sub>2</sub> in a  
50  
51 cellular environment? Superoxide anion radicals ( $\bullet\text{O}_2^-$ ) and hydrogen peroxide are  
52  
53 continuously produced by mitochondria, by certain enzymes, as well as by some autoxidation  
54  
55 reactions. The steady state concentration of both ROS normally is kept low, since superoxide  
56  
57 dismutases, catalase, and several peroxidases minimize their availability. Nevertheless, in the  
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59 presence of “free” transition metals, FENTON chemistry always produces a certain amount of  
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4 •OH from H<sub>2</sub>O<sub>2</sub>, and •O<sub>2</sub><sup>-</sup> help to re-reduce Fe(III) to its catalytically active form (Fe(II)). But  
5  
6 neither •O<sub>2</sub><sup>-</sup> nor H<sub>2</sub>O<sub>2</sub> seem to have a potential high enough to induce DCFH<sub>2</sub>-derived  
7  
8 fluorescence. Both need additional factors in order to oxidize DCFH<sub>2</sub> (Burkitt and Wardman  
9  
10 2001). On the other hand, •OH possesses this potential, but the probability of oxidizing  
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12 DCFH<sub>2</sub> should be rather low, since many alternative cellular targets compete with DCFH<sub>2</sub> for  
13  
14 this highly reactive oxidant. Scheme 1 summarizes the different effectors for DCFH<sub>2</sub>  
15  
16 oxidation.  
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19  
20 The primary derivatives of an attack of •OH on cellular compounds will be mostly again  
21  
22 radicals. Some of them, like e.g. the alkylperoxyl radicals formed during lipid peroxidation,  
23  
24 are obviously able to oxidize DCFH<sub>2</sub> (see Fig. 1B). Other radicals, like the ascorbate-radical  
25  
26 or carbon-centered radicals, will probably miss this ability.  
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28

29 Interestingly, this scavenging reaction of ethanol against hydroxyl radicals may be a potential  
30  
31 reason for some positive effects of low amounts of ethanol on the life span seen in humans.  
32  
33 For example, it could be demonstrated that moderate alcohol consumption can clearly reduce  
34  
35 the risk of cardiovascular diseases in populations throughout the world (Rimm and Ellison  
36  
37 1995). The current recommendation from the British Heart Foundation is a maximum  
38  
39 consumption of 30 g ethanol per day (Preedy and Richardson 1994). Epidemiological studies  
40  
41 have shown the protective effect of modest alcohol consumption on the incidence of  
42  
43 cardiovascular diseases which account for the majority of death in Western world (Redmond  
44  
45 et al., 2000; Tolstrup et al., 2006). We could recently demonstrate that at a final concentration  
46  
47 of about 0.1 promille (17 mM), ethanol effectively prevents degradation or modification of  
48  
49 chemical compounds by hydroxyl radicals (Brömme et al., 2002; Brömme et al., 2008).  
50  
51 Moreover, when ethanol was dissolved in the perfusion buffer of isolated rat hearts to a final  
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53 concentration of 21.7 mM (which corresponds to little more than 0.1 promille) a significant  
54  
55 reduction of cell injury was observed after reoxygenation (Ashraf and Rahamathulla 1989).  
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4 On the other hand, when ethanol was administered in much higher quantities many harmful  
5 effects have been reported by various authors (Ribiere et al., 1992; Wieland and Lauterburg  
6 1995; Mansouri et al., 1999). Above 80 g ethanol per day undoubtedly alcohol has age-  
7 accelerating and life-shortening properties (Laufs and Böhm 2003; Renaud et al., 1998).

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11 In summary, any transient decrease in the concentration of antioxidants (reductants) as well as  
12 any simultaneous increase in the formation of ROS as well as some ROS derived organic  
13 radicals will increase DCFH<sub>2</sub>-oxidation and thus fluorescence. On the other hand, hydroxyl  
14 radicals preferentially oxidize the organic solvents in cell-free batches whereas in cells mainly  
15 compounds in their immediate vicinity widely outcompete DCFH<sub>2</sub>. From experiments using  
16 DCFH<sub>2</sub>, one can therefore not conclude that increasing DCF fluorescence is indeed a proof for  
17 an increase of a particular radical formation. In ageing research, it will also be a problem to  
18 compare the oxidative stress in young and old cells due to changing cellular amounts of  
19 proteins like for example heme containing proteins. Even the release of cytochrome c during  
20 apoptosis will stimulate the generation of DCF and thus dramatically enhance the intensity of  
21 fluorescence, if hydrogen peroxide is available. So we can suppose that a generalized  
22 oxidative stress originating from this transient imbalance promotes the oxidation of  
23 fluorogens, like DCFH<sub>2</sub>.

## 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 **References**

- 47  
48 Al-Mehdi, A.B., Zhao, G., Dodia, C., Tozawa, K., Costa, K., Muzykantov, V., Ross, C.,  
49 Blecha, F., Dinauer, M. and Fisher, A.B., 1998. Endothelial NADPH oxidase as the source  
50 of oxidants in lungs exposed to ischemia or high K<sup>+</sup>. *Circ. Res.* 83, 730-737.  
51 Ashraf, M. and Rahamathulla, P.M., 1989. Beneficial effects of low doses of ethanol on  
52 reoxygenation injury following anoxia in rat hearts. *Basic Res Cardiol* 84, 378-387.  
53 Brömme, H., Peschke, E. and Israel, G., 2008. Photo-Degradation of Melatonin: Influence of  
54 Argon, Hydrogen peroxide, and Ethanol. *J Pineal Res*, in press,  
55 Brömme, H.J., Mörke, W. and Peschke, E., 2002. Transformation of barbituric acid into  
56 alloxan by hydroxyl radicals: interaction with melatonin and with other hydroxyl radical  
57 scavengers. *J Pineal Res* 33, 239-247.  
58 Buettner, G., 1993. The pecking order of free radicals and antioxidants: lipid peroxidation,  
59 alpha-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* 300, 535-543.  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4 Buettner, G.R., 1987. Spin trapping: ESR parameters of spin adducts. *Free Radic. Biol. Med.*  
5 3, 259-303.
- 6 Burkitt, M. and Wardman, P., 2001. Cytochrome C is a potent catalyst of dichlorofluorescein  
7 oxidation: implications for the role of reactive oxygen species in apoptosis. *Biochem.*  
8 *Biophys. Res. Commun.* 282, 329-333.
- 9 Burkitt, M., Jones, C., Lawrence, A. and Wardman, P., 2004. Activation of cytochrome c to a  
10 peroxidase compound I-type intermediate by H<sub>2</sub>O<sub>2</sub>: relevance to redox signalling in  
11 apoptosis. *Biochem. Soc. Symp.* 97-106.
- 12 Cahill, A., Wang, X. and Hoek, J.B., 1997. Increased oxidative damage to mitochondrial  
13 DNA following chronic ethanol consumption. *Biochem Biophys Res Commun* 235, 286-  
14 290.
- 15 Cartoni, A., Menna, P., Salvatorelli, E., Braghiroli, D., Giampietro, R., Animati, F., Urbani,  
16 A., Del Boccio, P. and Minotti, G., 2004. Oxidative degradation of cardiotoxic anticancer  
17 anthracyclines to phthalic acids. Novel function of ferrylmyoglobin. *J. Biol. Chem.* 279,  
18 5088-5099.
- 19 Cederbaum, A.I., Dicker, E., Rubin, E. and Cohen, G., 1977. The effect of dimethylsulfoxide  
20 and other hydroxyl radical scavengers on the oxidation of ethanol by rat liver microsomes.  
21 *Biochem. Biophys. Res. Commun.* 78, 1254-1262.
- 22 Epling, C.L., Stites, D.P., McHugh, T.M., Chong, H.O., Blackwood, L.L. and Wara, D.W.,  
23 1992. Neutrophil function screening in patients with chronic granulomatous disease by a  
24 flow cytometric method. *Cytometry* 13, 615-620.
- 25 Goldman, D.W., Breyer, R.J., 3rd, Yeh, D., Brockner-Ryan, B.A. and Alayash, A.I., 1998.  
26 Acellular hemoglobin-mediated oxidative stress toward endothelium: a role for ferryl iron.  
27 *Am. J. Physiol.* 275, H1046-1053.
- 28 Halliwell, B. and Cross, C.E., 1994. Oxygen-derived species: their relation to human disease  
29 and environmental stress. *Environ. Health Perspect* 102 Suppl 10, 5-12.
- 30 Halliwell, B. and Whiteman, M., 2004. Measuring reactive species and oxidative damage in  
31 vivo and in cell culture: how should you do it and what do the results mean? *Br. J.*  
32 *Pharmacol.* 142, 231-255.
- 33 Heikkila, R.E., Barden, H. and Cohen, G., 1974. Prevention of alloxan-induced diabetes by  
34 ethanol administration. *J. Pharmacol. Exp. Ther.* 190, 501-506.
- 35 Heikkila, R.E., 1977. The prevention of alloxan-induced diabetes in mice by dimethyl  
36 sulfoxide. *Eur. J. Pharmacol.* 44, 191-193.
- 37 Hempel, S.L., Buettner, G.R., O'Malley, Y.Q., Wessels, D.A. and Flaherty, D.M., 1999.  
38 Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison  
39 with 2',7'-dichlorodihydrofluorescein diacetate, 5-(and 6)-carboxy-2',7'-  
40 dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. *Free Radic. Biol. Med.*  
41 27, 146-159.
- 42 Kawano, T., Muto, S., Adachi, M., Hosoya, H. and Lapeyrie, F., 2002. Spectroscopic  
43 evidence in support of horseradish peroxidase compound II-catalyzed oxidation of  
44 salicylic acid but not of phenylethylamine. *Biosci. Biotechnol. Biochem.* 66, 651-654.
- 45 Kurose, I., Higuchi, H., Miura, S., Saito, H., Watanabe, N., Hokari, R., Hirokawa, M.,  
46 Takaishi, M., Zeki, S., Nakamura, T., Ebinuma, H., Kato, S. and Ishii, H., 1997. Oxidative  
47 stress-mediated apoptosis of hepatocytes exposed to acute ethanol intoxication.  
48 *Hepatology* 25, 368-378.
- 49 Landis, G.N. and Tower, J., 2005. Superoxide dismutase evolution and life span regulation.  
50 *Mech Ageing Dev* 126, 365-379.
- 51 Laufs, U. and Böhm, M., 2003. Neues vom Alkohol-Optimum in der Prophylaxe von Herz-  
52 Kreislauf-Erkrankungen. *Herz* 28, 787-790.
- 53 Lawrence, A., Jones, C.M., Wardman, P. and Burkitt, M.J., 2003. Evidence for the role of a  
54 peroxidase compound I-type intermediate in the oxidation of glutathione, NADH,  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

- ascorbate, and dichlorofluorescein by cytochrome c/H<sub>2</sub>O<sub>2</sub>. Implications for oxidative stress during apoptosis. *J. Biol. Chem.* 278, 29410-29419.
- LeBel, C.P., Ischiropoulos, H. and Bondy, S.C., 1992. Evaluation of the probe 2',7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* 5, 227-231.
- Mansouri, A., Gaou, I., De Kerguenec, C., Amsellem, S., Haouzi, D., Berson, A., Moreau, A., Feldmann, G., Letteron, P., Pessayre, D. and Fromenty, B., 1999. An alcoholic binge causes massive degradation of hepatic mitochondrial DNA in mice. *Gastroenterology* 117, 181-190.
- Mason, R.P., Hanna, P.M., Burkitt, M.J. and Kadiiska, M.B., 1994. Detection of oxygen-derived radicals in biological systems using electron spin resonance. *Environ. Health Perspect* 102 Suppl 10, 33-36.
- Niki, E., 1990. Free radical initiators as source of water- or lipid-soluble peroxy radicals. *Methods Enzymol.* 186, 100-108.
- Preedy, V.R. and Richardson, P.J., 1994. Ethanol induced cardiovascular disease. *Br Med Bull* 50, 152-163.
- Redmond, E.M., Sitzmann, J.V. and Cahill, P.A., 2000. Potential mechanisms for cardiovascular protective effect of ethanol. *Acta Pharmacol Sin* 21, 385-390.
- Renaud, S.C., Gueguen, R., Schenker, J. and d'Houtaud, A., 1998. Alcohol and mortality in middle-aged men from eastern France. *Epidemiology* 9, 184-188.
- Ribiere, C., Hininger, I., Rouach, H. and Nordmann, R., 1992. Effects of chronic ethanol administration on free radical defence in rat myocardium. *Biochem Pharmacol* 44, 1495-1500.
- Rimm, E.B. and Ellison, R.C., 1995. Alcohol in the Mediterranean diet. *Am J Clin Nutr* 61, 1378S-1382S.
- Schwarz, P., Turro, N., Bossmann, S., Braun, A. and al, e., 1997. A new method to determine the generation of hydroxyl radicals in illuminated TiO<sub>2</sub> suspensions. *J. Phys. Chem. B* 101, 7127-7134.
- Silveira, L., Pereira-Da-Silva, L., Juel, C. and Hellsten, Y., 2003. Formation of hydrogen peroxide in rat skeletal muscle cells during contraction. *Free Radic. Biol. Med.* 35, 455-464.
- Simm, A. and Brömme, H.J., 2005. Reactive oxygen species (ROS) and aging: Do we need them - can we measure them - should we block them? *Signal Transductions* 3, 115-125.
- Stadtman, E.R., Starke-Reed, P.E., Oliver, C.N., Carney, J.M. and Floyd, R.A., 1992. Protein modification in aging. *Exs* 62, 64-72.
- Tada, Y., Mori, T., Shinogi, T., Yao, N., Takahashi, S., Betsuyaku, S., Sakamoto, M., Park, P., Nakayashiki, H., Tosa, Y. and Mayama, S., 2004. Nitric oxide and reactive oxygen species do not elicit hypersensitive cell death but induce apoptosis in the adjacent cells during the defence response of oat. *Mol. Plant Microbe Interact.* 17, 245-253.
- Tarpey, M.M. and Fridovich, I., 2001. Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circ. Res.* 89, 224-236.
- Tolstrup, J., Jensen, M.K., Tjønneland, A., Overvad, K., Mukamal, K.J. and Gronbaek, M., 2006. Prospective study of alcohol drinking patterns and coronary heart disease in women and men. *Bmj* 332, 1244-1248.
- Udenfriend, S., Clark, C.T., Axelrod, J. and Brodie, B.B., 1954. Ascorbic acid in aromatic hydroxylation. I. A model system for aromatic hydroxylation. *J. Biol. Chem.* 208, 731-739.
- Watanabe, S., 1998. In vivo fluorometric measurement of cerebral oxidative stress using 2'-7'-dichlorofluorescein (DCF). *Keio J. Med.* 47, 92-98.
- Wieland, P. and Lauterburg, B.H., 1995. Oxidation of mitochondrial proteins and DNA following administration of ethanol. *Biochem Biophys Res Commun* 213, 815-819.

## Legends to figures

### Figure 1 Influence of different oxidants on the development of DCF-fluorescence

- A.) Sample composition: 150  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  of DCFH<sub>2</sub> (40  $\mu\text{M}$  stock dissolved in DMSO and ethanol), and 10  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> (4 to 40 mM stock). DCF-fluorescence was monitored over 2.5 min at 25°C. Wavelength of excitation: 485 nm and of emission: 520 nm.
- B.) Sample composition: 155  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  of DCFH<sub>2</sub> (40  $\mu\text{M}$  stock), and 5  $\mu\text{L}$  of AAPH (1 M stock). AAPH decomposition, formation of alkylperoxyl radicals, and DCFH<sub>2</sub>-oxidation was monitored over 20 min at 25°C. Wavelength of excitation: 485 nm and of emission: 520 nm.
- C.) Sample composition: 155  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  of DCFH<sub>2</sub> (40  $\mu\text{M}$  stock), and 5  $\mu\text{L}$  of KMnO<sub>4</sub> (1 to 20  $\mu\text{M}$  stock). DCF-fluorescence was monitored over 2.5 min at 25°C. Wavelength of excitation: 485 nm and of emission: 520 nm.

### Figure 2 Effect of different iron-chelators on DCF-fluorescence and ESR adduct intensity

- A.) Sample composition: 145  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  of DCFH<sub>2</sub> (40  $\mu\text{M}$  stock), 5  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> (20 mM stock) and 10  $\mu\text{L}$  of either EDTA-, NTA-, Citrate-Fe(II) or -Fe(III) (0.5 mM stock). For more details see Fig. 1A.
- B.) Sample composition ESR-experiments:  
*Trace a:* 85  $\mu\text{L}$  of water, 5  $\mu\text{L}$  of DMPO (1 M stock), 10  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> (20 mM stock).  
*Trace b:* 80  $\mu\text{L}$  of water, 5  $\mu\text{L}$  of DMPO (1 M stock), 10  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> (20 mM stock), 5  $\mu\text{L}$  of Fe(II) (0.5 mM stock).  
*Trace c:* 67.5  $\mu\text{L}$  of water, 20  $\mu\text{L}$  DMSO and ethanol (without DCFH<sub>2</sub>), 2.5  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> (40 mM stock), 5  $\mu\text{L}$  of Fe(II) (0.5 mM stock). After 5 min 5  $\mu\text{L}$  of DMPO (1 M stock) was added.  
*Trace d:* 60  $\mu\text{L}$  of water, 5  $\mu\text{L}$  of DMPO (1 M stock), 20  $\mu\text{L}$  of ethanol (96 % v/v), 10  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> (20 mM stock), 5  $\mu\text{L}$  of Fe(II) (0.5 mM stock).  
*Trace e:* Like in d), but instead of ethanol 20  $\mu\text{L}$  of DMSO (99.5 % v/v) was present.  
For further details see Material and Methods.
- C.) Sample composition: 140  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  of DCFH<sub>2</sub> (40  $\mu\text{M}$  stock), 5  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> (20 mM stock), 10  $\mu\text{L}$  of citrate-Fe(II) or -Fe(III) (0.5 mM stock)  $\pm$  5  $\mu\text{L}$  of ascorbate (0.5 mM stock). For more details see Fig. 1A.



**Figure 3** Influence of different heme-containing proteins on DCF-fluorescence

- A.) Sample composition: 150  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  of DCFH<sub>2</sub> (40  $\mu\text{M}$  stock), 5  $\mu\text{L}$  of cytochrome c (5 or 10  $\mu\text{M}$  stock)  $\pm$  H<sub>2</sub>O<sub>2</sub> (20 mM stock).
- B.) Sample composition: 150  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  of DCFH<sub>2</sub> (40  $\mu\text{M}$  stock), 5  $\mu\text{L}$  of hr-POD (0.1, 0.25, 0.5  $\mu\text{M}$  stock), 5  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> (20 mM stock).
- C.) Sample composition: 150  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  of DCFH<sub>2</sub> (40  $\mu\text{M}$  stock), 5  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> (20 mM stock), 5  $\mu\text{L}$  of myoglobin (1 to 10  $\mu\text{M}$  stock).
- D.) Sample composition: 150  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  of DCFH<sub>2</sub> (40  $\mu\text{M}$  stock), 5  $\mu\text{L}$  of H<sub>2</sub>O<sub>2</sub> (20 mM stock), 5  $\mu\text{L}$  of hemoglobin (1 to 5  $\mu\text{M}$  stock).
- For further details see Fig. 1A.

**Scheme 1:** Reaction scheme of the oxidation of DCFH<sub>2</sub>

Figure 1

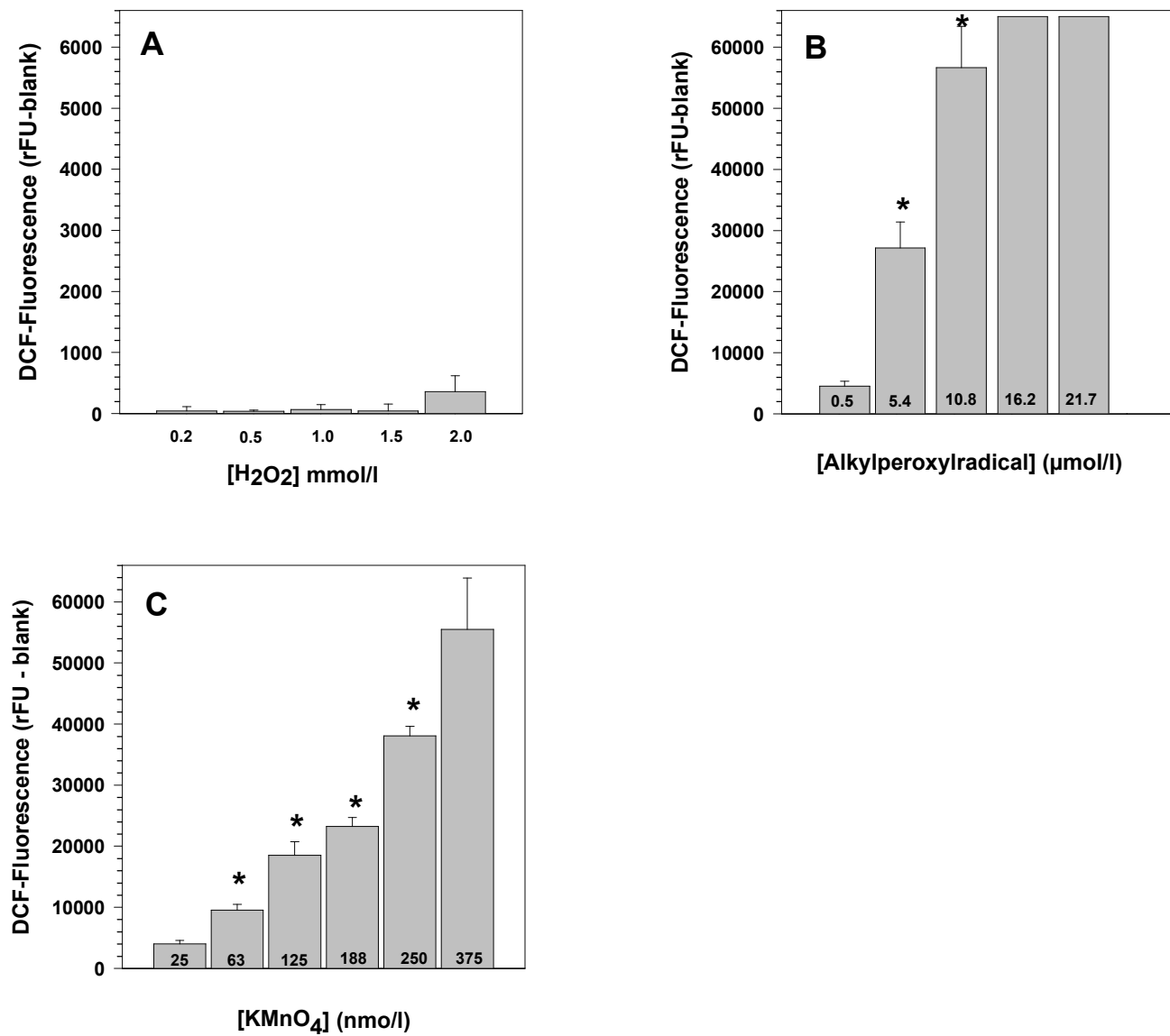


Figure 2

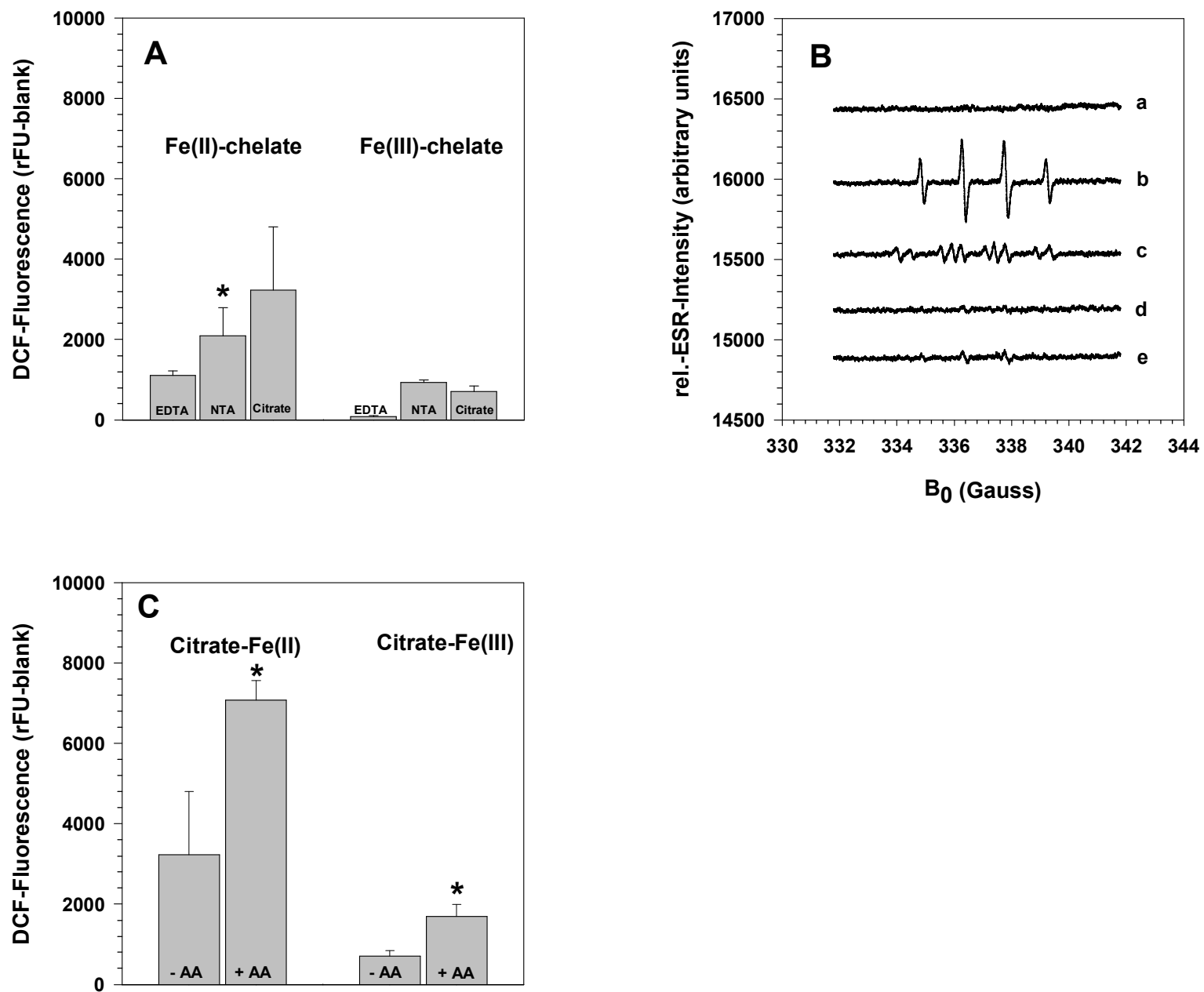


Figure 3

