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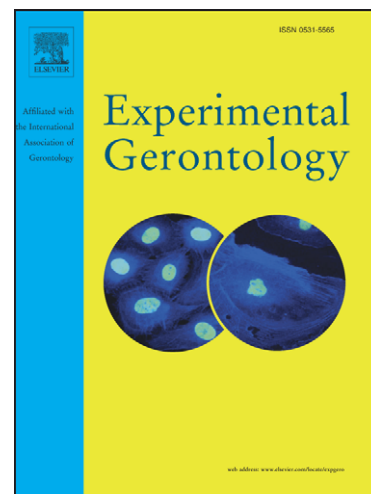
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**DCFH<sub>2</sub> interactions with hydroxyl radicals and other oxidants -  
influence of organic solvents.**

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**Keywords:** DCFH<sub>2</sub>; hydroxyl radicals; ethanol; dimethyl sulfoxide; hydrogen peroxide; heme-proteins

**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

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

## 2. Materials and methods

### 2.1. Material

2',7'-dichlorodihydrofluorescein diacetate (DCFH<sub>2</sub>-DA), ascorbic acid (AA), cytochrome c (Cyt c) from equine heart, dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), ferrous sulphate heptahydrate (FeSO<sub>4</sub> x 7 H<sub>2</sub>O), 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) from equine heart, nitrilotriacetic acid (NTA), and potassium permanganate (KMnO<sub>4</sub>) were purchased from Sigma (Taufkirchen, Germany), 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from Alexis (Grünberg, Germany), Citric acid and Water (HPLC grade) were from Merck (Darmstadt, Germany), hemoglobin (Hb), and peroxidase from horseradish (hr-POD) were purchased from ICN Biomedicals GmbH (Eschwege, Germany). 2',2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) was obtained from Polysciences Inc. (Warrington, PA, USA). All other chemicals were obtained from Fluka (Taufkirchen, Germany).

### 2.2. In vitro deacetylation of DCFH<sub>2</sub>-DA

5 µL of DCFH<sub>2</sub>-DA (10 mmol/L dissolved in DMSO or DMF) and 45 µL of ethanol (96 % v/v) were hydrolyzed in the presence of 200 µL of NaOH (10 mmol/L stock) and thereafter

neutralized with 1000  $\mu\text{L}$  of phosphate buffer (25 mmol/L stock). After neutralization, the final concentration of DCFH<sub>2</sub> (total 1250  $\mu\text{L}$ ) amounted to 40  $\mu\text{mol/L}$ . The concentration of DMSO and ethanol in the mixture was 56 and 616 mmol/L respectively.

### 2.3. Sample composition for fluorescence measurements

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 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  $\mu\text{L}$  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 Plates, Greiner, Omega Scientific (Venture, CA, USA). In the case of chemical oxidants, like KMnO<sub>4</sub> or AAPH, hydrogen peroxide was not present in the sample. When hemoproteins were used the composition of the sample was slightly different: To 150  $\mu\text{L}$  of buffer, 40  $\mu\text{L}$  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 consisted of 160  $\mu\text{L}$  of buffer and 40  $\mu\text{L}$  of neutralized DCFH<sub>2</sub>.

The fluorescence in the sample was monitored 2.5 min after starting DCFH<sub>2</sub>-oxidation in a fully automated microplate based multi-detection reader FLUOstar OPTIMA from BMG Labtech GmbH (Offenburg, Germany) using identical measuring conditions (excitation: 485 nm, emission: 520 nm, gain 1500, 25 °C).

### 2.4. Sample composition for electron spin resonance (ESR)-spectroscopy

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 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 tested the composition of the mixture was: 67.5  $\mu\text{L}$  of buffer, 20  $\mu\text{L}$  of organic solvent (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 of organic solvents the mixture consisted of 60  $\mu\text{L}$  of buffer, 20  $\mu\text{L}$  of ethanol or DMSO, 5  $\mu\text{L}$  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>.

50  $\mu\text{L}$  of each mixture were immediately placed into disposable microtubes Brand GmbH (Wertheim, Germany) and sealed carefully with plasticine. The tubes were then placed into the cavity of an ESR-spectrometer Miniscope MS 100 Magnettech GmbH (Berlin, Germany).

Spectrometer settings were: modulation amplitude: 1 G, field scan: 100 G, scan time: 30 sec, number of repeats: 5, B<sub>0</sub>-field: 3365 G, power attenuation: 12 dB.

### 2.5. Statistical analysis

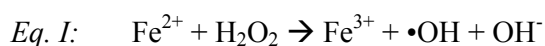
Data are expressed as mean  $\pm$  standard deviation (SD) of at least 5 independent experiments. For comparison of means the Student's *t*-test was applied. P-values less than 0.05 were considered significant.

## 3. Results

Several chemical oxidants, like e.g. alkylperoxyl radicals (decomposition products of AAPH formed in the presence of oxygen) (Niki 1990) or KMnO<sub>4</sub> are able to induce a concentration-dependent oxidation of DCFH<sub>2</sub>. Hydrogen peroxide, which is often used as a positive control in cell culture experiments, oxidizes DCFH<sub>2</sub> in the absence of additional factors only to a very small degree, if at all (Fig. 1A-C). However, if ferrous ion chelates of NTA, EDTA or citrate 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 chelates oxidize DCFH<sub>2</sub> only marginally (not shown). The oxidizing species in this type of experiments appear to be hydroxyl radicals ( $\bullet$ OH) which are formed by the FENTON reaction according to equation I. ESR spectroscopy is the only method that allows a direct measurement of radicals. Due to the short half-life of oxygen derived radicals their monitoring needs the presence of a spin trap like DMPO. A diamagnetic spin trap can be transformed by a given radical into a paramagnetic derivative or adduct, which can be characterized by its individual ESR-spectrum. An ESR-spectrum corresponding to a  $\bullet$ DMPO-OH adduct indicating the formation of hydroxyl radicals can be seen in Figure 2B, trace b. Without H<sub>2</sub>O<sub>2</sub> no  $\bullet$ OH are generated and no  $\bullet$ DMPO-OH adducts are formed (trace a). Ethanol as well as DMSO prevent greatly the formation of such adducts (trace d, e). But, if the spin trap (DMPO) is added after  $\bullet$ OH has already interacted with ethanol and DMSO, the formed carbon-centered radicals produce mixed adducts with DMPO (trace c). Figure 2C



shows the influence of ascorbate on DCFH<sub>2</sub> oxidation induced by •OH. Here ascorbate acts predominantly as a pro-oxidant. According to equation III, ascorbate reduces the produced ferric ions back into their catalytically active ferrous state. The surplus of the formed •OH significantly increases the oxidation of DCFH<sub>2</sub> as seen by the increased fluorescence in the presence of ascorbate.



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

#### 4. Discussion

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

How can this discrepancy be explained? The higher the oxidizing potential of a compound, the higher will be its chance to react with any other electron donating compound in its immediate vicinity. Although the composition of the reaction mixture is of low complexity, nevertheless, beside DCFH<sub>2</sub> the sample contains some additional targets for •OH. The most important of these supplementary targets are the organic solvents (ethanol and DMSO) in which DCFH<sub>2</sub> is dissolved. From both solvents it is known that they are excellent scavengers of •OH (Cederbaum et al., 1977; Heikkilä 1977; Heikkilä et al., 1974). This means that if such

additional targets are present, they compete with DCFH<sub>2</sub> for the •OH. The probability from which target a hydrogen atom will be abstracted depends on the local concentrations of all potential targets. The concentration ratio of ethanol, DMSO and DCFH<sub>2</sub> in the sample amounts to 15400 : 1400 : 8 µmol/L. Therefore, DCFH<sub>2</sub> should not be the favored target for •OH. Under the conditions applied, a maximum of 25 µmol/L •OH could be generated. However, the trapping efficiency (the chemical yield of the diamagnetic spin trap converted to the paramagnetic adduct) is normally less than 100 %. From DMPO it is known that its trapping efficiency amounts to 33 % (Schwarz et al., 1997). A corresponding trapping efficiency for DCFH<sub>2</sub> is not known. But it can be assumed that most of DCFH<sub>2</sub> present in the sample should be oxidized by this strong oxidant. Assuming an approximately similar electron donating potential for these three competing compounds, it can be expected that both organic solvents must be the preferred targets for •OH. This is indeed substantiated by the ESR experiments. As a result, the intensity of the •DMPO-OH adduct signal formed in the presence of DMSO or ethanol is remarkably lower than the signal intensity in the absence of organic solvents (see Fig. 2B). Regarding the preferred interaction of ethanol and DMSO with •OH the low efficiency of •OH to oxidize DCFH<sub>2</sub> can therefore be understood. The low efficiency of •OH to oxidize DCFH<sub>2</sub> was also reported by others (LeBel et al., 1992). However, until now it remains unclear whether the carbon-centered radicals derived from the •OH driven oxidation of organic solvents are able to participate also in the oxidation of DCFH<sub>2</sub>.

Under certain conditions the presence of ascorbate can intensify the formation of •OH from H<sub>2</sub>O<sub>2</sub> and iron chelates (Udenfriend et al., 1954). Under these conditions ascorbate probably acts as a pro-oxidant by reducing ferric ions back into their catalytically active state (redox-cycling of iron ions at the expense of ascorbate, see equations I-III).

On the other hand, oxidants less potent than  $\bullet\text{OH}$ , like e.g.  $\text{KMnO}_4$  or alkylperoxyl radicals, seem to be nearly ineffective in oxidizing DMSO or ethanol. Therefore they oxidize mainly DCFH<sub>2</sub> and produce significantly more DCF-fluorescence.

Heme-proteins, like cytochrome c (Burkitt et al., 2004; Burkitt and Wardman 2001; Lawrence et al., 2003), myoglobin or hemoglobin, can act as pseudoperoxidases. This heme-containing proteins, in general, lack substrate specificity. Hydrogen peroxide, organic peroxides, and molecular oxygen all can serve as oxidizing agents, while the list of reducing agents is even longer. The oxidizing entity of pseudoperoxidases appears to be *compound I*, a transiently formed heme intermediate (Tarpey and Fridovich 2001). It is believed that *compound I* consists of a ferryl ( $\text{Fe}^{\text{IV}}$ ) species and a porphyrin radical state of heme which abstracts electrons from the reductant, thereby regenerating the catalytic activity of the pseudoperoxidase (Cartoni et al., 2004; Goldman et al., 1998; Kawano et al., 2002). Under the *in vitro* conditions applied, the only available reductant seems to be DCFH<sub>2</sub>. Therefore, DCFH<sub>2</sub> is oxidized continuously by the pseudoperoxidases. The higher the amount of pseudoperoxidases, the more DCFH<sub>2</sub> is oxidized per unit of time. Thus DCFH<sub>2</sub>-oxidation is limited mainly by the availability of  $\text{H}_2\text{O}_2$ . Since in our batch natural substrates (cellular reductants) for peroxidases were omitted, horse radish peroxidase (hr-POD) utilizes only DCFH<sub>2</sub> as an artificial reductant. It might be expected that in the presence of e.g. GSH or other reductive antioxidants, the rate of oxidation of DCFH<sub>2</sub> would decline significantly as has been shown recently (Lawrence et al., 2003).

Which scenario can be expected in cells? Which oxidants could be able to oxidize DCFH<sub>2</sub> in a cellular environment? Superoxide anion radicals ( $\bullet\text{O}_2^-$ ) and hydrogen peroxide are continuously produced by mitochondria, by certain enzymes, as well as by some autoxidation reactions. The steady state concentration of both ROS normally is kept low, since superoxide dismutases, catalase, and several peroxidases minimize their availability. Nevertheless, in the presence of “free” transition metals, FENTON chemistry always produces a certain amount of

•OH from  $H_2O_2$ , and  $\bullet O_2^-$  help to re-reduce Fe(III) to its catalytically active form (Fe(II)). But neither  $\bullet O_2^-$  nor  $H_2O_2$  seem to have a potential high enough to induce DCFH<sub>2</sub>-derived fluorescence. Both need additional factors in order to oxidize DCFH<sub>2</sub> (Burkitt and Wardman 2001). On the other hand, •OH possesses this potential, but the probability of oxidizing DCFH<sub>2</sub> should be rather low, since many alternative cellular targets compete with DCFH<sub>2</sub> for this highly reactive oxidant. Scheme 1 summarizes the different effectors for DCFH<sub>2</sub> oxidation.

The primary derivatives of an attack of •OH on cellular compounds will be mostly again radicals. Some of them, like e.g. the alkylperoxyl radicals formed during lipid peroxidation, are obviously able to oxidize DCFH<sub>2</sub> (see Fig. 1B). Other radicals, like the ascorbate-radical or carbon-centered radicals, will probably miss this ability.

Interestingly, this scavenging reaction of ethanol against hydroxyl radicals may be a potential reason for some positive effects of low amounts of ethanol on the life span seen in humans. For example, it could be demonstrated that moderate alcohol consumption can clearly reduce the risk of cardiovascular diseases in populations throughout the world (Rimm and Ellison 1995). The current recommendation from the British Heart Foundation is a maximum consumption of 30 g ethanol per day (Preedy and Richardson 1994). Epidemiological studies have shown the protective effect of modest alcohol consumption on the incidence of cardiovascular diseases which account for the majority of death in Western world (Redmond et al., 2000; Tolstrup et al., 2006). We could recently demonstrate that at a final concentration of about 0.1 promille (17 mM), ethanol effectively prevents degradation or modification of chemical compounds by hydroxyl radicals (Brömme et al., 2002; Brömme et al., 2008). Moreover, when ethanol was dissolved in the perfusion buffer of isolated rat hearts to a final concentration of 21.7 mM (which corresponds to little more than 0.1 promille) a significant reduction of cell injury was observed after reoxygenation (Ashraf and Rahamathulla 1989).

On the other hand, when ethanol was administered in much higher quantities many harmful effects have been reported by various authors (Ribiere et al., 1992; Wieland and Lauterburg 1995; Mansouri et al., 1999). Above 80 g ethanol per day undoubtedly alcohol has age-accelerating and life-shortening properties (Laufs and Böhm 2003; Renaud et al., 1998).

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

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## 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

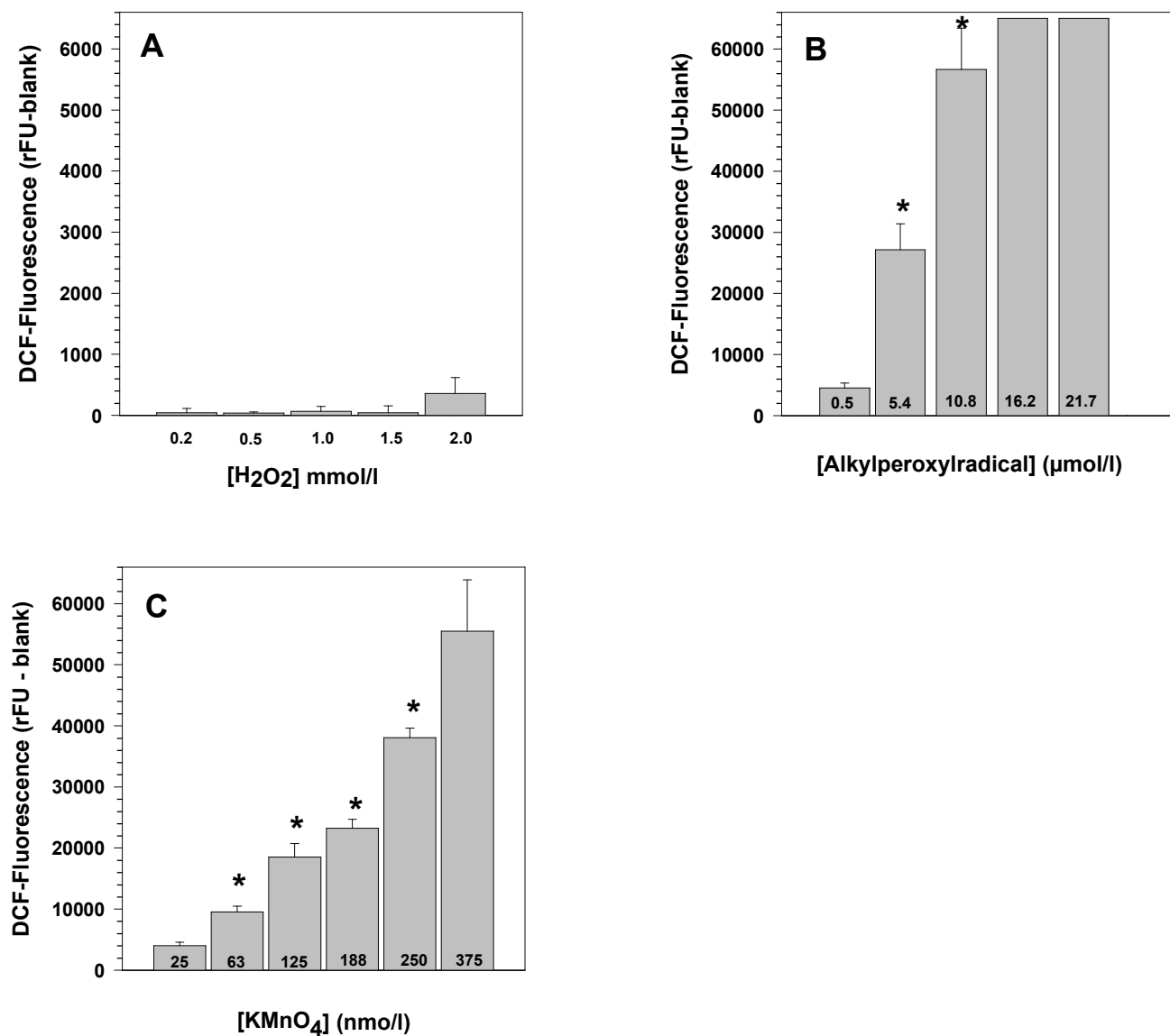


Figure 2

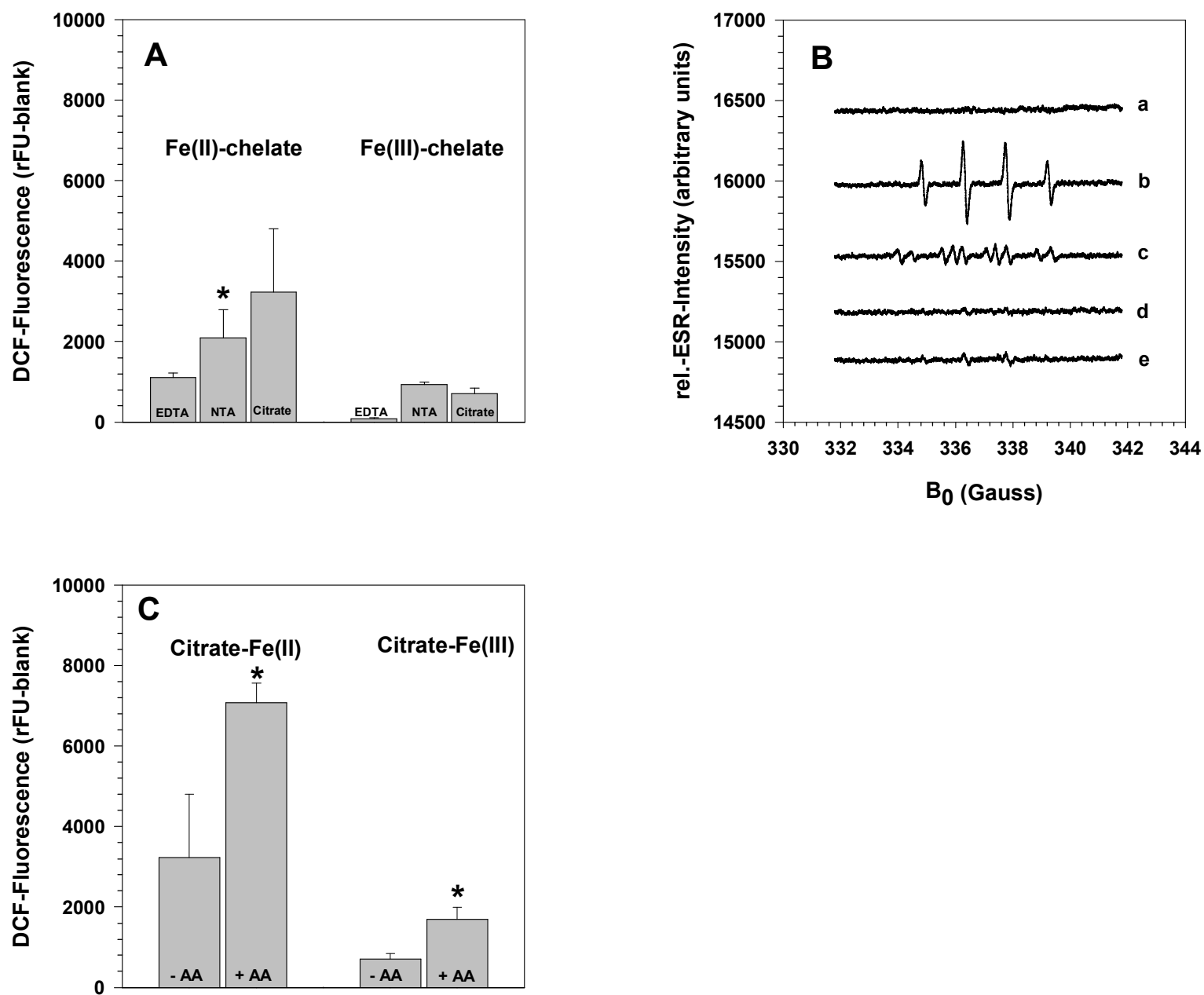


Figure 3

