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Analysis of Dichlorodihydrofluorescein and Dihydrocalcein as Probes for the Detection of Intracellular Reactive Oxygen Species

ALEXANDRA KELLER[a], ANNISUDDIN MOHAMED[a], STEFAN DRÖSE[b], ULRICH BRANDT[b], INGRID FLEMING[a] and RALF P. BRANDES[a,*]

[a]*Institut für Kardiovaskuläre Physiologie, Klinikum der J.W. Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany;* [b]*Institut für Biochemie I, Klinikum der J.W. Goethe-Universität, Frankfurt am Main, Germany*

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Dihydrocalcein (H_2-calcein) is recommended as a superior probe for intracellular radical (ROS) detection as different to dichlorodihydrofluorescein (H_2-DCF), its oxidation product calcein is thought not to leak out of cells. We determined whether H_2-calcein is a useful tool to measure ROS in vascular smooth muscle cells. *In vitro*, both compounds were oxidized by peroxynitrite, hydroxyl radicals and peroxidase, but not hydrogen peroxide or nitric oxide. The intracellular half-life of calcein was several hours whereas that of DCF was approximately 5 min. Intracellular ROS, as generated by the angiotensin II (Ang II)-activated NADPH oxidase, did not increase the oxidation of H_2-calcein but increased the oxidation of H_2-DCF by approximately 50%. Similar changes were detected using electron spin resonance spectroscopy. Inhibition of the NADPH oxidase using gp91ds-tat prevented the Ang II-induced increase in DCF fluorescence, without affecting cells loaded with H_2-calcein. Diphenylene iodonium (DPI), which inhibits all flavin-dependent enzymes, including those in the respiratory chain, had little effect on the basal but prevented the Ang II-induced oxidation of H_2-DCF. In contrast, DPI inhibited H_2-calcein oxidation in non-stimulated cells by almost 50%. Blockade of respiratory chain complex I inhibited H_2-calcein oxidation, whereas inhibitors of complex III were without effect. Calcein accumulated in the mitochondria, whereas DCF was localized in the cytoplasm. In submitochondrial particles, H_2-calcein, but not H_2-DCF inhibited complex I activity.

These observations indicate that H_2-DCF is an indicator for intracellular ROS, whereas the oxidation of H_2-calcein most likely occurs as a consequence of direct electron transfer to mitochondrial complex I.

Keywords: Oxygen-derived free radicals; Electron spin resonance; NADPH oxidase; Reactive oxygen species; gp91ds-tat

INTRODUCTION

Research into the generator- and effector-systems of reactive oxygen species (ROS) in biological systems, is hampered by difficulties in the measurement of ROS in living tissue.[27] Although, a multitude of different techniques has been developed, all of them lack either specificity or sensitivity.[27] Moreover, many assays are not quantitative since redox cycling,[3,15,16,23] or complex secondary reactions contribute to the signal finally detected.[24]

Among the techniques with the highest sensitivity that can be used in living cells, are fluorimetric assays. Cells are pre-treated or loaded with a non-fluorescent compound, which, after the reaction with the radical, turns into a fluorophor. The prototype of such a technique is the oxidation of dichlorodihydrofluorescein (H_2-DCF): H_2-DCF is administered as a diacetate ester (DA), which is rapidly taken up by cells. Once inside the cell, the diacetate residues are removed by esterases, liberating H_2-DCF, which accumulates intracellularly as a consequence of its low membrane permeability.[2] H_2-DCF reacts predominantly with highly oxidizing species of ROS such as hydroxyl radicals ($^{\cdot}OH$) and peroxynitrite ($ONOO^-$).[10,13] When H_2-DCF is used to detect other ROS, such as superoxide anions ($O_2^{\cdot-}$) or hydrogen peroxide (H_2O_2), the assay signal obtained represents only a indirect measure of the actual radical concentration.

*Corresponding author. Tel.: +49-69-6301-6995. Fax: +49-69-6301-7668. E-mail: r.brandes@em.uni-frankfurt.de

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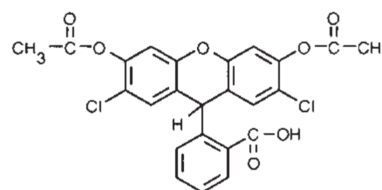
In the case of $O_2^{\cdot -}$, this has to either react with NO to form $ONOO^-$, which could oxidize the dye, or be dismuted to H_2O_2 by superoxide dismutase (SOD). The H_2O_2 generated is subsequently cleaved to $^{\cdot}OH$ via the Fenton reaction in the presence of Fe^{2+}, which then reacts with bicarbonate to form carbonate radicals. Alternatively, carbonate radicals can be formed by the reaction of H_2O_2 with intracellular peroxidases.[1] $^{\cdot}OH$ or, more likely, these carbonate radicals then react with H_2-DCF. These reactions compete for H_2O_2 with antioxidative enzymes, such as glutathione peroxides, thioredoxin peroxidase or catalase. Consequently, the antioxidative capacity of the cell under investigation and the concentration of NO are important determinants of the final amount of carbonate radical or $^{\cdot}OH$ that reacts with H_2-DCF. Thus, the formation of DCF in a cell, by no means allows a direct quantification of $O_2^{\cdot -}$.[20]

Despite these limitations, the DCF assay is the most frequently used assay for the determination of intracellular ROS formation.[9] As a result of the accumulation of the stable oxidation product DCF and the ease of detecting DCF by assessing its fluorescence, the sensitivity of this assay is superior to photometric methods, such as cytochrome *c* reduction, chemiluminescence and electron spin resonance (ESR).[19,27] Moreover, the DCF assay provides a measure for intracellular ROS generation, whereas the other techniques are suitable only for extracellular measurements (cytochrome *c*) or provide a mixed signal (ESR, chemiluminescence).[19,27]

A fundamental limitation of the DCF technique is that the compound leaks out of cells once it has been oxidized to the fluorescent form. Particularly in vascular cells, the intracellular half-life of DCF is in the range of only minutes, whereas in cardiac myocytes DCF can be retained intracellularly for more prolonged periods.[25,26] This particular aspect, however, limits the use of DCF in applications such as confocal microscopy or fluorescence-activated cell sorting (FACS), as well as for microplate reader assays, when measurements are performed in intact cells. In superfusion systems, a stable DCF signal cannot be obtained which results in substantial problems in situations with variable convection conditions, such as shear–stress experiments, pulsatile stretch or ischemia–reperfusion studies.

Given the need for ROS tracers with an improved intracellular retention, derivatives of DCF, such as 5,6-chloromethyl-H_2-DCF,[30] have been developed. This compound is, however, taken up into cells much more slowly than H_2-DCF and its fluorescence is less intense.[9] Recently, dihydrocalcein-diacetate (H_2calcein-DA) was introduced as a new probe for the intracellular detection of ROS. H_2-calcein is structurally tightly related to H_2-DCF (Fig. 1) and thus should be oxidized by ROS. The reaction

A Dichlorodihydrofluorescein-diacetate



B Dihydrocalcein-diacetate

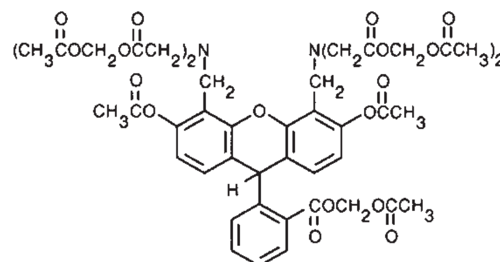


FIGURE 1 Structures of dichlorodihydrofluorescein-diacetate (H_2-DCF-DA) (A) and dihydrocalcein-acetoxymethylester (H_2-calcein-AM) (B).

product calcein, however, is known to be retained in cells for several days and has been used to label cells or to study cellular communication via gap junctions.[28] These properties of H_2-calcein render it an attractive new fluorogenic probe for ROS detection.

Since, the properties of H_2-calcein have not been studied extensively we set out to determine whether H_2-calcein is a suitable radical indicator and whether it is superior to H_2-DCF as a probe for intracellular ROS generation. To this end, we compared the signals generated by H_2-DCF and H_2-calcein *in vitro* as well as in intact, ROS-producing vascular smooth muscle cells.

MATERIALS AND METHODS

Reagents

H_2-DCF-DA, H_2-calcein-AM and mitotracker green were purchased from Molecular Probes (Leiden, The Netherlands). Diphenylene iodonium (DPI), $ONOO^-$ and the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5,-tetramethylpyrrolidine-HCl (CMH) were obtained from Alexis Biochemicals (Grünberg, Germany). gp91ds-tat[22] and the scrambled control peptide src-tat were synthesized by Eurogentec, Ourgée, Belgium. For *in vitro* experiments HPLC-grade ultra-pure water (J.T. Baker, Griesheim, Germany) and “suprapur” di-potassium hydrogen phosphate salt from Merck (Darmstadt, Germany) were used. All other compounds were purchased from Sigma (Deisenhofen, Germany).

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Cell Culture

Rat aortic vascular smooth muscle cells (RSMC) were obtained from Tebu (Frankfurt, Germany) and cultured in minimal essential medium (MEM) containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (50 μg/ml). All experiments were performed using serum-deprived cells (24 h, MEM + 0.1% bovine serum albumin) between passages 6 and 12 at 70% confluence.

Preparation of SMPs

Submitochondrial particles (SMPs) were prepared from bovine heart mitochondria as described previously[21] and were finally resuspended in 75 mmol/l Na-phosphate/1 mmol/l EDTA/1 mmol/l $MgCl_2$ pH 7.4. The protein content determined was 26.8 mg/ml and the cytochrome *b* and a/a_3 content was 17.24 and 19.78 μmol/l, respectively.

In Vitro Measurements

Deacetylation of H_2-DCF-diacetate and H_2-calcein-acetoxymethylester

Both, H_2-DCF and H_2-calcein are supplied as esters that are rapidly taken up by the cell and are subsequently deacetylated. For the *in vitro* studies, it was necessary to deacetylate both compounds to render them reactive to ROS. Deacetylation was performed according to the method of Cathcart *et al.*[5] In brief, 1 part of H_2-DCF-DA or H_2-calcein-AM solution (1 mmol/l dissolved in methanol) were mixed with 4 parts NaOH solution (0.01 mol/l, de-oxygenated by purging with argon) and incubated at room temperature in the dark for 30 min. Thereafter, the reaction was stopped by the addition of 20 parts of de-oxygenated potassium phosphate buffer (25 mmol/l, pH 7.4) and stored on ice.

In Vitro ROS Measurements

Deacetylated H_2-DCF or H_2-calcein (5 μmol/l, dissolved in deoxygenated phosphate buffer) were incubated in Eppendorf tubes in the presence or absence of the test substance (final volume 300 μl). Experiments were carried out to determine the oxidation of the probes by $ONOO^-$, H_2O_2, NO released from DEA-NONOate and horse radish peroxidase (HRP). Reaction mixtures, 200 μl, were transferred into 96-well plates and the fluorescence of DCF and calcein was measured using a microplate reader (Victor, Wallac-ADL) using an excitation wavelength of 488 nm and emission wavelength of 515 nm.

ROS Measurements in Living Cells

Determination of Dye Leakage

RSMC cultured in 12 well plates, were washed with HEPES (10 mmol/l)-modified Tyrode's solution (HT solution, pH 7.4, 37°C) and loaded with H_2-DCF-DA (10 μmol/l dissolved in HT solution, stock solution 10 mmol/l dissolved in dimethylsulfoxide) or H_2-calcein-AM (2 μmol/l, dissolved in HT solution, stock solution 1 mmol/l dissolved in dimethylsulfoxide) for 20 min in a final volume of 500 μl. Thereafter, the cells were washed twice and incubated with HT solution (300 μl) at 37°C in the dark for the times indicated. The supernatant containing the DCF and calcein which has leaked out of the cells (extracellular) was then removed and stored on ice. The cells were washed once and lysed by the addition of double distilled water (300 μl). The fluorescence of the extracellular and intracellular fraction was measured using a microplate reader (96-well plate, 300 μl).

Fluorimetric Measurements of Cellular ROS Formation

Basal and Agonist-induced ROS Formation

RSMC were cultured in 12-well plates. The culture medium was removed and replaced by fresh medium (MEM + 0.1% bovine serum albumin) containing either solvent or angiotensin II (Ang II, 100 nmol/l). After 45 min, cells were washed with HT solution and loaded for 20 min with either H_2-DCF or H_2-calcein. Thereafter, the cells were washed with HT solution and fresh HT solution (500 μl) was added to the wells. The fluorescence of the cells was measured using a microplate reader and the background fluorescence of wells containing no cells and no dye was subtracted from each signal. In some experiments, cells were visualized using an LSM 510 META (Zeiss, Jena) confocal microscope using a $\times 40$ long distance air objective and a pinhole setting of 3 airy units. For studies using gp91ds-tat, the peptides (100 μmol/l) were added to the cells 4 h before stimulation with Ang II. In the case of DPI (10 μmol/l), the compound was added during the incubation with Ang II and re-administered during loading.

In some experiments, the effect of the following respiratory chain inhibitors was assessed in RSMC: rotenone (10 μmol/l); an inhibitor of complex I, antimycin A (1 μmol/l), stigmatellin (100 nmol/l) and myxothiazol (100 nmol/l) inhibitors of complex III.

Redox Cycler

RSMC cultured in 12-well plates were washed with HT solution and loaded with H_2-DCF-DA or H_2-calcein-AM for 20 min in the presence or absence of 2,3-dimethoxy-1,4-naphthoquinone (DMNQ)

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(100 μmol/l). Thereafter, the fluorescence was measured as described above.

Electron Spin Resonance

RSMC were cultured in 6 cm dishes and stimulated with Ang II as described. Cells were then washed with ESR buffer (HT solution pre-treated with Chelax, containing diethylenetriaminepentaacetic acid, 100 μmol/l) and incubated in ESR buffer (1 ml) at 37°C for 30 min. CMH dissolved in argon purged 0.9% NaCl solution (final concentration 2 mmol/l) was added and after 5 min, 400 μl of the solution were withdrawn, transferred to Teflon cylinders and immediately frozen in liquid nitrogen. The formation of the stable spin label CM• was determined at 77 K in a liquid nitrogen cooled dewar using a Bruker EPR-EMX spectrometer. The instrument settings were as follows: microwave frequency, 9.468 GHz; power, 20.02 mW; receiver gain, 1×10^5; modulation amplitude, 2.010 G; modulation frequency, 100 KHz; conversion time, 81.92 ms; time constant, 81.92 ms; sweep time, 83.89 s; sweep width, 400 G; resolution, 1024 points and 3 scans.

Confocal Microscopy Imaging

RSMC were cultured on glass cover slips (9 × 9 mm) in 24 well plates. After washing with HT solution, cells were loaded with either H_2-DCF-DA, H_2-calcein-AM or mitotracker green (1 μmol/l) for 20 min. The glass cover slips were then transferred to a custom built chamber containing HT solution (500 μl) on the microscope and images were acquired using a 40 × oil immersion objective and a pinhole setting of 0.5 airy units. Scan speed and laser excitation were set to produce as little photo-oxidation of the dyes as possible.

Determination of NADH:Ubiquinone Activity in Submitochondrial Particles

NADH:DBQ (*n*-decylubiquinone) activity was assessed by monitoring NADH consumption in a buffer (25 mmol/l K-phosphate/2 mmol/l KCN, ph 7.4 at 30°C, 200 μl) containing 1.34 μg of submitochondrial particles using a Spectra Max Plus[384] microplate reader (Molecular Devices, Ismaning, Germany). DBQ (100 μmol/l) and NADH (100 μmol/l) were used as substrates and NADH-oxidation rates were calculated using $\varepsilon_{340-400\,nm} = 6.11\,mmol^{-1}\,cm^{-1}$ in the presence of different concentrations of H_2-DCF and H_2-calcein. Data shown are corrected for the effects of the solvents.

Statistical Analysis

Data presented are the mean ± standard error of the mean (SEM). Statistical analysis was carried out using paired or unpaired Student's *t*-test or analysis of variance (ANOVA) as appropriate. A *p*-value of less than 0.05 was considered significant.

RESULTS

H_2-DCF and H_2-calcein have Similar Chemical Properties

In order to assess the suitability of H_2-calcein as a probe for ROS, its reactivity to different compounds was studied in an *in vitro* assay and compared to that of H_2-DCF. In these experiments, the specificity of both probes was very similar. $ONOO^-$ readily oxidized both H_2-DCF and H_2-calcein, at concentrations as low as 0.1 μmol/l (Fig. 2A). H_2O_2 (100 μmol/l), in contrast, did not oxidize either substance (datfa not shown). Only very high concentrations (100 μmol/l) of the NO donor DEA-NONOate, which has a half life of approximately 15 min at neutral pH, also increased the oxidation of H_2-calcein and H_2-DCF, although this effect was less pronounced than its sensitivity to $ONOO^-$ (Fig. 2B). Therefore, it is possible that the NO donor-mediated oxidation reflects either a slight accumulation of $ONOO^-$, the formation of NO, or the formation of NO_2, which can be formed at high concentration of NO donors.[29] HRP, even at relatively low concentrations of 0.1 mU/ml increased the oxidation of H_2-calcein as well as H_2-DCF to similar extents (Fig. 2C). The effect of HRP was independent of the presence of H_2O_2, and thus not mediated by $^{\cdot}OH$, which is known to rapidly oxidize H_2-DCF.

Calcein but not DCF is Retained in Cells

In order to determine the flux of DCF and calcein, the accumulation of DCF and calcein in the assay medium and in the intracellular compartment was compared following loading of the cells with either H_2-DCF or H_2-calcein. Although this approach is complicated by the fact that the amount of oxidized probe increases during the assay, it provides useful information regarding membrane permeability. Direct measurement of this value would, however, require intracellular injection of calcein and DCF.

DCF rapidly leaked out of the cells, with a half-life of less than 10 min and accumulated in the extracellular space. In fact, intracellular DCF concentrations reached steady state levels of approximately 40% after 20 min, whereas extracellular DCF concentrations increased almost linearly during the course of the experiment (Fig. 3A). In contrast, only 20% of the total calcein leaked out of H_2-calcein-loaded cells over the first 60 min. The intracellular concentration of calcein continuously increased during the assay without reaching a plateau (Fig. 3B). The fluorescence of calcein was more intense than that of DCF. Since the volumes of the two compartments are very different, the high calcein fluorescence indicates practically negligible leakage of the fluorescent dye. Moreover, the continuous increase in the signal from the oxidized probes indicates that cellular loading with H_2-DCF

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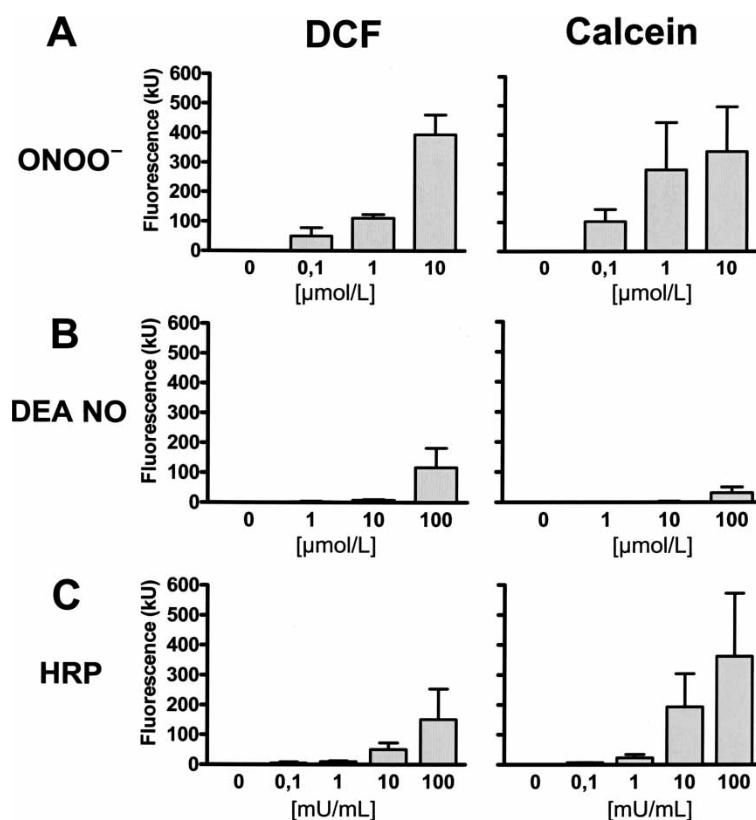

FIGURE 2 Reactivity of H_2-DCF and H_2-calcein *in vitro*. H_2-DCF-DA (5 µmol/l) and H_2-calcein-AM (5 µmol/l) were deacetylated by sodium hydroxide solution. Accumulation of the oxidation products DCF and calcein were measured in phosphate buffer (25 mmol/l) using a microplate reader (fluorescence expressed as arbitrary kilo units, kU) following the administration of the indicated concentrations of peroxynitrite ($ONOO^-$) (A), the NO-donor DEA-NONOate (DEA NO) (B) and horse-radish peroxidase (HRP) (C). $n = 3$.

and H_2-calcein was so efficient that even after 1 h there was no intracellular shortage of the non-oxidized compounds.

H_2-DCF is Superior to H_2-calcein in the Detection of Intracellular Oxidative Stress

In order to determine whether both compounds are suitable probes for intracellular ROS generation, the effect of DMNQ was studied. DMNQ is a semiquinone, which is taken up by cells and subsequently undergoes redox cycling with NADH to generate $O_2^{\cdot-}$. Treatment of cells with DMNQ significantly increased the oxidation of both H_2-DCF and H_2-calcein, so that no significant difference in the sensitivity of the two probes was observed (Fig. 4).

Redox cycling, however, does not reflect the physiological, enzymatic generation of ROS.

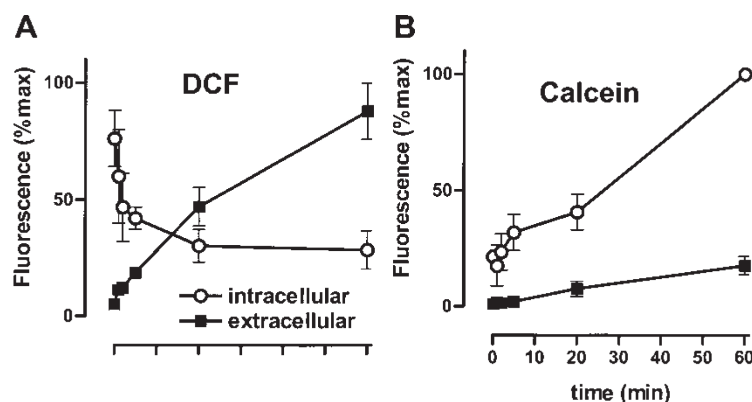

FIGURE 3 Intracellular retention of DCF and calcein in rat aortic vascular smooth muscle cells (RSMC). Cells grown on multi-well plates were loaded with H_2-DCF-DA (10 µmol/l) (A) or H_2-calcein-AM (2 µmol/l) (B) for 20 min. The cells were then washed and intracellular versus extracellular levels of the fluorophores were measured. For standardization, the maximal fluorescence signal obtained in the individual experiments was set to 100%. $n = 5$.

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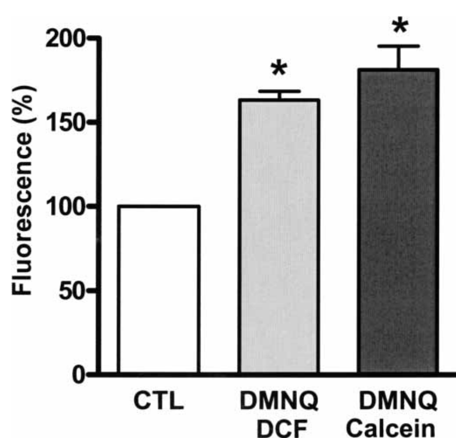

FIGURE 4 Comparison of H_2-DCF oxidation and H_2-calcein oxidation in response to a redox cycler in RSMC. RSMC were loaded with either H_2-DCF-DA (10 μmol/l) or H_2-calcein-AM (2 μmol/l) in the absence or presence of the semiquinone DMNQ (100 μmol/l). The relative effect of DMNQ on DCF and calcein accumulation was compared with that detected in unstimulated cells (CTL). $n = 3$, $*p < 0.05$.

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Therefore, the intracellular generation of ROS was increased by pretreating RSMC with Ang II, which increases ROS production by activating the vascular NADPH oxidase. While Ang II significantly increased the oxidation of H_2-DCF, it had absolutely no effect on the rate of H_2-calcein oxidation (Fig. 5). To ensure that this pronounced difference could not be attributed to an unspecific reaction of H_2-DCF, ROS production was also assessed by ESR spectroscopy using the spin trap CMH. The increase in CMH oxidation in response to Ang II was similar to that observed using H_2-DCF, indicating that H_2-calcein is indeed unable to detect ROS generated by the vascular NADPH oxidase in response to Ang II (Fig. 5A). In the next step, the effect of gp91ds-tat was compared to that of DPI. gp91ds-tat selectively inhibits the NADPH oxidase,[22] whereas DPI inhibits all flavin containing enzymes,[6] including microsomal cytochrome P450 monooxygenases,[18] NADPH oxidase[6] and the mitochondrial respiratory chain.[14] DPI and gp91ds-tat had little effect on basal ROS detection when H_2-DCF was used as a probe, whereas both compounds completely prevented the Ang II-induced increase in DCF accumulation. In contrast, the accumulation of calcein was completely unaffected by gp91ds-tat. DPI inhibited the basal oxidation of H_2-calcein by almost 50%, even in the absence of Ang II (Fig. 6).

H_2-DCF and H_2-calcein Detect ROS in Different Compartments of the Cell

The effectiveness of DPI under basal conditions may suggest that H_2-calcein predominantly detects constitutive flavin-dependent ROS formation in a cellular compartment distinct from that containing the NADPH oxidase. Indeed confocal microscopy revealed that DCF fluorescence is largely localized in the cytoplasm, whereas calcein fluorescence was almost exclusively restricted to the mitochondria (Fig. 7).

H_2-calcein but not H_2-DCF Directly Interacts with Complex I in Mitochondria

In order to determine the mechanism of mitochondrial H_2-calcein oxidation, the effects of different respiratory chain inhibitors on dye oxidation were assessed. In unstimulated RSMC, the mitochondrial complex III inhibitor antimycin A increased the fluorescence of DCF, whereas the complex III inhibitors stigmatellin and myxothiazol as well as the complex I inhibitor rotenone attenuated DCF fluorescence. In contrast, calcein fluorescence was inhibited only by rotenone (Fig. 8A). Since these data suggest that calcein specifically interacts with complex I we determined the effect of H_2-calcein on complex I activity in isolated submitochondrial particles. H_2-calcein (Fig. 8B) but not H_2-DCF (data not shown) inhibited the activity of complex I, demonstrating that H_2-calcein directly interacts with it. Accordingly, the oxidation of H_2-calcein by isolated mitochondria was independent of NADH (data not shown).

DISCUSSION

In the present study, we determined the suitability of H_2-DCF and H_2-calcein as indicators of intracellular ROS production. We found that both compounds possess a comparable sensitivity to different ROS *in vitro*, as well as to ROS generated intracellularly by the redox cycler DMNQ. However, when Ang II was used to increase intracellular ROS generation by the NADPH oxidase, the fluorescence of DCF but not calcein was increased. Calcein accumulated in the mitochondria and basal H_2-calcein oxidation was sensitive to an inhibitor of the mitochondrial complex I. However, stimulation of mitochondrial ROS production increased the oxidation of H_2-DCF without affecting that of H_2-calcein.

The structural similarities between H_2-DCF and H_2-calcein indicate that both compounds should exhibit a comparable sensitivity to ROS. Indeed, $ONOO^-$ as well as carbonate radicals and/or $^{\cdot}OH$ oxidized both compounds. Neither compound was oxidized by H_2O_2 and high concentrations of an NO donor were required to oxidize the probes (probably attributable to the formation of NO_2 radicals[29]). Moreover, both H_2-DCF and H_2-calcein were directly oxidized by HRP in a radical-independent manner, as reported previously for H_2-DCF.[23,24] Also, under certain conditions, H_2-DCF can act as reducing agent, donating protons under situations of

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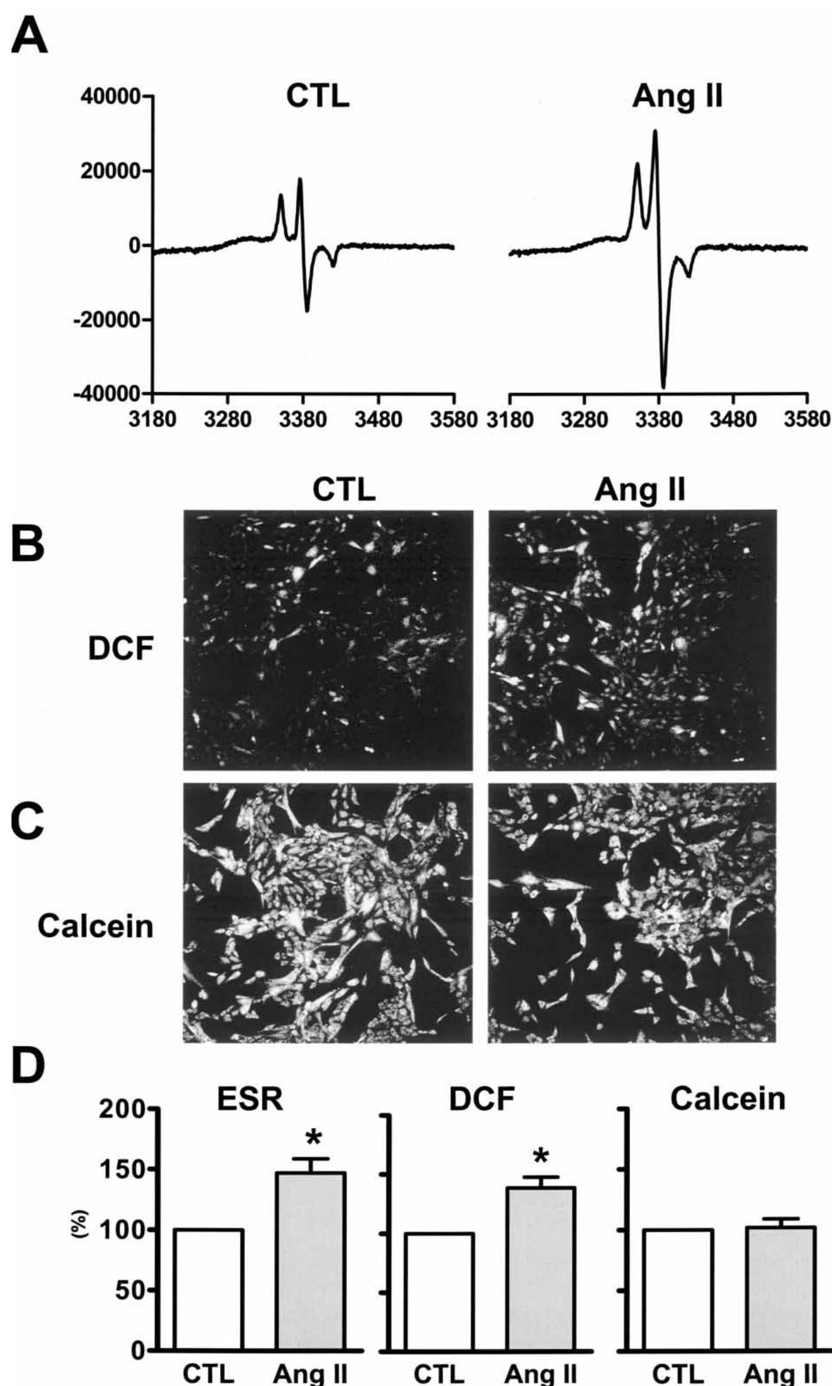

FIGURE 5 Determination of Ang II-induced radical generation in RSMC by electron spin resonance (ESR) and dye oxidation. The effect of Ang II (100 nmol/l)-induced radical generation on CMH-oxidation (2 mmol/l) as measured by ESR (A and D), H_2-DCF oxidation (B and D) and H_2-calcein oxidation (C and D) was determined in RSMC. (A–C): representative measurements using field sweep and laser scanning microscopy. (D) Statistical evaluation of ESR measurement and fluorometry using a microplate reader. $n = 5$, $*p < 0.05$. Data are expressed relative to the control (unstimulated) condition (CTL), (A) y-axis absorbance, x-axis gauss.

insufficient concentrations of either NADPH or glutathione.[24] This confounding reaction not only occurs in the presence of peroxidase, but also with cyclooxygenase[11] and gains importance in oxidizing environments, such as that associated with ischemia. Consequently, neither H_2-DCF nor H_2-calcein should be used to assess ROS production under conditions of ischemia-reperfusion.

In general, the specific fluorescence of calcein is greater than that of DCF.[28] This aspect might be advantageous under some conditions, since imaging DCF fluorescence by widefield fluorescence or laser scanning microscopy is difficult. H_2-DCF also has a very high tendency to undergo photo-oxidation.[17] Thus, the excitation light applied during microscopic assays rapidly but non-specifically increases the

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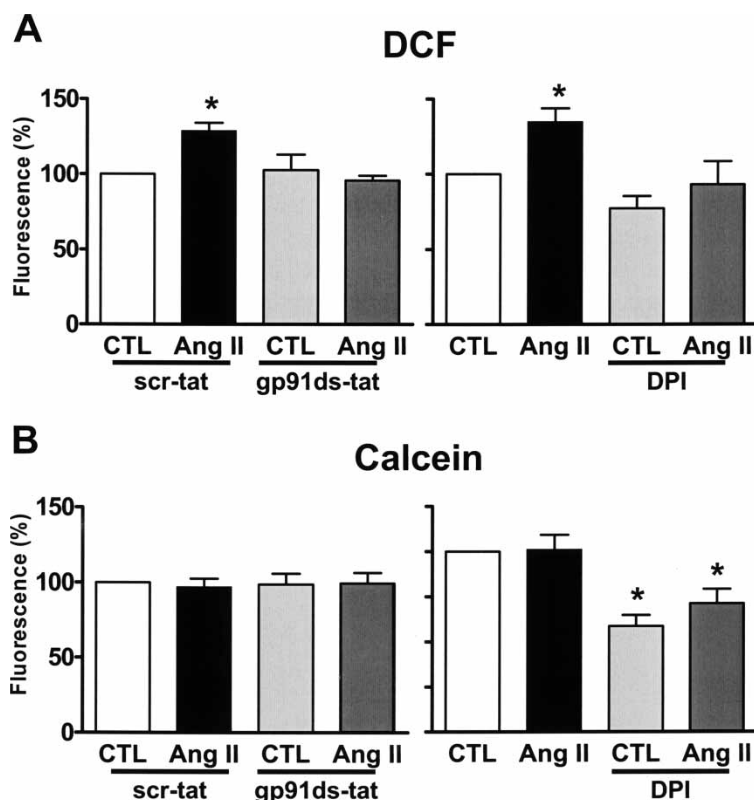

FIGURE 6 Comparison of the effect of inhibitors on H_2-DCF and H_2-calcein oxidation in Ang II-treated RSMC. Effects of the NADPH oxidase inhibitor gp91ds-tat (100 μmol/l), the control peptide scr-tat as well as the flavin-inhibitor diphenylene iodonium (DPI) (10 μmol/l) on the oxidation of H_2-DCF (A) and H_2-calcein (B) induced by Ang II (100 nmol/l) in RSMC. $n = 5$, $*p < 0.05$. Data are expressed relative to the control (CTL) condition.

oxidation of H_2-DCF. As a consequence, as little light as possible has to be used to image DCF, which results in low image quality and a low signal to noise ratio. Image quality is therefore much better with calcein, which also appears to be less susceptible to photo-oxidation (A.K. unpublished observation). It should, however, be mentioned that this point is particularly relevant to microscopic studies and not to the use of fluorescence microplate readers and flow cytometers in which the excitation energy and exposure time are much lower.

One important finding of the present study was that H_2-calcein failed to detect an increase in intracellular ROS in response to the Ang II-induced activation of the NADPH oxidase. Since cells are highly compartmentalized and ROS levels differ dramatically between different sub-cellular structures, it seemed logical to assume that DCF and calcein must accumulate in different sub-cellular compartments.

An oxidizing environment is present in the mitochondria and the stimulation of cellular energy consumption is thought to further increase mitochondrial oxidative stress.[4] In contrast, the generation of ROS following treatment with Ang II occurs at intracellular membranes, such as endosomes and the plasma membrane.[12] Consequently, the sub-cellular localization of the ROS-sensitive probe largely determines the outcome of the measurement. DCF has been suggested to partially accumulate in mitochondria, but substantial amounts of the compound are retained in the cytoplasm,[26] a finding confirmed in the present study. The flux of DCF to the extracellular space is therefore dependent on the volume of the cytoplasm relative to that of the mitochondria. Such a relationship may also account for the rapid loss of DCF from endothelial cells compared to cardiac myocytes, as the latter cell type has a much greater relative mitochondrial volume.[26] However, no information is available regarding the localization of calcein derived from H_2-calcein. The high polarity of the compound renders it almost completely membrane impermeable and thus facilitates its trapping in cellular organelles. That compartmentalization underlies the different properties of H_2-DCF and H_2-calcein can also be derived from the observations made with the flavin inhibitor DPI. Certainly, cells contain numerous flavin-dependent oxidases; in addition to the NADPH oxidase,[6] flavin-dependent ROS formation is most prominent in the mitochondrial respiratory chain, which is sensitive to DPI.[14] Our finding that DPI inhibited the oxidation of H_2-calcein but not H_2-DCF in resting cells, clearly demonstrated that

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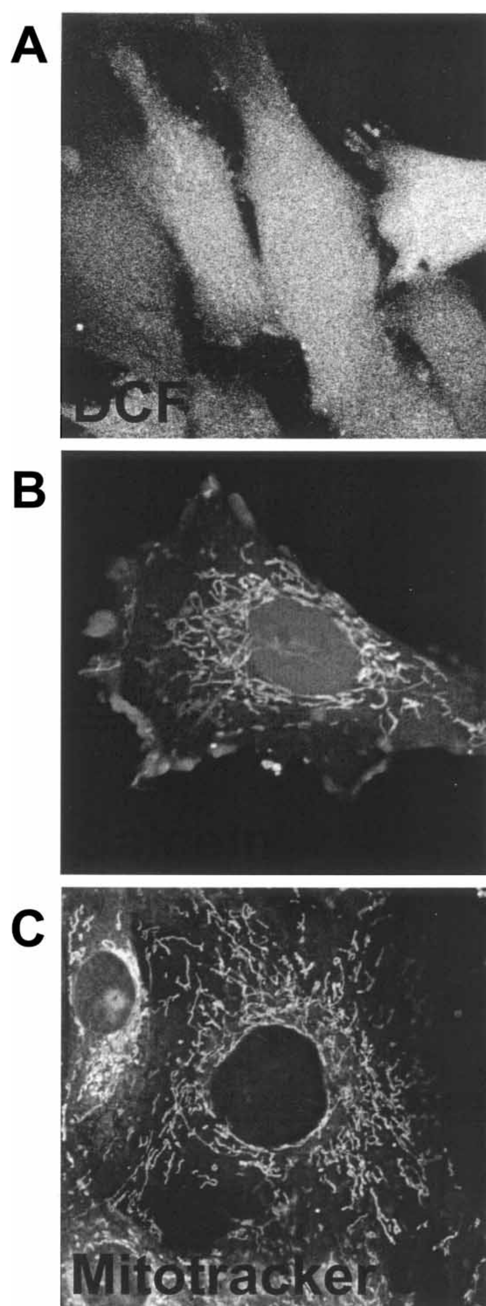

FIGURE 7 Intracellular localization of DCF and calcein in rat aortic smooth muscle cells. Confocal microscopy images of cells loaded with H_2-DCF-DA (5 μM) (A), H_2-calcein-AM (2 μM) (B) and mitrotracker green (100 nM) (C).

distinct flavin-dependent oxidases are responsible for the oxidation of the two compounds. Certainly, the mitochondrial trapping of H_2-calcein could account for the differential effects of DPI. Indeed, with the aid of confocal microscopy it was evident that calcein is almost exclusively localized to the mitochondria.

In order to determine the mechanism of mitochondrial-mediated dye oxidation, we assessed the effects of several inhibitors of complex I and III. Blockade of complex I decreased the oxidation of both dyes,

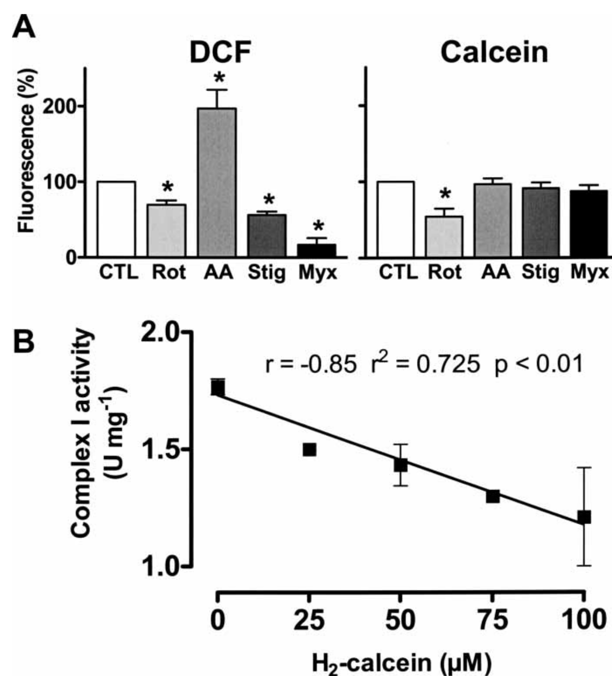

FIGURE 8 Involvement of mitochondria in H_2-DCF and H_2-calcein oxidation. (A) Effects of the complex I inhibitor rotenone (ROT, 10 μM) and the complex III inhibitors antimycin A (AA, 1 μM), stigmatellin (STIG, 100 nM) or myxothiazol (MYX, 100 nM) on the oxidation of H_2-DCF and H_2-calcein in RSMC. $n = 5$, $*p < 0.05$. Data are expressed relative to the control (CTL) condition. (B) Effect of H_2-calcein on mitochondrial complex I activity as determined by the NADH:ubiquinone assay in submitochondrial particles, $n = 3$.

whereas blockade of complex III had no effect on H_2-calcein oxidation. The fact that antimycin A increased H_2-DCF oxidation, whereas stigmatellin and myxothiazol decreased it, can be attributed to differences in the localization of the binding sites of these inhibitors on opposite sides of the membrane. Antimycin A blocks transfer of electrons at center Q_i, leading to the artificial formation of $O_2^{\cdot-}$ at center C_O in the mitochondrial intermembrane space, which is devoid of antioxidative defense.[8] The other two inhibitors, which prevent electron transfer at center Q_o, prevent this effect. On the basis of these observations it is possible to conclude that the oxidation of H_2-calcein by mitochondria is not mediated by $O_2^{\cdot-}$ in the intermembrane space, whereas complex III and $O_2^{\cdot-}$ contribute to the oxidation of H_2-DCF. Complex I is involved in H_2-calcein oxidation, although it is unclear whether this is facilitated by a radical-mediated effect or whether H_2-calcein acts as an substrate/electron donor for complex I. In order to dissect this point, we determined the effect of H_2-calcein on complex I NADH oxidase activity. H_2-calcein turned out to inhibit this enzyme with an estimated IC_{50} of 200 μmol/l, thus demonstrating a direct interaction between complex I and H_2-calcein. Complex I has been shown previously to interact with several different compounds. Indeed one class of compounds

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related to the fluorescein structure (erythrosin 5′-iodoacetamide) inhibits compound I with an IC_{50} of 20 nmol/l.[7] Nevertheless, using rotenone or DPI, inhibition of the oxidation rate of H_2-calcein of maximal 50% was reached, demonstrating that the complex I and flavin-dependent oxidation is not the only mechanism that oxidizes H_2-calcein in the cell. Certainly, we can only speculate about the mechanisms of this reaction. Given the high degree of autoxidation observed with hydrolyzed H_2-calcein, it can be speculated that unspecific oxidation may underlie this observation.

In summary, we have demonstrated that H_2-calcein can be used to assess ROS production *in vitro*. Although, the compound has similar chemical properties to established ROS indicators, it almost exclusively localizes to mitochondria where it may serve as electron donor for complex I of respiratory chain. Therefore, H_2-calcein is unsuitable as a probe for intracellular ROS formation and offers no alternative to H_2-DCF, despite the weak intracellular retention of the latter fluorophore.

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