Monitoring Intracellular Redox Changes in Ozone-Exposed Airway Epithelial Cells

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BACKGROUND: The toxicity of many xenobiotic compounds is believed to involve oxidative injury to cells. Direct assessment of mechanistic events involved in xenobiotic-induced oxidative stress is not easily achievable. Development of genetically encoded probes designed for monitoring intracellular redox changes represents a methodological advance with potential applications in toxicological studies.

OBJECTIVE: We tested the utility of redox-sensitive green fluorescent protein (roGFP)–based redox sensors for monitoring real-time intracellular redox changes induced by xenobiotics in toxicological studies.

METHODS: roGFP2, a reporter of the glutathione redox potential (E_GSH), was used to monitor E_GSH in cultured human airway epithelial cells (BEAS-2B cells) undergoing exposure to 0.15–1.0 ppm ozone (O3). Cells were imaged in real time using a custom-built O3 exposure system coupled to a confocal microscope.

RESULTS: O3 exposure induced a dose- and time-dependent increase of the cytosolic E_GSH. Additional experiments confirmed that roGFP2 is not directly oxidized, but properly equilibrates with the glutathione redox couple; Inhibition of endogenous glutathionin 1 (Grx1) disrupted roGFP2 responses to O3, and a Grx1-roGFP2 fusion protein responded more rapidly to O3 exposure. Selenite-induced up-regulation of GPx (glutathione peroxidase) expression–enhanced roGFP2 responsiveness to O3, suggesting that (hydro)peroxides are intermediates linking O3 exposure to glutathione oxidation.

CONCLUSION: Exposure to O3 induces a profound increase in the cytosolic E_GSH of airway epithelial cells that is indicative of an oxidant-dependent impairment of glutathione redox homeostasis. These studies demonstrate the utility of using genetically encoded redox reporters in making reliable assessments of cells undergoing exposure to xenobiotics with strong oxidizing properties.


The intracellular redox environment is a highly dynamic setting governed by the formation and degradation of various reactive species of oxygen and nitrogen. Under normal physiological conditions, the cytosol, the nucleus, and the mitochondrial matrix space maintain homeostatic conditions in favor of a highly reducing environment (Cannon and Remington 2008). Intracellular reducing conditions are largely maintained by millimolar concentrations of reduced glutathione and its accessory enzymes that together constitute the glutathione system (Anderson 1998). Ultimately, maintenance of the intracellular glutathione redox potential (E_GSH) comes from the metabolism of glucose because glutathione is reduced by glutathione reductase using NADPH (nicotinamide adenine dinucleotide phosphate) produced by the pentose phosphate pathway (PPP) (Wamelink et al. 2008).

A number of pathophysiological states are associated with changes in the E_GSH (Dubinina and Dadali 2010; Ma 2010; Yang and Omaye 2009). Such “oxidative stress” is commonly cited as a mechanistic feature of the toxicity of numerous xenobiotic compounds linked to adverse health outcomes (Bargagli et al. 2009; Cienciewicki et al. 2008; Kohen and Nyska 2002). For instance, the health effects of the potent ambient air pollutant ozone (O3) are understood to be mediated through an oxidative stress mechanism involving the oxidation of cellular biomolecules (Ballatori et al. 2009; Kelly et al. 1995; Mudway and Kelly 2000). In the lung, O3 exposure causes decreases in pulmonary function and induces inflammatory responses derived from the bronchial epithelium, a major target of O3 exposure (Ballinger et al. 2005; Cienciewicki et al. 2008; Kelly et al. 1995; Mudway and Kelly 2000; Pryor 1992; Pryor et al. 1995; Song et al. 2010). Because of its high reactivity, O3 interacts with cellular and extracellular biomolecules, resulting in multiple types of oxidative damage to lipids, proteins, and nucleic acids (Kelly et al. 1995; Laumbach 2010; Mudway and Kelly 2000; Srebot et al. 2009; van der Vliet et al. 1995; Yang and Omaye 2009). While numerous studies have established oxidant damage of biomolecules as a result of O3 exposure, direct measures of O3-mediated “oxidative stress” have been difficult to achieve; yet alteration of a defined intracellular redox couple like the glutathione redox pair (GSH/GSSG) would represent an important early indicator of the oxidative effects of O3 exposure.

Recent methodological advances have made it possible to focus studies on prooxidative changes to specific redox couples within defined subcellular compartments (Meyer and Dick 2010), potentially affording greater specificity in mechanistic investigations of the oxidative effects of xenobiotic exposures. A new generation of genetically encoded fluorophores permits direct assessment of the oxidative effects of xenobiotic compounds in relation to the GSH/GSSG redox pair with unprecedented spatial and temporal resolution (Cheng et al. 2012; Dooley et al. 2004; Meyer and Dick 2010). Redox-sensitive green fluorescent protein 2 (roGFP2) acts as a reporter of intracellular E_GSH by equilibrating with the GSH/GSSG redox pair (Gutsch et al. 2008; Meyer and Dick 2010; Morgan et al. 2011). In short, in a reaction that depends on catalysis by glutathioninins, roGFP2 responds to oxidation of reduced glutathione (GSH) to its oxidized form (GSSG) via the internal formation of a disulfide bond (Gutsch et al. 2008; Meyer 2008; Meyer and Dick 2010) (Figure 1). The formation of the disulfide bond alters the spectral characteristics of the GFP fluorophore causing the intensity of the emitted green fluorescence (~520 nm) induced by excitation at 488 nm to decrease, while causing the emitted fluorescence after excitation at 405 nm to increase, thus making this sensor a ratiometric reporter system for measuring changes in the intracellular redox environment.
probes. Further efforts to improve the responsiveness of roGFP2 have led to the conjugation of pathway-specific enzymes to create a chimeric fusion of proteins operating as redox relays. In particular, the conjugation of glutaredoxin 1 (Grx1) to roGFP2 has been shown to enhance the kinetics of the roGFP2 response to the oxidation of glutathione (Gutscher et al. 2008).

In the present study, we used live-cell microscopy to monitor the cytosolic $E_{GSH}$ of airway epithelial cells undergoing exposure to $O_3$ in real time. Here, we report an approach to validate the use of roGFP2-based redox sensors in toxicological studies of xenobiotics with strong oxidizing properties.

**Methods**

**Materials and reagents.** We purchased tissue culture media and supplements from Lonza (Walkersville, MD, USA), and Wilco Wells glass-bottom culture dishes from Ted Pella Inc. (Redding, CA, USA) and Warner Instruments (Hamden, CT, USA). Fugene 6 transfection reagent was acquired from Promega (Madison, WI, USA) and AbCam (Cambridge, MA, USA), respectively. Laboratory reagents and chemicals including hydrogen peroxide ($H_2O_2$), dihydrothreitol (DTT), 2-acetylamino-3-(4-(2-acetylamino-2-carboxyethyl)sulfanylthiocarbonyl)amino)methylbenzylpropionic acid (2-AAPA), buthionine sulfoximine (BSO), and sodium selenite were obtained from Sigma-Aldrich (St. Louis, MO, USA). Basic laboratory supplies were purchased from Fisher Scientific (Raleigh, NC, USA).

**Cell culture.** Transformed human airway epithelial cells [BEAS-2B, subclone S6 (Reddel et al. 1988)] were cultured as previously described (Tal’ et al. 2010) and maintained in serum-free keratinocyte growth medium (KGM; Lonza). The cells were incubated in a humidified incubator at 37°C in 5% CO$_2$ system maintained at 37°C with 1.5 L/min of balance air (O$_2$, 21%; CO$_2$, 5%; N$_2$ balance air). The entire exposure period typically consisted of three component intervals collectively lasting ≤ 1 hr. They included a) an initial untreated baseline period of 5 min; b) an exposure period of ≤ 45 min; and c) a 10-min control exposure period in which cells were oxidized by 0.1–1.0 mM H$_2$O$_2$ for 5 min and then reduced by 10 mM DTT for an additional 5 min. During these exposures, the O$_3$ concentration in the exposure chamber was monitored in real time using a Dasibi model 1003-AH O$_3$ analyzer (Dasibi Environmental Corporation, Glendale, CA, USA) sampling at a flow of 2.0 L/min. O$_3$ exposures for non-imaging assays were performed using exposure chambers operated by the U.S. Environmental Protection Agency’s Environmental Public Health Division.

**Imaging analysis.** All live-cell experiments done in real time were conducted using a Nikon Eclipse Ci4si spectral confocal imaging system equipped with an Eclipse Ti microscope, Perfect Focus System, and 404 nm, 488 nm, 561 nm, and 633 nm primary laser lines (Nikon Instruments Corporation, Redding, CA, USA).

**Figure 1.** roGFP2 interactions with the glutathione system (adapted from Meyer and Dick 2010). Glutathione peroxidases (GPx) oxidize GSH to GSSG in response to peroxides, including $H_2O_2$ and lipid hydroperoxides (LOOHs), thus increasing the glutathione redox potential ($E_{GSH}$). Abbreviations: LOOH, reduced lipid oxide; Se$,-$, reduced selenocyteine; SeOH, oxidized selenocyteine; SeSG, glutathionylated selenocyteine. In response to the increase in GSSG, one of the engineered vicinal cysteines of roGFP2 becomes $S$-glutathionylated by glutaredoxin (Grx). Glutathionylation in turn causes disulfide bond formation and alteration of the spectral properties of the GFP fluorophore. In the reductive pathway, Grx catalyzes the reduction of roGFP2 disulfide bonds through deglutathionylation as GSSG levels decrease and normal levels of GSH are reestablished by glutathione reductase (GR), at the expense of NADPH, causing a renormalization of $E_{GSH}$. Glucose and the pentose-phosphate pathway (PPP) create NADPH, which is used by GR to reduce GSSG to GSH.

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Melville, NY, USA). Images were acquired using a 60× Plan Apo lens. For experiments involving the genetically encoded fluorescent reporters, roGFP2 and HyPer, green fluorescence was observed via the use of independent excitations at 404 and 488 nm, and emitted light was collected for each using a 525/30 nm band-pass filter (Chroma, Bellows Falls, VT, USA). Results were calculated as ratios of the emissions excited by the 488 nm and 404 nm lasers scanned sequentially at a frequency of 1 min. All imaging data were acquired using Nikon EZ-C1 software.

**Measurement of intracellular NADPH.** After cells were equilibrated in fresh KGM or Locke solution (LS + G or LS – G) for 2 hr, the intracellular levels of total NADPH were assessed using an AbCam (Cambridge, MA, USA) NADP/NADPH assay kit according to the manufacturer’s instructions. Following the equilibration period, cells were immediately placed on ice and washed with cold 1× PBS just before the initial lysis step. Absorbance was read at 450 nm using a PolarStar Optima microplate reader (BMG Labtech, Durham, NC, USA).

**Statistical analysis.** All imaging data were quantified using NIS-Elements AR software (Nikon). For each experiment, the responses of 5–10 cells were collected as regions of interest and then averaged to derive an overall response. Data are expressed as the mean of at least three repeated experiments. Pairwise comparisons of control and treatment groups were performed using analysis of variance and linear regression, with some cells responding with strongly

**Results**

O3 exposure induces an increase in the cytosolic E<sub>GSH</sub>. The presence of glucose in the exposure media is known to shorten the half-life of O3 (Taylor-Clark and Undem 2010). Therefore, in these experiments, cells were first equilibrated in LS + G for 2 hr, and then switched to LS – G for the exposure. Exposure of BEAS-2B cells expressing cytosolic roGFP2 to 0.15–0.50 ppm O3 resulted in a dose- and time-dependent probe response, reflecting an increase in the cytosolic E<sub>GSH</sub> [Figure 2; see also Supplemental Material, Figure S5 (http://dx.doi.org/10.1289/ehp.1206039)]. Increasing O3 concentration hastened the onset while elevating the magnitude of the oxidative response reported by roGFP2 (Figure 2). Adding 0.1 mM H<sub>2</sub>O<sub>2</sub> at the end of each O3 exposure produced a maximal response, which was fully reversible with the addition of 10 mM DTT.

**Glucose deprivation potentiates the elevation of E<sub>GSH</sub> induced by O3 exposure.** On an individual basis, BEAS-2B cells equilibrated in the presence of glucose (i.e., in LS + G) were observed to respond variably to a given concentration of O3, suggesting substantial heterogeneity of redox homeostasis within the cellular population under the given conditions. Figure 3A shows the individual responses of seven BEAS-2B cells in the same field of view being exposed to 0.5 ppm O3, with some cells responding with strongly increasing E<sub>GSH</sub>, while others responded only minimally. Furthermore, some cells exhibited an intermediate response followed by a recovery of E<sub>GSH</sub> despite continued O3 exposure. Given the importance of PPP-generated NADPH in maintaining intracellular GSH levels, we hypothesized that glucose deprivation would sensitize the cells to a subsequent O3 exposure. As shown in Figure 3B, depriving the cells of glucose for 2 hr before exposure homogenized the magnitude, time of onset, and rate of response of the cells to O3. Glucose status did not affect the probe response to the addition of H<sub>2</sub>O<sub>2</sub> and DTT (Figure 3A,B). As expected, glucose deprivation led to decreased cellular NADPH levels [see Supplemental Material, Figure S2 (http://dx.doi.org/10.1289/ehp.1206039)].

**Validation of glutathione-dependent roGFP2 responses to O3 exposure.** Given the extreme reactivity of O3 with biomolecules, we considered the possibility that the spectral changes of the probe interpreted as changes in E<sub>GSH</sub> are the result of a direct oxidation of roGFP2 by O3 itself or by an O3-generated secondary oxidant. We therefore undertook a series of experiments to determine whether O3-induced changes in the roGFP2 fluorescence intensity ratio involve components of the glutathione system through which roGFP2 has been demonstrated to respond (Figure 1). We first aimed to confirm that O3 exposure leads to increased levels of GSSG. To this end we assessed the extent of intracellular glutathione oxidation in control and O3-treated cells. We observed ≤3-fold increases in GSSG following 1 ppm O3 exposure in LS – G as compared with air controls [see Supplemental Material, Figure S3 (http://dx.doi.org/10.1289/ehp.1206039)], which agrees with previous reports of the effect of O3 exposure on intracellular glutathione (Chalfant and Bernd 2011; Todokoro et al. 2004).

Next, we asked whether the roGFP2 response to O3 is influenced by glutaredoxin (Grx) activity. Grx is essential to mediate roGFP2 oxidation by GSSG (Figure 1), but Grx should play no role if roGFP2 is directly oxidized by O3. On the one hand, we compared the response of roGFP2 (which interacts with endogenous Grx) with that of Grx1-roGFP2, a translational fusion of Grx1 and roGFP2. The fusion of these components is known to kinetically improve the equilibration between roGFP2 and GSSG in a highly specific manner (Gutsch et al. 2008; Meyer and Dick 2010). We found that the O3-induced increase of E<sub>GSH</sub> in BEAS-2B cells expressing Grx1-roGFP2 occurred earlier and at a faster rate relative to that reported by cells expressing unlinked roGFP2, thus indicating...
a GSSG/Grx–specific response (Figure 4A). In contrast, we investigated the effect of glutaredoxin inhibition by pretreating cells with the diethiocarbamate derivative 2-AAPA (Sadhu et al. 2012). The inhibitor completely blocked roGFP2 responses to O₃ and H₂O₂ (which also act indirectly through glutathione oxidation and reduction, respectively) (Figure 4B), thus confirming the role of Grx as the catalyst necessary for roGFP2 responsiveness to O₃. Together these observations confirm that O₃ is not simply sensed by the probe through direct oxidation, but rather by its specific effects on the glutathione redox couple.

Having confirmed that O₃ induces formation of GSSG, which is then detected by the roGFP2 probe, we asked whether glutathione peroxidases (GPx), major generators of GSSG, are involved in the O₃ response. Here we investigated the role of GPx activity in O₃-induced roGFP2 redox changes by pretreating BEAS-2B cells with 1 µM sodium selenite for 48 hr before O₃ exposure. Previous studies have used selenium supplementation as an effective means of increasing GPx expression, a finding that we also observed in preliminary studies with BEAS-2B cells [see Supplemental Material, Figure S4 (http://dx.doi.org/10.1289/ehp.1206039)] (Helmy et al. 2000; Holben and Smith 1999; Leist et al. 1996). Selenium-induced overexpression of GPx accelerated roGFP2 oxidation during a 0.5 ppm O₃ exposure (Figure 4C), suggesting that O₃ gives rise to peroxides, which are then converted by GPx to GSSG, which is in turn reported by roGFP2 through the intervention of Grx.

Investigating the role of secondary products in O₃-induced redox changes. Because the data shown in Figure 4C suggested the involvement of peroxides in the O₃ response, we asked whether there is a specific role for H₂O₂. For these experiments we examined H₂O₂ generation as a consequence of O₃ exposure using the cytosolic-targeted H₂O₂ sensor, HyPer. O₃ caused a relatively modest increase in the HyPer response during the exposure period (Figure 5). However, the observed HyPer response did not precede nor match the magnitude of the roGFP2 response, making it unlikely that the observed increase in E₆₅₀ is primarily caused by H₂O₂ generation.

Mitochondrial oxidant production, frequently associated with increased oxidation of mitochondrial glutathione, has been implicated as a contributing factor in the cellular response to xenobiotics (Cheng et al. 2010, 2012; Hanson et al. 2004). Therefore, we next used mitochondrial targeted roGFP2 (roGFP2-mito) to assess the impact of O₃ exposure on the mitochondrial E₆₅₀ of BEAS-2B cells. As shown in Figure 6, exposure to 1 ppm O₃ twice the amount used for cytosolic assessments, induced an increase in mitochondrial E₆₅₀. However, relative to the cytosolic roGFP2 response, the increase in mitochondrial E₆₅₀ occurred at a slower rate and achieved a lower magnitude, suggesting that mitochondrial oxidants are not the primary source of the oxidants that lead to increased cytosolic E₆₅₀.

**Discussion**

“Oxidative stress” is a frequently cited mechanistic component of the adverse health effects induced by numerous xenobiotic compounds (Bargagli et al. 2009; Chung and Marwick 2010; Ciencwewicki et al. 2008; Jones 2008; Kohne and Nysk 2002; MacNee 2001; Ward 2010; Yang and Omaye 2009). However, the term “oxidative stress” is a very broad concept and the detection of early and specific indices of oxidant stress has proven to be methodologically difficult. The advent of genetically encoded fluorescent reporters that are sensitive to their redox environment has enabled real-time imaging-based assessments of oxidant outcomes in living cells with unprecedented spatial and temporal resolution. In this study, we validated the use of one such reporter, roGFP2, for the specific assessment of xenobiotic-induced changes in the E₆₅₀.

Using O₃ as a model oxidant and BEAS-2B cells as a model of the human bronchial epithelium.

The prooxidative change in E₆₅₀ observed in this study represents an early event in the oxidant injury caused by O₃. O₃ is a potent oxidant gas that has the potential to interact directly with virtually any cellular component, potentially including fluorescent reporter molecules such as roGFP2. Thus, in interpreting the probe response observed in O₃-exposed BEAS-2B cells, we had to consider the possibility that O₃ could be bypassing the glutathione system through which roGFP2 sensors normally respond (Gutschker et al. 2008; Meyer and Dick 2010).

Our findings strongly suggest that even in the presence of a strong oxidant like O₃, roGFP2 is oxidized only indirectly through its known coupling to the glutathione system. This conclusion is supported by several observations: First, glucose deprivation increased O₃-mediated roGFP2 oxidation, consistent with the requirement for NADPH in robustly maintaining E₆₅₀, the lack of glucose preventing regeneration of reducing equivalents throughout the exposure period. NADPH levels were approximately 70% lower in cells equilibrated in the absence of glucose, which appears to be sufficient to sensitize cells uniformly. In addition, it is important to bear in mind that other cellular processes also draw on the NADPH pool, and the continued lack of glucose largely prevents active regeneration of reducing equivalents throughout the exposure period.

Second, we confirmed the role of glutaredoxin in mediating the roGFP2 response to O₃. Grx1 is required to transfer oxidizing equivalents from the glutathione pool to roGFP2. In previous studies using Grx1-roGFP2, the chimeric linkage of Grx1 to roGFP2 enhanced responses to physiological oxidants such as H₂O₂ (Gutschker et al. 2008; Meyer and Dick 2010). Importantly, Grx1-roGFP2 also accelerated the roGFP2

![Figure 4](http://dx.doi.org/10.1289/ehp.1206039)

**Figure 4.** Manipulation of the glutathione system modulates roGFP2 responses to O₃. (A) Changes in cytosolic E₆₅₀ induced by 0.5 ppm O₃ exposure as reported by BEAS-2B cells expressing either roGFP2 or Grx1-roGFP2. (B) BEAS-2B cells were pretreated with 100 µM 2-AAPA, a glutaredoxin inhibitor, before exposure to 0.5 ppm O₃; the responses shown are the normalized 404/488 ratios plotted relative to their established baseline. (C) BEAS-2B cells were pretreated with 1 µM sodium selenite for 48 hr before 0.5 ppm O₃ exposure. To facilitate comparison of the responses in (A) and (C), the normalized ratios were plotted as a percentage of the signals obtained at maximal oxidizing and reducing conditions achieved using 1 mM H₂O₂ and 10 mM DTT, respectively. Other experimental conditions were as described for Figure 2. Values shown are mean ± SE (n ≥ 3).

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response to O₂, whereas inhibition of endogenous Grx with 2-AAAPA prevented roGFP2 oxidation in the presence of O₂. Although early reports describe 2-AAAPA as being an inhibitor of glutathione reductase, a more recent study reports that this dithiocarbamate derivative acts as a direct inhibitor of Grx as well (Sadhu et al. 2012). Thus, the finding that 2-AAAPA-treated cells failed to respond to O₂ supports the involvement of Grx and is consistent with the claim that 2-AAAPA is an inhibitor of Grx. Importantly, the fact that use of this inhibitor was effective at disconnecting the glutathione pool from the redox reporter argues against a nonspecific interaction between O₂, or a secondary oxidant, and the roGFP2 sensor.

Last, findings from experiments examining the effect of overexpression of glutathione peroxidases (GPx) using prolonged selenite supplemented preparations are consistent with an upstream role of GPx in the oxidative pathway leading to O₂-induced roGFP2 oxidation. GPx couple the reduction of (hydro)peroxides to the generation of GSSG (Arbur 2000; Matsé and Sánchez-Jiménez 1999; Meyer and Dick 2010). In our system, the increased expression of GPx-enhanced roGFP2 oxidation, suggesting that O₂ exposure generates (hydro)peroxides, which then drive the formation of GSSG. In fact, O₂ has been shown to produce many types of lipid hydroperoxides upon exposure (Mudway and Kelly 2000; Yang and Omaye 2009). Moreover, cells exposed to O₂ may have an increased H₂O₂ burden as well. Overall, the results from GPx-overexpressing cells suggest that the increased activity of these enzymes leads to an enhanced catalytic destruction of peroxides with concomitant GSH oxidation, leading to the roGFP2 response to O₂.

Using the H₂O₂ probe HyPer, our initial assessments suggested slightly elevated H₂O₂ production following O₂ exposure of BEAS-2B cells. The quantitative interpretation of HyPer responses is, however, difficult. It is not clear to what extent the OxyR domain of HyPer may be outcompeted by endogenous peroxidases. In addition, HyPer is highly pH sensitive, as much as the cpFP module on which it is based (Schwarzlender et al. 2012). Thus, an O₂-induced intracellular acidification could dampen the HyPer response to H₂O₂. Nevertheless, following O₂ exposure, HyPer responded to exogenously applied H₂O₂ and DTT as expected, which demonstrates the general functionality of the probe. Taken together, the delayed time of onset, the slow rates of response, and the relatively low magnitude of the HyPer responses seem to suggest that H₂O₂ production is not a major factor in the total O₂-induced EGH₂₀₁₀₀ effects. Likewise, the measurements in mitochondrial targeted roGFP do not support a mitochondrial source for the O₂-induced increase in cytosolic EGH₂₀₁₀₀. These results suggest that the relevant oxidant species, potentially a hydroperoxide, is primarily generated in the cytosol, or within the outer plasma membrane, and the mitochondrial EGH₂₀₁₀₀ response would be expected to lag behind that of the cytosol. Additionally, differences in peroxidase composition and activity may contribute to the lag in the O₂-induced mitochondrial EGH₂₀₁₀₀ response relative to the cytosol.

The studies presented herein cannot completely exclude a partial contribution of direct interactions between O₂, or its secondary by-products, with the thiols of the roGFP2 sensor. In addition, because the roGFP2 fluorescence ratio reflects EGH₂₀₁₀₀, which is a function of both the GSSG:GSH ratio and the total glutathione concentration, it is possible that O₂-induced electrophilic attack mediates the changes reported by roGFP2 by consuming reduced GSH. It should, however, be noted that the EGH₂₀₁₀₀ in the cytosol or mitochondrial matrix is much more sensitive to an increase in GSSG than to depletion of GSH. This is because the EGH₂₀₁₀₀ in the cytosol (around –320 mV, or even lower, in mitochondria) represents nanomolar GSSG in a millimolar pool of GSH. To deflect the roGFP2 signal from –320 mV to about –260 mV only requires the concentration of GSSG to increase from 200 nm to 20 μM (in a 10 mM total glutathione pool). To achieve the same magnitude of EGH₂₀₁₀₀ response by depleting GSH exclusively would require a loss of 90% of GSH (e.g., from 10 mM to 1 mM) (Meyer and Dick 2010). Such a massive depletion of GSH by parts-per-million concentrations of O₂ (which could generate only limited amounts of electrophiles) seems stoichiometrically unlikely. If one also considers the relative kinetics of GSSG generation and consumption (GSSG generation by GPx exhibits second order rate constants in the range of 1 × 10⁻⁶ M⁻¹ sec⁻¹), it appears reasonable to suggest that the O₂ effect reported in our study is primarily due to GSSG generation and that the contribution of glutathionylated electrophiles (E) formation is minor.

Thus, although several different O₂-induced processes may together drive glutathione oxidation, including lipid peroxidation, H₂O₂ generation, and other oxidizing processes, the available evidence strongly suggests that the cytosolic roGFP2 responses to O₂ exposure are appropriately reporting the EGH₂₀₁₀₀. Taken together, these results demonstrate that roGFP2-based sensors can be used to monitor shifts in glutathione redox homeostasis in O₂-exposed cells. Furthermore, the experimental approach we used may be utilized for the validation of “oxidant stress” induced by other reactive xenobiotics in living cells.

Conclusion

Our results demonstrate the utility of using genetically encoded fluorescent reporters in making reliable assessments of cells undergoing exposure to xenobiotics with strong oxidizing properties.

References


