Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 1575–1582
Commentary
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Evaluation of the probes 2′,7′-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation

Oddvar Myhre[a,b], Jannike M. Andersen[a], Halvor Aarnes[c], Frode Fonnum[a,*]

[a]*Norwegian Defence Research Establishment, Division for Protection and Materiel, P.O. Box 25, NO-2027 Kjeller, Norway*
[b]*VISTA (The Norwegian Academy of Science and Letters/Statoil), Oslo, Norway*
[c]*Department of Biology, University of Oslo, Oslo, Norway*

Abstract

This study attempts to provide a critical assessment of three different common approaches to identifying reactive species formed in biological systems: the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay, and the luminol- and lucigenin-amplified chemiluminescence assays. There have been several contradictory reports about the specificity of these methods. Our results show that DCFH is oxidized to the fluorescent compound 2′,7′-dichlorofluorescin (DCF) in human neutrophils exposed to the following compounds: Aroclor (A)1242, hydrogen peroxide (H_2O_2), nitric oxide (NO), and $FeSO_4$. Use of a cell-free DCFH system showed increased formation of DCF by peroxynitrite ($ONOO^-$), horseradish peroxidase (HRP) alone, and HRP in combination with H_2O_2, $FeSO_4$ alone, and a mixture of $FeSO_4$ and H_2O_2. The hydroxyl radical ($^{\bullet}OH$) scavenger formate and the iron ion chelator deferoxamine reduced the DCF formation induced by $FeSO_4$ in combination with H_2O_2. DCFH was insensitive to NO and H_2O_2 in the cell-free system. In the presence of neutrophils, the A1242-induced luminol chemiluminescence was decreased by the superoxide dismutase inhibitor diethyldithiocarbamic acid (DDC) and the myeloperoxidase inhibitor salicylhydroxamic acid (SHA). Exposure of the neutrophils to NO, $FeSO_4$, or H_2O_2 alone did not have any effect. A1242-induced lucigenin chemiluminescence in the neutrophils was increased slightly by DDC, but was not affected by SHA, NO, $FeSO_4$, or H_2O_2. In conclusion, we suggest that the DCF assay is only suitable for measurements of $ONOO^-$, H_2O_2 in combination with cellular peroxidases, and $^{\bullet}OH$. Luminol is sensitive towards HOCl, while lucigenin is oxidized by $O_2^{\bullet -}$.

Keywords: 2′,7′-Dichlorofluorescin diacetate; Luminol; Lucigenin; Neutrophils; Cell-free DCFH system

1. Introduction

In principle, oxidative stress could result from either reduced levels of antioxidants or increased production of reactive oxygen species and reactive nitrogen species (denoted RONS). Regardless of how and where they are generated, a rise in intracellular oxidant levels has potentially two important effects: damage to biological molecules and activation of specific signaling pathways. RONS have been implicated as an important causative factor in cell damage during inflammation, ischemia, cancer, and aging [1,2], as well as in the mechanisms of action of several environmental contaminants [3–8]. In specialized cell types involved in defense against invading microorganisms, NADPH oxidase and NOS have been identified as major sources of RONS. The reactive molecules produced include nitric oxide (NO), peroxynitrite ($ONOO^-$), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), as well as the hydroxyl radical ($^{\bullet}OH$). These various radical species can be generated exogenously or produced intracellularly from different sources. However, evaluation of the hazardous actions of these species has proved difficult due to their short life spans, so their individual roles in cell damage are still controversial. Thus, this calls for sensitive and specific methods for the measurement of RONS.

The fluorogenic compound DCFH-DA has been utilized extensively as a marker for oxidative stress, and is suggested to reflect the overall oxidative status of the cell [9].

* Corresponding author. Tel.: +47-63807803; fax: +47-63807509.
E-mail address: Frode.Fonnum@ffi.no (F. Fonnum).

Abbreviations: A1242, Aroclor 1242; DCF, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DDC, diethyldithiocarbamic acid; 3,4-DHBA, 3,4-dihydroxybenzoic acid; 4-HBA, 4-hydroxybenzoic acid; HBSS, Hanks' Balanced Salt Solution; HRP, horseradish peroxidase; NOS, nitric oxide synthase; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; SHA, salicylhydroxamic acid; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine.

0006-2952/03/$ – see front matter © 2003 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00083-2

However, conclusions drawn of the specificity of DCFH-DA are contradictory. DCFH-DA was initially described as an assay for the measurement of H_2O_2 in the presence of peroxidase [10]. HRP alone, and to a larger extent in combination with H_2O_2, is capable of oxidizing DCFH into DCF [11]. It is claimed that DCF formation is due to NO or H_2O_2 in cells or in cell-free systems [12–15]. Others have found that DCFH shows rather low sensitivity towards oxidation by NO, $O_2^{\bullet -}$, or H_2O_2, but displays a much greater sensitivity for $ONOO^-$ [16–18]. However, increased DCF formation is also suggested to implicate $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$ [19,20]. Furthermore, there are conflicting findings regarding the inhibition of DCF formation by superoxide dismutase [19,21–23].

Lucigenin and luminol are used frequently in studies of ROS produced by activated phagocytes. Lucigenin appears rather specific for $O_2^{\bullet -}$, but its specificity is questioned since the probe itself may act as a source of $O_2^{\bullet -}$ generation [24,25]. Also, $ONOO^-$ has been reported previously to induce lucigenin-amplified chemiluminescence [26]. Luminol seems to largely detect HOCl production. However, addition of $ONOO^-$ but not $^{\bullet}OH$ is also claimed to generate light from this probe [27,28], while others suggest that $^{\bullet}OH$ or $O_2^{\bullet -}$ may oxidize luminol [29–31].

It is important to know the limitations and strengths of oxidative stress sensitive probes. Therefore, the present work attempts to give a review of the specificity of DCFH, luminol, and lucigenin. In addition, we have supplemented the present knowledge about the specificity of these three probes by use of two model systems: human neutrophils *in vitro* and a cell-free DCFH system.

2. Identification of RONS in biological systems

One problem when dealing with the true biological consequences of the formation of short-lived RONS has been that they are difficult to measure *in vivo*. However, several ROS-trapping methods have been developed for use *in vitro*. In the present study, we have evaluated and discussed DCF-amplified fluorescence, and luminol- and lucigenin-amplified chemiluminiscence, since the specificity of these light-emission-enhancing compounds is controversial. We have supplemented the present knowledge about the specificity of these assays by the use of human neutrophil granulocytes *in vitro* and a cell-free DCFH assay as model systems. For the benefit of the reader, Fig. 1 summarizes pathways leading to the formation of

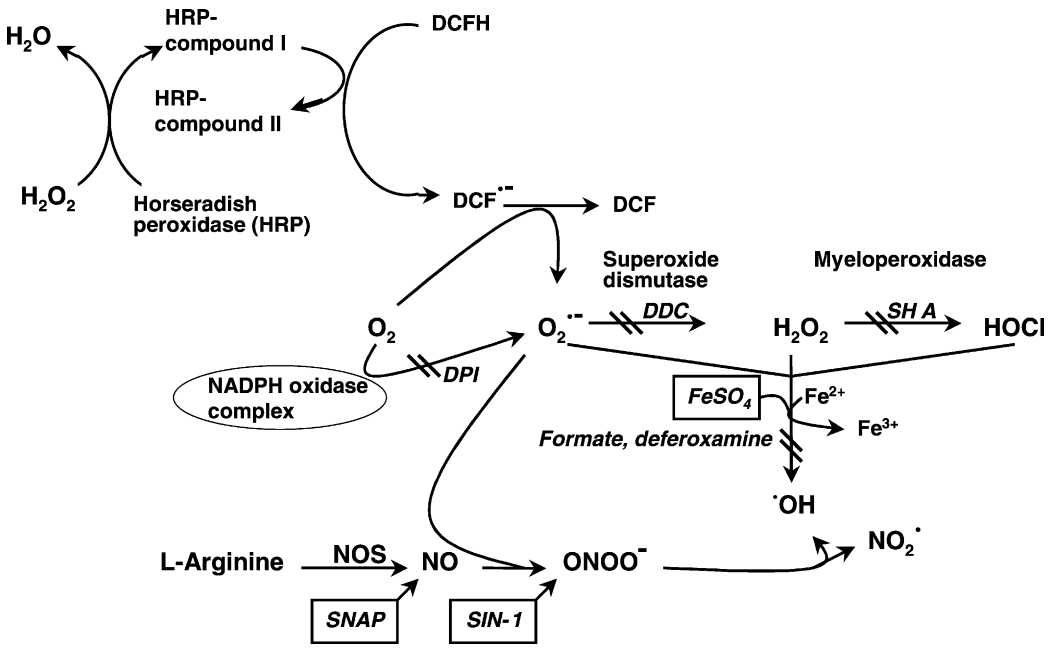


Fig. 1. Generation of RONS. Transfer of one electron to oxygen leads to the formation of multiple reactive species through the superoxide radical ($O_2^{\bullet -}$), which dismutates to hydrogen peroxide (H_2O_2), either spontaneously or by superoxide dismutase. Hypochlorous acid (HOCl) is generated enzymatically by myeloperoxidase in neutrophils. The reaction between horseradish peroxidase (HRP) and H_2O_2 (upper part) generates HRP-compound I, and then DCFH reduces HRP-compound I to HRP-compound II, with the formation of the $DCF^{\bullet -}$ semiquinone free radical. $DCF^{\bullet -}$ is oxidized to the fluorescent compound DCF by oxygen, concomitantly forming $O_2^{\bullet -}$. Nitric oxide (NO) is generated by NOS inside cells. NO may combine with $O_2^{\bullet -}$ to form peroxynitrite ($ONOO^-$). The highly reactive hydroxyl radical ($^{\bullet}OH$) can be formed through iron ion (Fe^{2+}) catalyzed cleavage of the oxygen atoms in H_2O_2, from HOCl reacting with $O_2^{\bullet -}$, or through decomposition of $ONOO^-$, leading to nitrogen dioxide radicals ($NO_2^{\bullet}$) as well. The following pharmacological inhibitors are denoted in the figure: the NADPH oxidase inhibitor diphenylene iodonium (DPI), the superoxide dismutase inhibitor diethyldithiocarbamic acid (DDC), and the myeloperoxidase inhibitor salicylhydroxamic acid (SHA). Addition of SNAP to aquatic solutions forms NO, SIN-1 gives $ONOO^-$, and $FeSO_4$ generates $^{\bullet}OH$ when added in combination with H_2O_2. Formation of $^{\bullet}OH$ can be prevented by the scavenger formate or by the iron ion chelator deferoxamine.

reactive species. The mechanisms of action of the inhibitors and the free radical donors used in the present work are denoted.

2.1. Strengths and limitations of the use of the DCFH-DA assay

The oxidative stress sensitive dye DCFH-DA diffuses passively through the cellular membrane. Intracellular esterase activity results in the formation of DCFH, a nonfluorescent compound, which emits fluorescence when it is oxidized to DCF. DCFH-DA has been widely used to measure the formation of reactive species in cells. It is not clear, however, which oxidative species are responsible for oxidation of DCFH to DCF in cells, or in assays using cell-free systems.

Our results show that human neutrophils exposed to the polychlorinated biphenyl mixture A1242 exhibit a strong activation of the respiratory burst response (400% of cell control), in agreement with previous findings [32]. As shown in Fig. 2, the A1242-stimulated DCF fluorescence in the neutrophils decreased (by 55%) when added in combination with the copper–zinc superoxide dismutase inhibitor DDC [33], resulting in reduced H_2O_2 production. The myeloperoxidase inhibitor SHA, which inhibits the formation of HOCl [34], did not have any effect. Exposure of the neutrophils to H_2O_2 alone increased DCF fluorescence by 265% compared to the cell control. However, addition of H_2O_2 alone to our cell-free system (buffer + DCFH) did not lead to increased DCF formation, while HRP alone and to a larger extent in combination with H_2O_2 oxidized DCFH to DCF (Fig. 3A). The mechanism for the HRP-catalyzed DCFH oxidation to DCF was proposed previously [35]. HRP, when reacting with H_2O_2, is converted to HRP-compound I. HRP-compound I oxidizes DCFH to the $DCF^{\bullet -}$ semiquinone free radical, which is oxidized to the fluorescent compound DCF by oxygen, with the production of $O_2^{\bullet -}$ (Fig. 1). Electron paramagnetic spin resonance (ESR) spectroscopy in combination with spin-trapping investigations demonstrated the formation of both $O_2^{\bullet -}$ and $^{\bullet}OH$ adducts of DMPO in the system containing DCFH, H_2O_2, and HRP [35]. The previous suggestion that DCF formation in neutrophils may be due to H_2O_2 alone [13,19] was probably due to the fact that these authors did not consider the presence of cellular peroxidases.

There are conflicting findings regarding the inhibition of cellular DCF formation by superoxide dismutase. Some

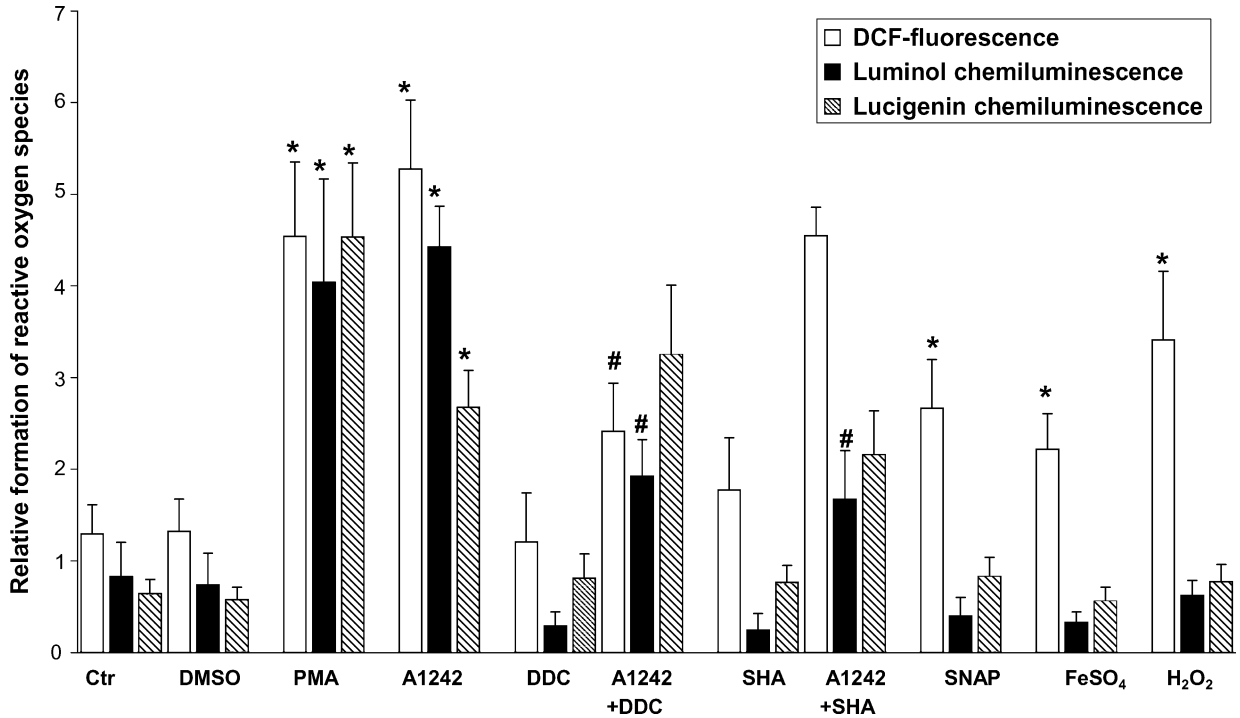
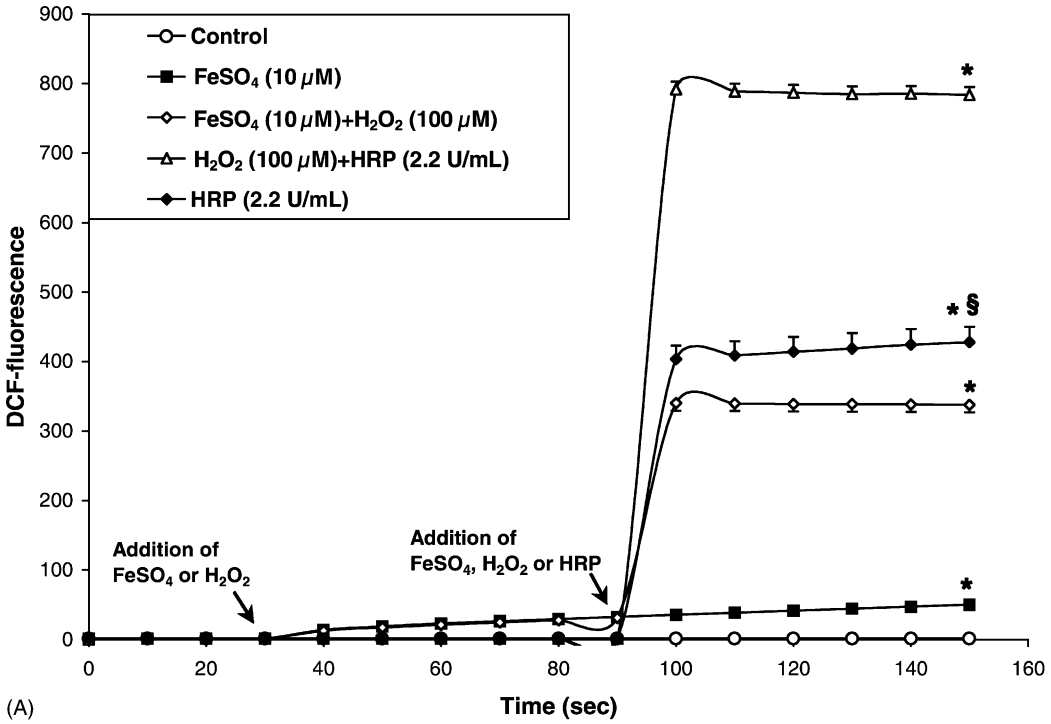


Fig. 2. Effect of phorbol 12-myristate 13-acetate (PMA; 10^{-8} M), Aroclor (A)1242 (25 μM) (purity tech. mix., AccuStandard Inc., USA), the superoxide dismutase inhibitor DDC (100 μM), the myeloperoxidase inhibitor SHA (50 μM), the NO donor SNAP (1 mM), $FeSO_4$ (10 μM), and H_2O_2 (100 μM) on DCF fluorescence, luminol-amplified chemiluminescence, and lucigenin-amplified chemiluminescence in human neutrophils. The first three columns show the unstimulated activity of the neutrophils (Ctr). DMSO (0.3%, v/v) (columns 3–6) was used as a solvent for A1242. Fresh blood samples were collected in the morning from healthy, adult males, and neutrophils were prepared by the standard density-gradient centrifugation method [52]. The neutrophils were diluted in HEPES-buffered (20 mM) HBSS (pH 7.4) with glucose (5.55 mM) to a density of 10^6 cells/mL (final cell concentration), and assayed on microplates with the probes DCFH-DA (2 μM) [6], luminol (0.1 mM) [4], or lucigenin (0.1 mM) [53]. All the reactions were carried out at 37°. Unless otherwise stated, the chemicals were purchased from Sigma–Aldrich (USA). Values are means ± SEM, N = 4–6 assayed in triplicate. Key: (*) statistically different from the cell control (Ctr), and (#) statistically different from A1242 (25 μM) ($P < 0.05$) (one-way ANOVA, Newman–Keuls).



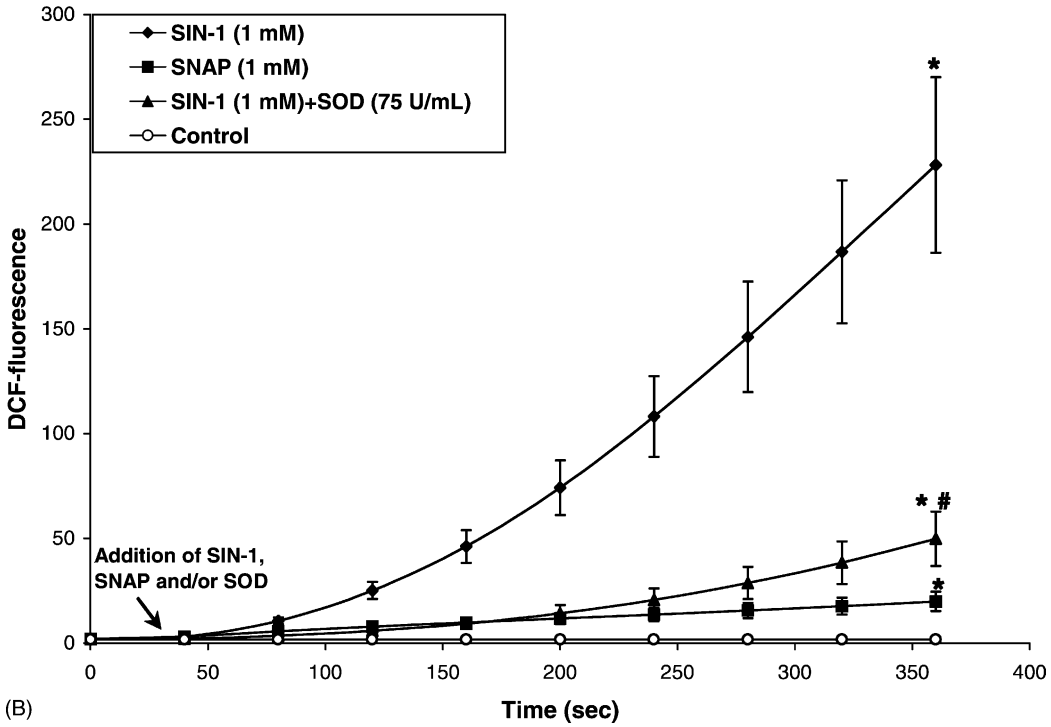


Fig. 3. Analysis of the ability of different RONS to oxidize DCFH in a cell-free system. DCFH was prepared from DCFH-DA by mixing 0.5 mL of 1 mM DCFH-DA (in methanol) with 2 mL of 10 mM NaOH at room temperature for 30 min [42]. The mixture was then neutralized to pH 7.4 with 10 mL of 25 mM NaH_2PO_4. The solution was kept in the dark on ice until used. The reactions were performed in a cuvette with HEPES-buffered (20 mM) HBSS (pH 7.4, total volume of 2 mL) containing DCFH (5 μM, final concentration). The chemicals were purchased from Sigma–Aldrich (USA). (A) DCFH displayed a higher sensitivity for H_2O_2 (100 μM) + HRP (2.2 U/mL) than for HRP alone, followed by Fe^{2+} (10 μM, from $FeSO_4$) + H_2O_2 (100 μM), and Fe^{2+} (10 μM, from $FeSO_4$) alone, respectively. Values are means ± SEM, N = 3. Key: (*) statistically different from control (buffer + DCFH), and (§) statistically different from H_2O_2 + HRP ($P < 0.05$) (one-way ANOVA, Newman–Keuls). (B) The $ONOO^-$ donor SIN-1 (1 mM) induced a much stronger and faster oxidation of DCFH into DCF than the NO donor SNAP (1 mM). Addition of SIN-1 (1 mM) in combination with superoxide dismutase (75 U/mL) lowered the response considerably. Values are means ± SEM, N = 3. Key: (*) statistically different from control (buffer + DCFH), and (#) statistically different from SIN-1 ($P < 0.05$) (one-way ANOVA, Newman–Keuls).

studies report an inhibitory effect of superoxide dismutase on DCF formation [19,21], while other studies show no effects [22,23]. The interpretation of the results when adding extracellular superoxide dismutase is dependent on the cell type. In neutrophils, ROS are generated either extracellularly or intraphagosomaly [36]. We suggest that extracellular addition of superoxide dismutase to stimulated neutrophils will lead to increased formation of H_2O_2, which may diffuse across the plasma membrane, leading to further elevation of intracellular DCF fluorescence (in the presence of cellular peroxidases). ROS formation is located mainly inside of non-phagocytic cell types. In these cases, we expect that there should be less or no effect of extracellular added superoxide dismutase.

Scott *et al.* [19] suggest that increased cellular DCF formation in response to xanthine oxidase exposure implicates $^{\bullet}OH$ because the $^{\bullet}OH$ scavenger DMSO inhibits the response. This is in agreement with Zhu *et al.* [20], who claim that $^{\bullet}OH$ is responsible for iron/peroxide-mediated oxidation of DCFH in a cell-free system. The $^{\bullet}OH$ scavenger formate (8 mM) in our cell-free system lowered the $Fe^{2+} + H_2O_2$-stimulated DCF formation by about 50% (when formate was added prior to $Fe^{2+} + H_2O_2$) (not shown). The iron ion chelator deferoxamine (8 mM) totally ameliorated the DCF fluorescence when added prior to $Fe^{2+} + H_2O_2$ (not shown). Addition of Fe^{2+} alone also increased the DCF fluorescence to a small extent in our cell-free system (Fig. 3A). Exposure of the neutrophils to $FeSO_4$ alone increased the DCF fluorescence (175% of cell control) (Fig. 2), possibly because of a reaction between H_2O_2 and Fe^{2+} (the Fenton's reaction), leading to $^{\bullet}OH$ formation. A1242-induced formation of $^{\bullet}OH$ in our neutrophils was indicated by use of an aromatic acid hydroxylation assay (Table 1). This is shown for the first time in human neutrophils exposed to A1242. Hydroxylation of 4-HBA is a highly sensitive technique used to detect $^{\bullet}OH$ down to picomole levels [37,38]. Addition of DMSO ($\leq$0.3%, v/v) to the neutrophils resulted in a small decrease of 3,4-DHBA formation, which is not surprising, since DMSO is a well-known $^{\bullet}OH$ scavenger. Previously, we have also shown that $^{\bullet}OH$ can be formed in neutrophils by use of ESR spectroscopy in combination with the spin trap DEPMPO [6]. The susceptibility of 2,3-DHBA to be degraded by HOCl questions the alternative salicylate (2-HBA) assay for measurement of $^{\bullet}OH$ generation by neutrophils [39]. Important sources of $^{\bullet}OH$ formation in phagocytes are due to HOCl reacting with $O_2^{\bullet-}$, and the reaction between HOCl and catalytic iron ions [40] (Fig. 2). Also, $ONOO^-$ decomposition generates a strong oxidant similar to $^{\bullet}OH$ [41]. DCFH has been used to measure lipid hydroperoxides [42], which may be formed by the action of Fe^{2+} and Fe^{3+}, or by $^{\bullet}OH$ [43]. Even if caution should be used in the interpretation of our results, we propose that $^{\bullet}OH$ can oxidize DCFH to DCF.

Table 1
Formation of 3,4-DHBA as a measure for $^{\bullet}OH$ formation in human neutrophil granulocytes exposed to the polychlorinated biphenyl mixture A1242 and the positive control PMA

Treatment[a]	3,4-DHBA formed[b] (nmol/120 min)
Cell control	0.126 ± 0.02
DMSO control	0.11 ± 0.05
A1242	0.24 ± 0.11*
PMA	0.711 ± 0.06*

Fresh blood samples were collected in the morning from healthy, adult males, and neutrophils were prepared by the standard density-gradient centrifugation method [52]. The aromatic acid hydroxylation assay was carried out as previously described [6].

[a] The complete reaction mixture consisted of neutrophils (4×10^6 cells/mL) incubated with 4-HBA (2 mM) in HEPES-buffered (20 mM) HBSS (pH 7.4) with glucose (5.55 mM) and the test compounds A1242 (25 μM) or PMA (10^{-7} M). The control mixtures contained neutrophils in HEPES-buffer (cell control) or 0.3% (v/v) DMSO (DMSO control), the solvent for A1242.

[b] Values represent the averages ± SD of 3–4 independent experiments.

* Statistically different from cell control, $P \leq 0.05$ (one-way ANOVA, Newman–Keuls).

It is claimed that a high concentration of NO (0.8–6.4 mM/mL) increases DCF formation in a cell-free system as well as in cells [14]. Also, DCF formation in neutrophils is suggested to be due to NO [12]. In contrast, others have found that DCFH shows rather low reactivity towards NO, while it is more sensitive towards $ONOO^-$ in cell-free systems and in cells [17,18]. In agreement, Crow [44] revealed that $ONOO^-$, HOCl, and H_2O_2 plus peroxidase oxidize DCFH to varying degrees in a cell-free assay. However, neither $O_2^{\bullet-}$, H_2O_2, nor a physiological level of NO was capable of dye oxidation in that system. In agreement, addition of a 1 mM concentration of the NO donor SNAP to our cell-free system did not increase the DCF fluorescence to any significant extent within a 6-min period (Fig. 3B). On the other hand, addition of the $ONOO^-$ donor SIN-1 to our cell-free system induced a robust and fast increase of DCF formation (Fig. 3B), which reached a steady state after about 10 min (not shown). SIN-1 releases $O_2^{\bullet-}$ and NO, the combination of which results in the rapid generation of $ONOO^-$ [45]. Thus, the authors who suggest that NO is responsible for increased DCF formation in cellular systems may not have taken the formation of $ONOO^-$ into account. For example, Gabriel *et al.* [15] proposed that the addition of SIN-1 to DCFH-loaded dissociated cerebellar granule cells led to an NO-amplified fluorescence. To assure that SIN-1 actually released $ONOO^-$ in our cell-free system, we incubated the suspension with superoxide dismutase, which removes $O_2^{\bullet-}$ and thereby $ONOO^-$. This decreased the DCF fluorescence by about 75% (Fig. 3B). Exposure of the neutrophils to the NO donor SNAP alone increased the DCF fluorescence by 205% (Fig. 2). As shown by the background lucigenin-amplified chemiluminiscence in the cell control (Fig. 2), these cells form a small but detectable amount of $O_2^{\bullet-}$ also in the absence of any stimulation. Thus, addition of NO to the cells will combine with $O_2^{\bullet-}$ and form $ONOO^-$, a strong inducer of DCFH oxidation.

Some of the background fluorescence (in the cell control) observed in Fig. 2 could, in principle, be due to autooxidation of DCFH, or be caused by intracellular peroxidases alone. Also, removal of the diacetate groups of DCFH-DA by cellular esterases may result in trace H_2O_2 production [35]. However, we suggest that these are quantitatively insignificant sources of error in a cellular system, because the background levels of the DCF fluorescence, and the luminol- and lucigenin-amplified chemiluminescence are almost ameliorated when the neutrophils are incubated with the NADPH-oxidase inhibitor diphenyleneiodonium chloride [46] (not shown). Therefore, we conclude that the background light emission in our system (Fig. 2) is due mainly to a small, but detectable production of reactive species by the NADPH oxidase.

2.2. *Specificity of the chemiluminescent probes luminol and lucigenin*

Lucigenin and luminol are used frequently in studies of ROS production by activated phagocytes. Lucigenin appears rather specific for $O_2^{\bullet -}$ [47]. Also $ONOO^-$ was claimed previously to induce lucigenin-amplified chemiluminescence in a cell-free system [26]. However, exposure of the neutrophils in our system to the NO donor SNAP or $FeSO_4$ alone indicates insensitivity of lucigenin towards oxidation by NO, $ONOO^-$, or $^{\bullet}OH$ (Fig. 2). Preincubation of the neutrophils with the superoxide dismutase inhibitor DDC led to a small increase of lucigenin chemiluminescence, indicating that lucigenin is sensitive towards $O_2^{\bullet -}$ but not towards H_2O_2 or HOCl. This hypothesis was supported by exposure of the neutrophils to the myeloperoxidase inhibitor SHA in combination with A1242, or to H_2O_2 alone (Fig. 2), which did not influence the lucigenin chemiluminescence. To detect $O_2^{\bullet -}$, lucigenin must be reduced to the lucigenin cation radical. The validity of lucigenin as a chemiluminescence probe has been questioned though, because in several *in vitro* assays lucigenin may itself act as a source of $O_2^{\bullet -}$ via autooxidation of the lucigenin cation radical [24,25]. However, it is suggested that redox cycling of lucigenin is of no importance [48], in particular, in cellular systems that produce significant amounts of $O_2^{\bullet -}$ [49].

Enhanced chemiluminescence in the presence of luminol appears to depend largely on the myeloperoxidase–H_2O_2–Cl^- system in cells [50]. This was confirmed by the use of neutrophils in our system, since the luminol-amplified chemiluminescence was decreased by the myeloperoxidase inhibitor SHA and by the superoxide dismutase inhibitor DDC (Fig. 2). The effect of DDC is probably due to lowered formation of H_2O_2, and thereby less HOCl. However, it is suggested that $ONOO^-$, but not $^{\bullet}OH$, also oxidizes luminol in cellular systems [27,28,51]. Others have reported that luminol-enhanced chemiluminescence may be due to the generation of $^{\bullet}OH$ *in vivo* [30], in neutrophils *in vitro* [31], or in a cell-free system [29]. However, as seen in Fig. 2, production of $^{\bullet}OH$ after the addition of $FeSO_4$ or H_2O_2, or the formation of NO/$ONOO^-$ by the addition of SNAP did not elevate the luminol-amplified chemiluminescence in the neutrophils.

3. Conclusion

We suggest that DCFH is sensitive towards oxidation by $ONOO^-$, H_2O_2 (in combination with cellular peroxidases), peroxidases alone, and $^{\bullet}OH$, while it is not suitable for measurement of NO, HOCl, or $O_2^{\bullet -}$ in biological systems. Importantly, $^{\bullet}OH$ and $ONOO^-$ may be the only oxidants that oxidize DCFH within seconds or a few minutes. Small quantities of these oxidants will rapidly increase DCF formation, whereas other oxidants may need higher concentrations and more time. Also, the simultaneous presence of cellular peroxidases is necessary for DCFH oxidation by H_2O_2; H_2O_2 alone does not oxidize DCFH into DCF. The increased oxidation of DCFH by HRP in a cell-free system raises the possibility that the presence of peroxidases in biological systems exposed to H_2O_2 or other reactive species may have led to wrong conclusions.

The lucigenin and luminol probes are made chemiluminescent by $O_2^{\bullet -}$ and HOCl, respectively, but are not suitable for detection of NO, $ONOO^-$, H_2O_2, or $^{\bullet}OH$ in cellular systems. Because of their individual unique specificities, several methods should be combined when dealing with identification of reactive species formed in biological systems.

References

[1] Beal MF. Aging, energy, and oxidative stress in neurodegenerative diseases. Ann Neurol 1995;38:357–66.
[2] Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature 2000;408:239–47.
[3] Ali SF, LeBel CP, Bondy SC. Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. Neurotoxicology 1992;13:637–48.
[4] Voie ØA, Wiik P, Fonnum F. *Ortho*-substituted polychlorinated biphenyls activate respiratory burst measured as luminol-amplified chemoluminescence in human granulocytes. Toxicol Appl Pharmacol 1998;150:369–75.
[5] Schulz JB, Henshaw DR, MacGarvey U, Beal MF. Involvement of oxidative stress in 3-nitropropionic acid neurotoxicity. Neurochem Int 1996;29:167–71.
[6] Myhre O, Vestad TA, Sagstuen E, Aarnes H, Fonnum F. The effects of aliphatic (*n*-nonane), naphthenic (1,2,4-trimethylcyclohexane), and aromatic (1,2,4-trimethylbenzene) hydrocarbons on respiratory burst in human neutrophil granulocytes. Toxicol Appl Pharmacol 2000;167:222–30.
[7] Myhre O, Bjugan B, Fonnum F. Toxic effect of L-2-chloropropionate on cultured rat cerebellar granule cells is ameliorated after inhibition of reactive oxygen species formation. J Neurosci Res 2001;66:992–7.
[8] Mariussen E, Myhre O, Reistad T, Fonnum F. The polychlorinated biphenyl mixture Aroclor 1254 induces death of rat cerebellar granule cells: the involvement of the *N*-methyl-D-aspartate receptor

and reactive oxygen species. Toxicol Appl Pharmacol 2002;179: 137–44.
[9] Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 1999;27:612–6.
[10] Keston AS, Brandt R. The fluorometric analysis of ultramicro quantities of hydrogen peroxide. Anal Biochem 1965;11:1–5.
[11] LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2′,7′-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 1992;5:227–31.
[12] Rao KM, Padmanabhan J, Kilby DL, Cohen HJ, Currie MS, Weinberg JB. Flow cytometric analysis of nitric oxide production in human neutrophils using dichlorofluorescein diacetate in the presence of a calmodulin inhibitor. J Leukoc Biol 1992;51:496–500.
[13] Vowells SJ, Sekhsaria S, Malech HL, Shalit M, Fleisher TA. Flow cytometric analysis of the granulocyte respiratory burst: a comparison study of fluorescent probes. J Immunol Methods 1995;178:89–97.
[14] Gunasekar PG, Kanthasamy AG, Borowitz JL, Isom GE. Monitoring intracellular nitric oxide formation by dichlorofluorescein in neuronal cells. J Neurosci Methods 1995;61:15–21.
[15] Gabriel C, Camins A, Sureda FX, Aquirre L, Escubedo E, Pallas M, Camarasa J. Determination of nitric oxide generation in mammalian neurons using dichlorofluorescein diacetate and flow cytometry. J Pharmacol Toxicol Methods 1997;38:93–8.
[16] Royall JA, Ischiropoulos H. Evaluation of 2′,7′-dichlorofluorescein and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch Biochem Biophys 1993;302:348–55.
[17] Kooy NW, Royall JA, Ischiropoulos H. Oxidation of 2′,7′-dichlorofluorescein by peroxynitrite. Free Radic Res 1997;27:245–54.
[18] Possel H, Noack H, Augustin W, Keilhoff G, Wolf G. 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation. FEBS Lett 1997;416:175–8.
[19] Scott JA, Homcy CJ, Khaw BA, Rabito CA. Quantitation of intracellular oxidation in a renal epithelial cell line. Free Radic Biol Med 1988;4:79–83.
[20] Zhu H, Bannenberg GL, Moldeus P, Shertzer HG. Oxidation pathways for the intracellular probe 2′,7′-dichlorofluorescein. Arch Toxicol 1994;68:582–7.
[21] Atlante A, Gagliardi S, Minervini GM, Ciotti MT, Marra E, Calissano P. Glutamate neurotoxicity in rat cerebellar granule cells: a major role for xanthine oxidase in oxygen radical formation. J Neurochem 1997;68:2038–45.
[22] Takeuchi T, Nakajima M, Morimoto K. Relationship between the intracellular reactive oxygen species and the induction of oxidative DNA damage in human neutrophil-like cells. Carcinogenesis 1996;17:1543–8.
[23] Yang CF, Shen HM, Shen Y, Zhuang ZX, Ong CN. Cadmium-induced oxidative cellular damage in human fetal lung fibroblasts (MRC-5 cells). Environ Health Perspect 1997;105:712–6.
[24] Liochev SI, Fridovich I. Lucigenin (bis-*N*-methylacridinium) as a mediator of superoxide anion production. Arch Biochem Biophys 1997;337:115–20.
[25] Vasquez-Vivar J, Hogg N, Pritchard Jr KA, Martasek P, Kalyanaraman B. Superoxide anion formation from lucigenin: an electron spin resonance spin-trapping study. FEBS Lett 1997;403:127–30.
[26] Gunaydin B, Demiryurek AT. Interaction of lidocaine with reactive oxygen and nitrogen species. Eur J Anaesthesiol 2001;18:816–22.
[27] Kooy NW, Royall JA. Agonist-induced peroxynitrite production from endothelial cells. Arch Biochem Biophys 1994;310:352–9.
[28] Castro L, Alvarez MN, Radi R. Modulatory role of nitric oxide on superoxide-dependent luminol chemiluminescence. Arch Biochem Biophys 1996;333:179–88.
[29] Yildiz G, Demiryurek AT. Ferrous iron-induced luminol chemiluminescence: a method for hydroxyl radical study. J Pharmacol Toxicol Methods 1998;39:179–84.
[30] Oldenburg B, van Kats-Renaud H, Koningsberger JC, van Berge Henegouwen GP, van Asbeck BS. Chemiluminescence in inflammatory bowel disease patients: a parameter of inflammatory activity. Clin Chim Acta 2001;310:151–6.
[31] Nemeth K, Furesz J, Csikor K, Schweitzer K, Lakatos S. Luminol-dependent chemiluminescence is related to the extracellularly released reactive oxygen intermediates in the case of rat neutrophils activated by formyl-methionyl-leucyl-phenylalanine. Haematologia 2002;31: 277–85.
[32] Tithof PK, Watts S, Ganey PE. Protein tyrosine kinase involvement in the production of superoxide anion by neutrophils exposed to Aroclor 1242, a mixture of polychlorinated biphenyls. Biochem Pharmacol 1997;53:1833–42.
[33] Misra HP. Reaction of copper–zinc superoxide dismutase with diethyldithiocarbamate. J Biol Chem 1979;254:11623–8.
[34] Davies B, Edwards SW. Inhibition of myeloperoxidase by salicylhydroxamic acid. Biochem J 1989;258:801–6.
[35] Rota C, Fann YC, Mason RP. Phenoxyl free radical formation during the oxidation of the fluorescent dye 2′,7′-dichlorofluorescein by horseradish peroxidase. Possible consequences for oxidative stress measurements. J Biol Chem 1999;274:28161–8.
[36] Kettle AJ, Winterbourn CC. Myeloperoxidase: a key regulator of neutrophil oxidant production. Redox Rep 1997;3:3–15.
[37] Ste-Marie L, Boismenu D, Vachon L, Montgomery J. Evaluation of sodium 4-hydroxybenzoate as an hydroxyl radical trap using gas chromatography–mass spectrometry and high-performance liquid chromatography with electrochemical detection. Anal Biochem 1996;241:67–74.
[38] Coudray C, Talla M, Martin S, Fatome M, Favier A. High-performance liquid chromatography—electrochemical determination of salicylate hydroxylation products as an *in vivo* marker of oxidative stress. Anal Biochem 1995;227:101–11.
[39] Blackburn AC, Doe WF, Buffinton GD. Salicylate hydroxylation as an indicator of hydroxyl radical generation in dextran sulfate-induced colitis. Free Radic Biol Med 1998;25:305–13.
[40] Candeias LP, Patel KB, Stratford MR, Wardman P. Free hydroxyl radicals are formed on reaction between the neutrophil-derived species superoxide anion and hypochlorous acid. FEBS Lett 1993; 333:151–3.
[41] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620–4.
[42] Cathcart R, Schwiers E, Ames BN. Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. Anal Biochem 1983;134:111–6.
[43] Minotti G, Aust SD. The role of iron in oxygen radical mediated lipid peroxidation. Chem Biol Interact 1989;71:1–19.
[44] Crow JP. Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite *in vitro*: implications for intracellular measurement of reactive nitrogen and oxygen species. Nitric Oxide 1997;1:145–57.
[45] Huie RE, Padmaja S. The reaction of NO with superoxide. Free Radic Res Commun 1993;18:195–9.
[46] O'Donnell VB, Tew DG, Jones OTG, England PJ. Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. Biochem J 1993;290:41–9.
[47] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. New York: Oxford University Press; 1999.
[48] Afanas'ev IB, Ostrachovitch EA, Korkina LG. Lucigenin is a mediator of cytochrome *c* reduction but not of superoxide production. Arch Biochem Biophys 1999;366:267–74.
[49] Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA. Validation of lucigenin (bis-*N*-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. J Biol Chem 1998;273:2015–23.

[50] McNally JA, Bell AL. Myeloperoxidase-based chemiluminescence of polymorphonuclear leukocytes and monocytes. J Biolumin Chemilumin 1996;11:99–106.

[51] Carreras MC, Pargament GA, Catz SD, Poderoso JJ, Boveris A. Kinetics of nitric oxide and hydrogen peroxide production and formation of peroxynitrite during the respiratory burst of human neutrophils. FEBS Lett 1994;341:65–8.

[52] Bøyum A, Løvhaug D, Tresland L, Nordlie EM. Separation of leucocytes: improved cell purity by fine adjustment of gradient medium density and osmolality. Scand J Immunol 1991;34:697–712.

[53] Minkenberg I, Ferber EJ. Lucigenin-dependent chemiluminescence as a new assay for NAD(P)H-oxidase activity in particulate fractions of human polymorphonuclear leukocytes. J Immunol Methods 1984;71:61–7.