RAPID COMMUNICATION

Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells

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Abstract: One of the objectives of studying endothelial cells in vitro is to evaluate neutrophil-endothelial cell interactions including potential consequences of oxidant-mediated damage to the endothelial cell. Current understanding of endothelial cell oxidative function is derived primarily from the measurement of extracellular products. We utilized 2 dyes, 2',7'-dichlorofluorescin diacetate (DCFH-DA) and hydroethidine (HE), which measure hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) respectively, for their suitability to monitor oxidative mechanisms in endothelial cells and to provide a reliable measure of intracellular oxidants. Endothelial cells stained with DCFH-DA and stimulated with H_2O_2 exhibited an increase in the fluorescent product 2',7'-dichlorofluorescein (DCF) (measure of intracellular H_2O_2) which peaked at 10 min. Endothelial cells stained with HE and stimulated with H_2O_2 exhibited an increase in the fluorescent product ethidium bromide (EB) (measure of intracellular O_2^-) which lasted for approximately 60 min. Superoxide dismutase increased DCF fluorescence in endothelial cells stimulated with H_2O_2 by 158%. Allopurinol (xanthine oxidase inhibitor) reduced DCF and EB fluorescence by 48% and 37% respectively in endothelial cells stimulated with H_2O_2. Catalase completely inhibited an increase in DCF or EB fluorescence in endothelial cells stimulated with H_2O_2. There was a direct correlation between mean DCF and EB fluorescence intensity and the concentration of H_2O_2 or the number of phorbol 12-myristate 13-acetate-activated neutrophils added to endothelial cells. We conclude from these studies that DCFH-DA and HE can be used to measure intracellular H_2O_2 and O_2^- in endothelial cells and that the xanthine oxidase pathway for intracellular O_2^- production accounts for approximately 40% of the total intracellular O_2^- generated in endothelial cells after stimulation with H_2O_2. The combination of image cytometry and flow cytometry will be important for future evaluations of endothelial cell function. *J. Leukoc. Biol.* 55: 253-258; 1994.

Key Words: *fluorochrome · xanthine oxidase · hydroethidine · ethidium bromide · 2',7'-dichlorofluorescin diacetate · 2',7'-dichlorofluorescein*

INTRODUCTION

Endothelial cells have been grown in culture since the 1970s. One of the objectives of endothelial studies has been to evaluate the oxidant stress on endothelial cells invoked by inflammatory cells such as the neutrophil and monocyte. The neutrophil is particularly important in the induction of oxidative damage on the endothelial cell [1-4]. The neutrophil must interact directly with the endothelial cell to initiate margination of the neutrophil out of the circulating pool. Eventually the neutrophil migrates through the endothelium and into tissues. Many disease states such as adult respiratory distress syndrome are manifestations of this neutrophil-endothelial interaction [5]. Understanding the ability of the endothelial cell to withstand oxidant stress induced by the neutrophil and other cells may clarify the response of the endothelial cell in disease states. However, our current understanding of endothelial cell oxidative function is based primarily on measurement of extracellular products [6-8].

2',7'-dichlorofluorescin diacetate (DCFH-DA) and hydroethidine (HE) are used to evaluate oxidative burst in neutrophils and monocytes [9-11]. DCFH-DA is freely permeable across cell membranes and is incorporated into hydrophobic lipid regions of the cell [12]. The acetate moiety is cleaved off by cellular esterases leaving the nonfluorescent 2',7'-dichlorofluorescin (DCFH). Hydrogen peroxide and peroxidases produced by the cell oxidize DCFH to 2',7'-dichlorofluorescein (DCF) which is fluorescent (530 nm). The green fluorescence measured is thus proportional to the H_2O_2 produced. HE is the sodium borohydride-reduced form of ethidium bromide (EB) [13, 14]. HE is freely permeable to cells and can be directly oxidized to EB by superoxide anion produced by the cell [9]. Intracellular EB is fluorescent (610 nm) with 488 nm excitation.

MATERIALS AND METHODS

Cell-free assay

Cell-free assays were performed to evaluate the oxidation of HE with several oxidants. Ten μM HE (Polysciences, Inc., Warrington, PA) was added to sterile-filtered 37°C Hanks's balanced salt solution with 0.22% glucose, 2 mM glutamine, and 1% bovine serum albumin (hereafter referred to as HBSS). Hydrogen peroxide (200 μM), 200 units/ml horseradish peroxidase (HRP) (Sigma Chemical Co., St. Louis, MO), and 7.51 mM potassium superoxide (Aldrich Chemical Co., Milwaukee, WI) (equivalent to 200 μM O_2^-) [15] were used to evaluate oxidation of HE to EB. Cell-free analysis was performed on a Perkin Elmer LS50B spectrofluorometer with kinetic measurements every 2 s at 37°C for 15 min. Excitation was 488 nm with a 5 nm slit and emission was collected at 610 nm with a 20 nm slit.

Abbreviations: 90LS, 90 degree light scatter; DCF, 2',7'-dichlorofluorescein; DCFH, 2',7'-dichlorofluorescin; DCFH-DA, DCFH-diacetate; EB, ethidium bromide; FS, forward angle light scatter; HBSS, Hanks's balanced salt solution; HE, hydroethidine; HRP, horseradish peroxidase; PBS, physiologic buffered saline; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; XO, xanthine oxidase.

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Staining of nonadherent endothelial cells

Dye incorporation studies and inhibitor studies were performed on endothelial cells in suspension. Rat pulmonary artery endothelial cells were isolated from a 350–400 g Long Evans male rat by an enzyme-free technique as previously described [16]. Endothelial cells (passage 4–20) were grown to confluence in T-25 culture flasks. After removal of the culture medium, cells were washed one time with sterile-filtered physiologic buffered saline (PBS), followed by the addition of 5 ml of 2 mM EDTA in PBS to the adherent endothelial cells. Following a 5–10 min incubation at 37°C, the cells were suspended by gentle pipetting followed by centrifugation (100*g* for 4 min). The endothelial cells were resuspended in sterile-filtered HBSS to a final concentration of approximately 1×10^6/ml.

For inhibitor studies, 1000 U/ml superoxide dismutase (SOD) (Sigma), 1000 U/ml catalase (Boehringer Mannheim, Indianapolis, IN), or 5 mM allopurinol (Sigma) was used to modulate cell oxidation. To confirm that the only modulatory role of SOD and catalase was enzyme-induced, control studies were performed with enzyme heat-inactivation (100°C for 15 min). The inhibitors were incubated with the endothelial cells at 37°C for 15 min. DCFH-DA (20 μM) (Molecular Probes Inc., Eugene, OR) or 10 μM HE was added to the cell suspension and sample collection was started immediately on the EPICS Elite flow cytometer (Coulter Corp., Hialeah, FL) using 488 nm excitation. Emission filters were 525 nm bandpass for DCF and 610 nm longpass for EB. Additionally, simultaneous UV-visible excitation was used for dye incorporation studies. HE was excited at 352 nm and emission collected with a 434 nm bandpass filter. Forward angle light scatter (FS) and 90 degree light scatter (90LS) were also collected. Time interval gating was used to allow several samples to be run concurrently on the flow cytometer with measurements taken every 10 min for 2 h [17]. Forty min after addition of DCFH-DA or HE, 500 μl of cells (approximately half the total cell volume in each tube) was transferred to a separate tube and H_2O_2 added to a final concentration of 200 μM. Cell analysis was continued on both the unstimulated and H_2O_2-stimulated sample. Cells were maintained at 37°C between sampling intervals on the cytometer.

Endothelial cell viability was determined by staining cells with 10 μg/ml propidium iodide (PI). Cells within a negative control gate for PI fluorescence (live cells) were back-gated on FS and 90LS. A bitmap gate was created around live cells in the FS and 90LS histogram which was applied to all fluorescence histograms so that only live cells were evaluated for DCF, HE, and EB fluorescence.

Staining of adherent endothelial cells

Adherent endothelial studies were performed with pulmonary artery endothelial cells (passage 4–20) grown to confluence in 24-well tissue culture plates or 35 mm tissue culture petri dishes (Costar Corp., Cambridge, MA). The RPMI culture medium was removed and the adherent endothelial cells were washed with HBSS. Twenty μM DCFH-DA or 10 μM HE was used to stain the endothelial cells. Adherent endothelial cells were incubated with DCFH-DA or HE at 37°C for 45 min and then washed 2 times with sterile-filtered HBSS. One ml of HBSS was added to each culture well or petri dish. Various concentrations (0–500 μM) of H_2O_2 were added to the endothelial cell cultures and incubated for 2 h. Alternatively, neutrophil concentrations ranging from 0–3.2 $\times$ 10^6/ml were stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA), added to the endothelial cell cultures and incubated for 4 h at 37°C. The neutrophils were collected from the peritoneal cavity of Sprague-Dawley rats after 6 h, 0.5% glycogen stimulation [18]. After incubation, the HBSS was removed, each well or petri dish was washed, and cells were resuspended as before in HBSS at 4°C. The cells were evaluated immediately on an EPICS Elite flow cytometer as described above. All endothelial cell experiments were performed in triplicate.

RESULTS

Cell-free assay

The cell free assay was performed to determine the relative potential of various agents to oxidize HE to EB. The relative intensity of EB fluorescence is represented in **Figure 1** as percentages with potassium superoxide (KO_2) as a 100% positive control. A relatively small degree of HE oxidation to EB occurred in HBSS alone; in HBSS with 200 μM H_2O_2; in HBSS with 200 U/ml HRP; and in HBSS with HRP and H_2O_2. A ten-fold increase in EB fluorescence intensity was evident when KO_2 was added in comparison to all other oxidants tested.

Non-adherent endothelial cells

Dye incorporation studies were performed with cells in suspension. Stable intracellular DCF fluorescence was evident 20–25 min after the addition of DCFH-DA to the endothelial cell suspension (**Fig. 2B**). Stable intracellular HE fluorescence was evident approximately 5 min after the addition of HE to the endothelial cell suspension followed by stable intracellular EB fluorescence at 10–15 min (Fig. 2A).

Stimulation of endothelial cells, as discussed below, was achieved by the addition of 200 μM H_2O_2 unless otherwise indicated. To test the intracellular oxidation of HE in endothelial cells, 200 μM H_2O_2 was added to endothelial cells 40 min after HE. A marked increase in mean EB fluorescence was evident that continued for approximately 60 min (**Fig. 3**). After this time, the increase in mean EB fluorescence in stimulated endothelial cells paralleled the increase in non-stimulated (control) cells. This same duration of increased EB fluorescence was observed for endothelial cells

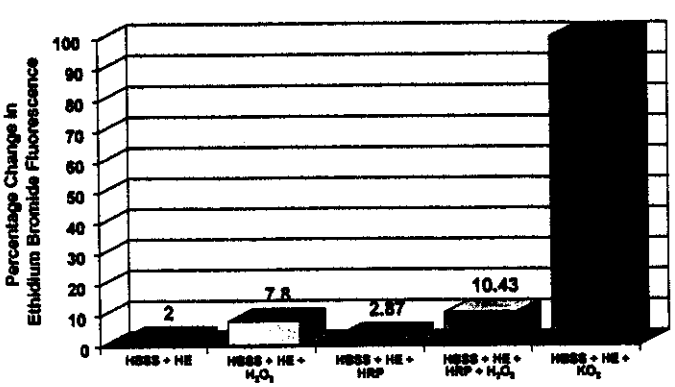

Fig. 1. Relative percentage of EB fluorescence in a cell-free assay after incubation at 37°C for 15 min. HRP (200 U/ml), 200 μM H_2O_2, or 7.511 mM KO_2 (equivalent to 200 μM O_2^-) was added to HBSS and 10 μM HE. Excitation was 488 nm and emission was collected at 610 nm. The KO_2 result was set at 100%. The O_2^- generated by KO_2 resulted in a 10-fold increase in EB fluorescence intensity when compared to any other combination tested in the cell-free assay.

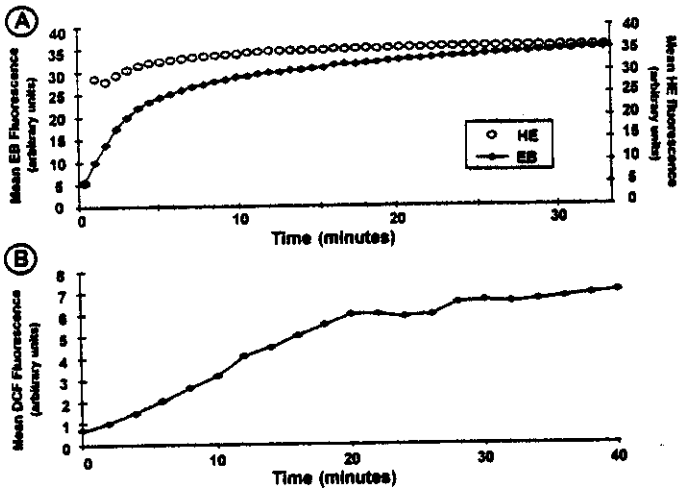

Fig. 2. Kinetics of (A) mean HE intracellular incorporation and simultaneous oxidation to EB and (B) mean DCF intracellular fluorescence. HE was excited at 352 nm and emission collected at 434 nm. EB was excited at 488 nm and emission collected at 610 nm. DCF was excited at 488 nm and emission collected at 525 nm.

pre-treated with 5 mM allopurinol. Catalase (1000 U/ml) had no effect on mean EB fluorescence in unstimulated cells but completely inhibited an increase in EB fluorescence in endothelial cells treated with H_2O_2. Unstimulated endothelial cells incubated with 5 mM allopurinol prior to addition of HE exhibited a slight decrease in EB fluorescence compared to cells without allopurinol. Allopurinol decreased mean intracellular EB fluorescence in H_2O_2-stimulated endothelial cells by 37% (Fig. 3).

Unstimulated endothelial cells incubated with SOD, catalase, or allopurinol exhibited no marked change in mean intracellular DCF fluorescence in comparison to untreated endothelial cells. Endothelial cells pre-treated with 1000 U/ml SOD exhibited a 158% increase in mean intracellular DCF 10 min after addition of H_2O_2 compared to untreated endothelial cells (Fig. 4). Endothelial cells pre-treated with 5 mM allopurinol exhibited 52% of the intracellular DCF fluorescence 10 minutes after addition of H_2O_2 in comparison to untreated endothelial cells.

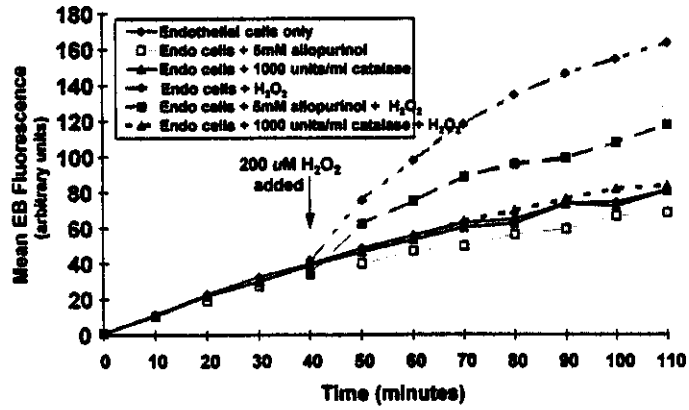

Fig. 3. Mean intracellular EB fluorescence (O_2^- production) in rat pulmonary artery endothelial cells versus time in min. Endothelial cells were incubated with allopurinol or catalase for 15 min at 37°C and analyzed every 10 min. After 40 min, 500 μl of cell suspension was placed in a separate tube and 200 μM H_2O_2 was added. Cell analysis continued every 10 min with cells incubated at 37°C between measurements. In comparison to untreated endothelial cells, allopurinol-treated endothelial cells exhibited 60% of O_2^- production after H_2O_2 stimulation. Catalase completely suppressed the H_2O_2-induced increase in EB fluorescence.

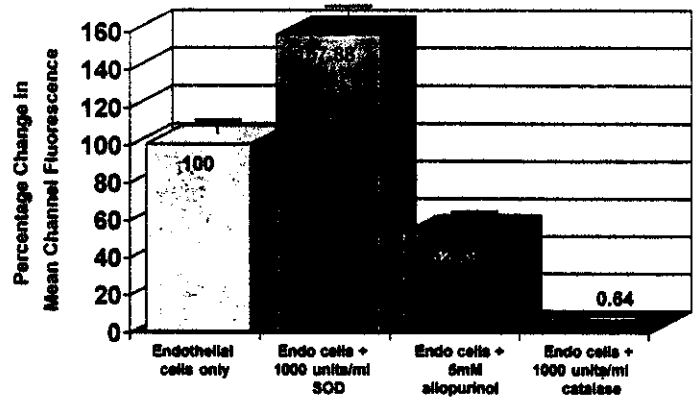

Fig. 4. Relative percentage of mean intracellular DCF fluorescence (H_2O_2 measurement) in rat pulmonary artery endothelial cells 10 min. after stimulation with 200 μM H_2O_2. Allopurinol, catalase, and SOD were incubated with the endothelial cells for 15 min at 37°C prior to the addition of DCFH-DA. Endothelial cells were incubated at 37°C for 40 min prior to the addition of 200 μM H_2O_2. SOD increased intracellular H_2O_2 due to the dismutation of O_2^- into H_2O_2. Allopurinol decreased intracellular H_2O_2 by inhibiting O_2^- production. Data shown are means ± 1SD; $n = 3$. All points are significantly different to the endothelial cells only control ($P < .01$).

As a control, heat-inactivated SOD or catalase had no effect on intracellular DCF fluorescence in unstimulated or H_2O_2-stimulated cells. Additionally, heat-inactivated catalase had no effect on EB fluorescence in unstimulated or stimulated endothelial cells. Endothelial cells incubated with 5000 U/ml SOD and stimulated with H_2O_2, exhibited no increase in mean intracellular EB fluorescence. However, heat-inactivated SOD also inhibited an H_2O_2-stimulated increase in endothelial EB fluorescence. Similar results were seen with SOD or heat-inactivated SOD in cell-free EB assays.

Adherent endothelial cells

Similar studies of adherent endothelial cells stained with DCF or EB exhibited a direct correlation between the concentration of H_2O_2 added and the mean intracellular fluorescence (data not shown). The intensity of the intracellular fluorescence was less in adherent cells compared to non-adherent endothelial cells. A marked decrease in cell viability, as determined by PI fluorescence, was evident when the concentration of H_2O_2 exceeded 200 μM.

Adherent endothelial cells stained with DCF or EB exhibited a direct correlation between the mean intracellular fluorescence and the number of PMA-activated neutrophils added to endothelial cells (Fig. 5). Similar to that of the H_2O_2-stimulated adherent endothelial cells, the fluorescence intensity was less in the adherent endothelial cell-neutrophil experiments than the non-adherent endothelial experiments. Endothelial cell viability (as determined by PI fluorescence) after 4-h incubation with neutrophils was not significantly affected even at the highest neutrophil concentration (3.2×10^6/ml). No increase in mean intracellular fluorescence intensity was evident when endothelial cells were incubated with unstimulated neutrophils.

DISCUSSION

DCFH-DA and HE are commonly used to evaluate oxidative burst mechanisms in neutrophils [9–11, 19]. The oxidative or respiratory burst in neutrophils results from activation of the membrane-bound NADPH oxidase via an electron transfer reaction. Superoxide anion is produced and then dismutates

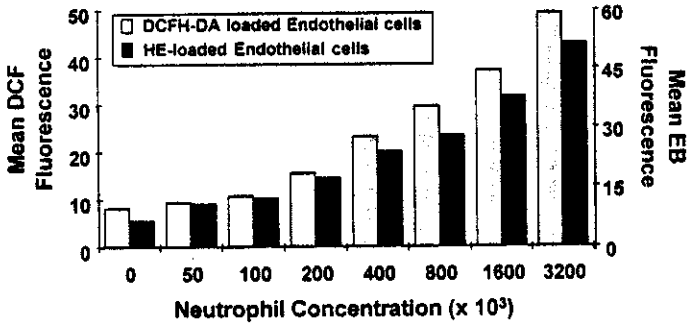

Fig. 5. Mean intracellular DCF fluorescence (H_2O_2 measurement) and EB fluorescence (O_2^- measurement) in rat pulmonary artery endothelial cells after 4-h incubation with PMA-activated rat neutrophils (100 ng/ml). Adherent endothelial cells were stained for 45 min with either DCFH-DA or HE, washed and maintained in HBSS. Various concentrations of PMA-activated neutrophils were added to the adherent endothelial cells and incubated for 4 h at 37°C. Cells were washed to remove the neutrophils, placed into suspension, and evaluated on the flow cytometer. Both DCF and EB endothelial cell fluorescence increased proportional to the neutrophil concentration. This was a representative data series of several experiments.

to H_2O_2 either spontaneously or via SOD. The reactive oxygen species and H_2O_2 produced are necessary for normal bactericidal mechanisms in the neutrophil.

HE is the sodium borohydride reduced product of EB and was initially developed as a vital dye which would cross cell membranes and label DNA [13, 14]. Therefore, visual assessment of the EB staining in a cell after oxidation of HE is primarily nuclear fluorescence as indicated in **Figure 6** (confocal image).

The spectrofluorometric assay used to evaluate the oxidation of HE in a cell-free assay was a modification of a similar experiment by Rothe and Valet [9]. Our results were similar to those of that study, which demonstrated that superoxide anion resulted in 10-fold increase in fluorescence intensity of EB compared to all other oxidizing agents tested.

Many publications discuss endothelial cell oxidation; however, all reported studies have used extracellular techniques to measure oxidation products [6–8]. There are no reports of measured intracellular oxidation products using DCF and EB in viable endothelial cells. With flow and image cytometry and the expanding availability of fluorescent probes, the measurement of intracellular physiologic compounds offers a more direct detection system than extracellular techniques. Additionally, image cytometry allows for intracellular measurements in functional, adherent endothelial cell monolayers.

To evaluate cells with a flow cytometer, the cells obviously have to be in suspension; therefore, the dye incorporation studies were performed with cells in suspension. However, endothelial cells are naturally adherent in vivo and in vitro, and placing them in suspension and performing physiologic experiments in a fluidic measurement system requires cautious interpretation. HE and EB attained stable intracellular fluorescence within 5 and 15 min respectively in nonadherent endothelial cells. Mean intracellular DCF fluorescence was stable within 20–25 min. In studies involving adherent endothelial cells, a 45-min dye incorporation time was used to ensure stable loading. No adverse effects were noted with extended dye incorporation times; thus, these probes appear stable and non-activating in endothelial cells.

Hydrogen peroxide was used to simulate oxidant released from phagocytic cells. Two hundred μM H_2O_2 was chosen because of previous results demonstrating greater than 90% cell viability (PI exclusion) and maximum DCF fluorescence in treated endothelial cells. This was also the maximum concentration that could be used before a rapid decrease in cell viability was observed. In nonadherent endothelial cells stained with HE, the addition of H_2O_2 caused a significant increase in EB fluorescence, which persisted for 60 min, compared to control cells. This suggests activation of an enzyme system that results in the production of O_2^- for a significant period of time. Earlier studies in phagocytic cells demonstrated that EB fluorescence was an indicator of superoxide anion generation and that O_2^- was acting to oxidize the HE to EB [9]. In our experiments, to verify O_2^- was oxidizing HE to EB, SOD was used to dismutate O_2^-. However, SOD and heat-inactivated SOD interfered with HE by an unknown mechanism. Potassium superoxide resulted in cell lysis and thus prevented its usefulness in generating O_2^- to evaluate intracellular oxidation of HE to EB. Catalase had no effect on the production of EB fluorescence in unstimulated cells but completely inhibited the generation of EB fluorescence in H_2O_2-stimulated endothelial cells presumably by removal of H_2O_2 prior to activating the generation of O_2^- inside the cell. In this system we only evaluated the role of catalase in removing extracellular H_2O_2. It is possible that catalase may enter the cell via pinocytosis and exert an intracellular effect. This effect was not evaluated in our system.

The source of all oxidation capacity in endothelial cells is unknown. Xanthine oxidase (XO) is an enzyme in endothelial cells that leads to the generation of O_2^- [20, 21]. In the presence of xanthine, XO reduces O_2 to O_2^- [22]. Additionally, H_2O_2 can be produced by either spontaneous or SOD-catalyzed dismutation of O_2^-. Allopurinol, an inhibitor of XO, partially inhibited the formation of O_2^- (EB fluorescence) in H_2O_2-stimulated endothelial cells. Allopurinol decreased the intracellular EB fluorescence by approximately 40%, suggesting that XO accounted for less than half of O_2^- generated by H_2O_2-stimulated endothelial cells. A

Fig. 6. Confocal microscope image of 4 endothelial cells stained with hydroethidine (z axis 2.0 μm). The image was collected 30 min after addition of 200 μM H_2O_2. The colors represent intensities of intracellular EB (excitation 488 nm, emission 610 nm). Once EB is formed it is rapidly intercalated into DNA, thus the nuclei are the primary fluorescent structures labeled.

previous report indicated that XO activity in endothelial cells was not increased by H_2O_2 [20]. While we did not measure XO directly, our data support an increase in XO activity in H_2O_2-stimulated endothelial cells inhibitable by allopurinol. Hydrogen peroxide is not normally considered a "stimulant"; however, in addition to our results, Bradley et al. previously reported that H_2O_2 activated ICAM-1 and MHC-1 expression in human umbilical vein endothelial cells [23]. Additionally, H_2O_2 has been shown to activate the transcription factor nuclear factor κB in Jurkat T cells [24]. It is possible that the endothelial cells may have been "primed" to respond to H_2O_2 by placing them in suspension or by the action of the fluorochrome (HE). Nitric oxide is another potential source of oxidant in endothelial cells. Additionally, nitric oxide has been shown to react with O_2^- to form peroxynitrite anion, another potential oxidant [25]. Depending on the rate of the various reactions, including dismutation of O_2^- by SOD, oxidation of HE to EB, or formation of peroxynitrite anion, it is possible that an increase in nitric oxide may directly or indirectly alter EB fluorescence. We are currently investigating the possible interactions of nitric oxide in this measurement system.

DCFH-DA has been used to measure intracellular H_2O_2 in a variety of cells including phagocytic cells, chondrocytes, renal epithelial cells, and HL-60 cells [9, 10, 26–28]. In experiments with nonadherent endothelial cells, peak DCF fluorescence was attained 10 min after the addition of H_2O_2. Endothelial cells incubated with SOD prior to staining with DCFH-DA exhibited 58% greater DCF fluorescence (in comparison to non-SOD treated cells) 10 min after the addition of H_2O_2. Superoxide dismutase can be endocytosed by cells, as reported previously, and was likely endocytosed by the endothelial cells [26, 29]. Thus, dismutation of O_2^- resulted in the increased generation of H_2O_2. Allopurinol, as shown above, decreased the O_2^- produced by H_2O_2-stimulated cells and resulted in a similar decrease in DCF fluorescence (48%) in H_2O_2-stimulated endothelial cells compared to cells without allopurinol treatment. These studies suggest that in addition to the direct oxidation of DCFH to DCF by the added H_2O_2, O_2^- dismutation to H_2O_2 contributed a significant amount of the intracellular endothelial cell H_2O_2 generated by this methodology.

In experiments with adherent endothelial cells, there was a direct correlation to the DCF or EB fluorescence and the concentration of H_2O_2 added to cultures. Cell viability, as determined by PI fluorescence, decreased significantly when the concentration of H_2O_2 increased above 200 μM. The intensity of fluorescence was less in adherent cells than in nonadherent cells, suggesting that the fluorescence intensity may be related to differences in cellular dye incorporation between adherent and nonadherent cells. However, the primary difference may be related to methodology since the stained, adherent endothelial cells were washed several times and resuspended prior to evaluation on the flow cytometer. Cells in suspension were not washed after dye loading. One factor explaining the degree of fluorescence attained in the endothelial cell suspension was related to an equilibrium of the dye in the intracellular and extracellular fluid. This equilibrium was probably obtained with the adherent endothelial cells, but was disrupted when the cells were repeatedly washed.

Adherent endothelial cells incubated with PMA-activated neutrophils exhibited an increase in H_2O_2 and O_2^- production (DCF or EB fluorescence) directly proportional to the number of PMA-activated neutrophils. To avoid overt staining of neutrophils added to the adherent endothelial cells, the endothelial cells were washed after staining with DCFH-DA or HE prior to addition of unstained neutrophils. It is conceivable that the neutrophils incorporated a small amount of the fluorescent dye. For this reason, every effort was made to separate the endothelial cells from the neutrophils and neutrophils adhered to endothelial cells by light scatter discrimination, and thus to isolate the endothelial cell fluorescence from any neutrophil fluorescence. The unique capacity of the flow cytometer to analyze only intracellular fluorescence allowed careful control of these studies. No change in fluorescence intensity was noted when unstimulated neutrophils were incubated with endothelial cells.

The present study has clearly demonstrated the utility of these intracellular fluorochromes in the evaluation of endothelial cell oxidation reactions. DCFH-DA and HE can be used to evaluate oxidant damage to endothelial cells invoked by several mechanisms including direct damage by neutrophils. Because of the recognized substrate specificities for each of the dyes, differences in fluorescence intensity between DCF and EB may provide additional information regarding endothelial cell function and response to oxidants. These dyes can probably be used for a variety of cell types to evaluate oxidative mechanisms and stresses on cells. While very useful for flow cytometric studies, DCFH-DA and HE will likely prove very beneficial for image cytometry studies of endothelial cells. Because of the different emission spectra, these dyes can also be used for dual color staining of cells for simultaneous oxidation product measurement.

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