The GOX/CAT system: A novel enzymatic method to independently control hydrogen peroxide and hypoxia in cell culture

Mueller S¹*, Millonig G¹, Waite GN¹,²

¹ Department of Medicine and Center for Alcohol Research, Liver Disease and Nutrition, Salem Medical Center, University of Heidelberg, Heidelberg, Germany
² Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Terre Haute, IN, USA

ABSTRACT

The increasing demand in studying cellular functions in cultured cells under various levels of oxygen and hydrogen peroxide \((\text{H}_2\text{O}_2)\) is only partly fulfilled by conventional approaches such as hypoxia chambers, bolus additions of \(\text{H}_2\text{O}_2\) or redox-cycling drugs. This article describes the recently developed enzymatic GOX/CAT system consisting of glucose oxidase (GOX) and catalase (CAT) that allows the independent control and maintenance of both \(\text{H}_2\text{O}_2\) and hypoxia in cell culture. In contrast to hypoxia chambers, the GOX/CAT system more rapidly induces hypoxia within minutes at a defined rate. The degree of hypoxia is dependent on the GOX activity and the diffusion distance of oxygen from the medium surface to the adherent cells. In contrast, \(\text{H}_2\text{O}_2\) levels are solely controlled by the ratio of GOX and CAT activities. They can be adjusted at non-toxic or toxic dosages over 24 hours. Thus, the GOX/CAT system mimics a non-phosphorylating respiratory chain and allows to adjust \(\text{H}_2\text{O}_2\) levels under hypoxic conditions truly simulating \(\text{H}_2\text{O}_2\) release e.g. by inflammatory cells or intracellular sources. GOX/CAT can be employed to address many questions ranging from redox signaling to ischemia/reperfusion studies in transplantation medicine. Factors such as HIF1alpha that respond both to hypoxia and \(\text{H}_2\text{O}_2\) are an especially attractive target for the novel methodology. Several applications are discussed in detail to demonstrate the technical requirements and potentials. In addition, simplified protocols are presented for cell or molecular biology labs without dedicated biophysical equipment.

Key words: hypoxia, hydrogen peroxide, catalase, glucose oxidase, redox signaling, hypoxia inducible factor

INTRODUCTION

Oxygen and \(\text{H}_2\text{O}_2\) are interdependent molecules of the cellular energy metabolism controlling a multitude of cellular functions. However, both molecules can be highly toxic depending on factors such as the co-presence of transition metals. During evolution, cells have developed sophisticated detoxification mechanisms to eliminate so-called reactive oxygen species (ROS) including \(\text{H}_2\text{O}_2\). In addition to their role in cell metabolism and survival, \(\text{H}_2\text{O}_2\) and oxygen have recently gained much attention as cellular signal transduction molecules. This opened an additional realm of studies aimed at investigating their effects on many cellular functions [1-8].

The increasing need to study \(\text{H}_2\text{O}_2\) and hypoxia under several experimental conditions is only partly fulfilled by conventional approaches such as hypoxia chambers and \(\text{H}_2\text{O}_2\) bolus additions. Hypoxia chambers show a slow onset of hypoxia [9] and prevent the co-exposure to \(\text{H}_2\text{O}_2\) during hypoxia. \(\text{H}_2\text{O}_2\) boli (usually between 50 and 500 µmol/l) expose cells to artificially high concentrations of \(\text{H}_2\text{O}_2\) as compared to (patho) physiological \(\text{H}_2\text{O}_2\) levels e.g. the release by inflammatory cells below 10 µmol/l[10]. Another drawback of \(\text{H}_2\text{O}_2\) boli is the short half-life time of approximately 10 min under cell culture conditions [11] which prevents long term exposure to \(\text{H}_2\text{O}_2\) for hours to days.

These insufficiencies have led us to develop a completely different approach in studying the effects of hypoxia and \(\text{H}_2\text{O}_2\) alone or in combination. The oxygen depleting/\(\text{H}_2\text{O}_2\) producing system consists of a combination of GOX and CAT mixed into the cell culture medium. Due to its specific kinetic
properties, the GOX/CAT system allows the independent and rapid control and maintenance of hypoxia and \( \text{H}_2\text{O}_2 \) in cultured cells. It has first been successfully used to study signaling functions of \( \text{H}_2\text{O}_2 \) in iron homeostasis [12-18] and, recently, it has been extended to induce hypoxia [19].

The structure of this article is designed to offer enough background for all readers to use the system. At the same time, precise and practical comments for the use at the bench are given, including detailed protocols in Appendix A and supplemental tables to facilitate the calculation of GOX/CAT conditions. We also briefly describe real time measurements of very low \( \text{H}_2\text{O}_2 \) levels in an additional Appendix B, since they have been instrumental in developing the GOX/CAT system and may be necessary to validate or adjust experimental conditions. However, since we have made great effort to provide robust protocols, it will not be necessary to set up the \( \text{H}_2\text{O}_2 \)-assay in any lab that intends to use the GOX/CAT system under most circumstances.

For general orientation, section 2 is recommended. Sections 3 and 4 discuss separately and in more detail the conditions required to control \( \text{H}_2\text{O}_2 \) and hypoxia. Section 5 demonstrates the application of the GOX/CAT system giving three different examples. Taken together, the GOX/CAT system is an attractive approach for all researchers that would like to study their individual experimental culture system under close to realistic \textit{in vivo} conditions such as less than atmospheric oxygen pressures and physiological \( \text{H}_2\text{O}_2 \) levels.

Due to a large size of the full article its Supplemental Tables and Appendices are available only online at: http://dx.doi.org/10.2478/v10039-009-0042-3.

2. General principles of the GOX/CAT system

2.1. Overview

The GOX/CAT system is established by the addition of glucose oxidase (GOX) and catalase (CAT) to buffered solutions containing at least 5 mmol/l D-glucose. Fig. 1 shows the overall stoichiometry. GOX generates \( \text{H}_2\text{O}_2 \) by consuming oxygen while catalase degrades \( \text{H}_2\text{O}_2 \) back to water and half a molecule of oxygen. Thus, the overall reaction consumes oxygen which is the prerequisite for generating hypoxia. Due to the special kinetic properties of GOX and catalase which will be discussed later in sections 2.3 and 2.4, the system generates stable \( \text{H}_2\text{O}_2 \) concentrations that only depend on the ratio of the enzyme activities. In contrast, hypoxia is mainly controlled by the GOX activity and the medium volume since it defines the diffusion distance for gaseous oxygen to reach the cell culture bottom with adherent cells. The GOX/CAT system offers the unique opportunity to independently control both hypoxia and \( \text{H}_2\text{O}_2 \) in cell culture. All other molecules involved in the system are either physiological (water, glucose) or physiologically inert (gluconolactone) under cell culture conditions. Some limitations of the system will be discussed in sections 3.2. and 4.2. For those readers who are mainly interested in the application of the GOX/CAT system, we recommend to start straight with sections 3.2., 4.2. and Appendix A. These sections have been designed in a way that not too much theoretical background is required. Readers who are interested in only the \( \text{H}_2\text{O}_2 \)-or in only the hypoxia-aspect of the system may also directly jump to the respective sections.

2.2. History

The study and application of both GOX and catalase have a long history in biochemistry, cell biology and biotechnology. GOX has first been isolated by Müller in 1928 from the fungus \textit{Aspergillus niger} and the bacterium \textit{Penicillium glaucum} [20]. It has been intensively studied several decades ago [21] and finally cloned in 1989 [22,23]. Interestingly, GOX is also produced in the hypopharyngeal gland of the honey bee and secreted into the honey [24]. The role of GOX in all of these organisms is thought to be antibacterial via \( \text{H}_2\text{O}_2 \) as an oxidant. Commercially available GOX preparations are purified from \textit{Aspergillus niger}. In industry, GOX has been broadly used to remove glucose and oxygen for a better preservation of food,
as essential part of biosensors [25] and in clinical analysis for blood glucose measurements [26].

Catalase was one of the first enzymes to be isolated and purified [27,28]. Its activity was first noticed in 1811 when Louis Jacques Thénard discovered H2O2. In 1900, Oscar Loew named the enzyme catalase, and in 1937 catalase from beef liver was crystallized by James B. Sumner. In 1969 the amino acid sequence of bovine catalase was identified and it took another 10 years (1981) to obtain the 3D structure. Catalase is an evolutionary highly conserved enzyme found in all aerobic microorganisms, plants and animal cells [29, 30]. It is extremely efficient in degrading H2O2 since it cannot be saturated by H2O2. In other words, turnover rates solely and linearly depend on the concentration of H2O2 up to molar concentrations. In mammalian cells, catalase is normally localized in peroxisomes rendering it less efficient in removing extraperoxisomal H2O2 [31]. In the food industry, catalase is used to remove H2O2 from milk prior to cheese production.

The first combined applications for GOX and catalase have been used to avoid accumulation of H2O2, to prevent oxidation reactions, to develop H2O2 sensors [32] and to induce hypoxia in cultured bacteria [33]. In addition, GOX has been used as oxidative stress model for in vitro and in vivo studies [34-38]. Based on a previously developed ultra-sensitive real time assay for H2O2 [10,13], we were then able to adapt large-scale industrial GOX/CAT systems to a low-scale cell culture system in a true quantitative manner [12]. The GOX/CAT system has been first successfully used in our laboratory to study signaling reactions, to develop H2O2 sensors [32] and to induce hypoxia in cultured bacteria [33]. In addition, GOX has been used as oxidative stress model for in vitro and in vivo studies [34-38]. Based on a previously developed ultra-sensitive real time assay for H2O2 [10,13], we were then able to adapt large-scale industrial GOX/CAT systems to a low-scale cell culture system in a true quantitative manner [12].

Figure 2. Formation of steady-state H2O2 concentrations in a GOX/CAT system under saturating glucose conditions.

\[ \frac{\Delta[H_2O_2]}{\Delta t} = k_{GOX} \times [H_2O_2] \]

\[ k_{GOX} = k_{CAT} \times [H_2O_2] \]

\[ [H_2O_2]_{ss} = k_{GOX} / k_{CAT} \]

H2O2 generation by GOX follows a zero order kinetics, while catalase removes H2O2 by first order kinetics. H2O2 steady-state concentration is reached when the GOX activity equals the CAT activity and can numerically be obtained by the ratio of the two activities.

There are several reasons why GOX is a reliable H2O2 source for cell culture applications:

a) In contrast to other oxidases such as xanthine oxidase, GOX exclusively converts oxygen to H2O2 in a stoichiometrically simple 1:1 relationship, without producing appreciable amounts of other biologically relevant oxidants such as the superoxide anion.

b) GOX works well at physiological pH of 7.4 although its optimal activity lays between pH 4.5 and 6.

c) GOX is highly substrate specific. It uses primarily D-glucose; other sugars such as D-mannose and D-fructose are oxidized at a much reduced rate.

d) Since most cell culture media have glucose concentrations between 5 and 25 mmol/l, GOX can work in these media near saturated conditions (Km of 9.8 mmol/l) allowing almost constant turnover independent of glucose levels [43].

e) GOX is a stable enzyme that remains fully active over 24 hours at 37°C.

f) GOX does not exist in mammalian cells; therefore it does not interfere with endogenous enzyme expression.

The activity of GOX also leads to the production of D-gluconolactone which may cause acidification of culture media. In water, gluconolactone slowly, within minutes to hours, hydrolyzes to gluconic acid/gluconate with a pKa of 3.5 to 3.8. In Aspergillus niger, the acidification is avoided by the presence of gluconolactones [44]. Accumulation of gluconolactone and concomitant acidification of the medium are not relevant as long as the GOX/CAT system is solely used for H2O2 generation. Under such conditions, only small activities of GOX are used that do not result in high level of gluconolactone. However, the accumulation of gluconolactone/ gluconate and the depletion of glucose can become critical at high GOX activities usually requiring replacement of culture medium (see also section 4.2.).
The GOX/CAT system: A novel enzymatic method to independently control hydrogen peroxide and hypoxia in cell culture

2.4. Control of H$_2$O$_2$ by catalase

Catalase (E.C. 1.11.1.6) is added to the system to control H$_2$O$_2$ levels for several reasons. It decomposes H$_2$O$_2$ to water and oxygen without requiring further substrates. Second, it is not saturable even at molar H$_2$O$_2$ concentrations. This results in a typical exponential decomposition kinetics of H$_2$O$_2$ as is shown in Fig. 20. This is also the reason why H$_2$O$_2$ degradation directly depends on the concentration of H$_2$O$_2$ (Fig. 2).

This kinetic property of catalase is an important prerequisite to generate hydrogen peroxide steady-state, [H$_2$O$_2$]$_{ss}$ levels in combination with GOX. Although other H$_2$O$_2$-degrading enzymes exist, they are less suitable as enzymatic partners for GOX in an H$_2$O$_2$-producing system. Thus, glutathione peroxidase (GPO) becomes saturated at concentrations higher than 1 µmol/l. In addition, H$_2$O$_2$ affects cellular glutathione levels preventing GPO to form stable [H$_2$O$_2$]$_{ss}$ in combination with GOX [45]. Although the H$_2$O$_2$ degradation capacity of culture media and cultured cells is typically very low, the GOX/CAT system may also work without the addition of external catalase. Catalase activity of cultured cells should then be determined e.g. as shown in Fig. 12 and only very small amounts of GOX are required (Tab. 1).

3. GOX/CAT as steady-state H$_2$O$_2$ system

In general, the GOX/CAT system can be used in three ways. First, it can be used to expose cells physiologically relevant and sustained H$_2$O$_2$ concentrations which will be discussed in more details in the next two sections 3.1 and 3.2, respectively. Second, the system can be used to induce hypoxia, and, third, combinations between various degrees of hypoxia and H$_2$O$_2$ concentrations are possible which will be discussed in section 4.

3.1. Control of H$_2$O$_2$

Fortunately, generation of H$_2$O$_2$ by GOX is almost independent of oxygen, glucose and gluconolactone (Fig. 2) under saturating conditions. As is shown in Fig. 3, GOX is saturated due to a K_m for glucose at 9.7 mmol/l in high glucose media [43]. Steady state levels of H$_2$O$_2$ are reached as soon as the H$_2$O$_2$ degradation rate of catalase equals the H$_2$O$_2$ production by GOX (Fig. 2). Fig. 4 shows in real time the formation of a H$_2$O$_2$ steady state after addition of GOX to a solution containing catalase and glucose. It can be seen that [H$_2$O$_2$]$_{ss}$ forms within seconds. Fig. 6 demonstrates that such sustained H$_2$O$_2$ levels can be maintained for many hours.

The ratio of GOX and catalase activity determines the concentration of H$_2$O$_2$. Under equilibrium conditions, H$_2$O$_2$ steady state concentrations can be directly calculated as [H$_2$O$_2$]$_{ss}$ = $K_{GOX}/K_{CAT}$. Since [H$_2$O$_2$]$_{ss}$ concentrations are

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<th>GOX dilution*</th>
<th>Oxygen (%)</th>
<th>Catalase dilution**</th>
<th>24h Glucose consumption (mmol/l)</th>
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</table>

* GOX activity of stock solution at 25 mmol/l glucose and in the presence of catalase
** Value takes into account the endogenous cellular catalase activity

Figure 3. GOX activity as a function of glucose concentration.

GOX activity has been determined as rate of H$_2$O$_2$ generation using the luminol/hypochlorite assay. Since high glucose culture media contain 25 mM glucose, GOX remains rather stable despite slow glucose depletion. Depletion of glucose from 25 to 5 mmol/l will decrease GOX activity by about 50%.

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The ratio of GOX and catalase activity determines the concentration of H$_2$O$_2$. Under equilibrium conditions, H$_2$O$_2$ steady state concentrations can be directly calculated as [H$_2$O$_2$]$_{ss}$ = $K_{GOX}/K_{CAT}$. Since [H$_2$O$_2$]$_{ss}$ concentrations are
described as a mere ratio between GOX and catalase activity, the same [H$_2$O$_2$]ss can be achieved at various absolute enzyme concentrations. Thus, 1 μmol/l H$_2$O$_2$ will be achieved when combining a $K_{GOX}$ of 1.5 x 10^{-6} M s$^{-1}$ with a $K_{CAT}$ of 1.5 s$^{-1}$ but also when combining $K_{GOX}$ of 5 x 10^{-6} M s$^{-1}$ and $K_{CAT}$ of 5 s$^{-1}$. Fig. 6 demonstrates that the resulting [H$_2$O$_2$]ss concentration is a function of catalase activity over a wide range. Tab. 1 can be used to compare GOX activity and CAT activity (presented as enzyme dilutions), as well as [H$_2$O$_2$]ss level and the time needed until a stable H$_2$O$_2$ concentration is reached.

Especially in culture media with 25 mmol/l glucose can be provided in such excess that $V_{max}$ is maintained over 24 hours. As a rule of thumb, depletion of glucose from 25 to 5 mmol/l lowers GOX activity by about 50% which needs to be considered when calculating H$_2$O$_2$ ss conditions (Tab. 1). On the other hand, GOX activity almost linearly depends on the oxygen concentration under typical physiological concentrations below 200 μmol/l oxygen (not shown). Thus, if used as a mere H$_2$O$_2$ source, conditions should be avoided that may result in hypoxia (see also section 4). We have identified culture conditions (Tab. 1) that will not result in significant decrease of oxygen and no expression of hypoxia-sensitive factors such as HIF1 is observed under these conditions [18]. In order to maintain oxygen at circa 200 μmol/l, rather low GOX concentrations and small media volumes should be used.

An important prerequisite to develop and validate the GOX/CAT system was the ability to measure micro- and nanomolar H$_2$O$_2$ levels in real time using the luminol/hypochlorite assay [10,13]. This assay is a powerful tool to determine enzyme activities and to confirm [H$_2$O$_2$]ss concentrations. Detailed protocols are presented in Appendix B. Alternative assays e.g. by Dringen et al., [46] can be used to confirm H$_2$O$_2$ ss levels. However, they do not allow real time measurements which can become a limiting factor in case of fast changes of H$_2$O$_2$. GOX and catalase activities can also be determined by oxygen electrodes although no H$_2$O$_2$ ss can be validated with such methods [47]. Finally, it should be noted that the time of equilibrium formation of [H$_2$O$_2$]ss depends on the absolute activities of enzymes and estimates are provided in the right column of Tab. 1.

3.2. Practical considerations
Tab. 1 provides examples of GOX and CAT dilutions for using GOX/CAT as an H$_2$O$_2$ source. In addition, the glucose consumed over 24 h and the time necessary to achieve the [H$_2$O$_2$]ss are indicated. The supplemental Tab. IS allows to enter cellular catalase activity as e.g. determined in Fig. 17. Although other H$_2$O$_2$ degrading mechanisms e.g. by GPO or peroxiredoxins have been neglected, the pragmatic focus on cellular catalase activity has been feasible and accurate enough for practical applications. Small variations in [H$_2$O$_2$]ss are due to a variety of factors such as cell type, cellular metabolic state, and cell density. In general, it is recommended to test not only one condition but various [H$_2$O$_2$]ss within one experiment.
The GOX/CAT system: A novel enzymatic method to independently control hydrogen peroxide and hypoxia in cell culture

We have earlier shown that the cellular toxicity in a GOX/CAT system is mainly determined by the level of H$_2$O$_2$ and not other factors such as hypoxia, glucose depletion etc. Therefore, it is advisable to know exactly the H$_2$O$_2$ mediated cytotoxicity threshold for individual cell lines. If new cell lines are explored, we recommend to identify toxic and subtoxic conditions over 24 hours by using cytotoxicity assays such as cell counts, trypan blue dye exclusion, or the MTT assay. An example is shown in Fig. 13.

Tab. 1 can then be used to define GOX/CAT combinations that provide for several [H$_2$O$_2$]ss within the biologically identified boundaries.

To avoid unwanted hypoxia, GOX has to be used at high dilutions. As a general rule, GOX dilutions of higher than 1:100,000 at normal cell culture medium volume (e.g. 1 ml in 12 well plate or 10 ml in 10 cm dish) will not cause significant hypoxia. The use of low GOX concentrations has the additional advantages of slow glucose depletion from the medium and slow production of gluconolactone and concomitant decrease of the pH. GOX should never be incubated without catalase in culture media since H$_2$O$_2$ can rapidly accumulate to such high levels that are per se toxic to cells even during short exposure times. On a final note, any novel experimental set-up has to be checked for the presence of potential interferences with the GOX/CAT system. For instance, sodium azide, monoethyl peroxide, sulphide, and cyanide are known inhibitors of GOX/CAT. Also, metal ions such as Ag$^+$, Hg$^{2+}$, Cu$^+$ can interfere with the system. Such potential interferences should be tested, for instance by using the luminol/hypochlorite assay.

### 4. GOX/CAT as steady-state hypoxia system

We will now mainly focus on the conditions of the GOX/CAT system to induce hypoxia in cultured cells. In general, as shown in Fig. 1, the GOX/CAT system is an oxygen consuming system since the catalase reaction recycles only 50% of oxygen. Hypoxia develops under conditions where oxygen consumption is larger than its replacement from air. Fig. 7A illustrates this principle in a cell culture dish, filled with medium to a height x, with access to atmospheric oxygen at the top, and with settled or attached cells at the bottom. Oxygen will be consumed by GOX and by the cells at the bottom of the dish, at the same time as oxygen will dissolve from air into medium, from where it diffuses along its concentration gradient. Comparable to the [H$_2$O$_2$]ss formation described in Section 3, an oxygen equilibrium will form when oxygen consumption by GOX equals oxygen supply by diffusion.

Fig. 7B shows the principle of the classical hypoxia chamber. The cell culture dish is hermetically separated from the environment of 21% oxygen when flushed with a low oxygen gas mixture such as of 2% oxygen. It has been shown that it takes 4 to 24 hours for the cells to be stably exposed to the desired conditions [9]. For comparison, our system can reach stable hypoxia by 20 minutes as explained in Section 4.1 and 4.3. In addition, the decrease of oxygen is well controlled and defined by the GOX activity.

#### 4.1. Control of hypoxia

GOX activity and medium volume which determines the diffusion distance of oxygen from the surface to the bottom of the culture dish are the two major factors that affect hypoxia in a GOX/CAT system. Fig. 8 shows that a high activity of GOX (1:10,000 dilution) causes a more rapid and pronounced hypoxia of about 1% oxygen as compared to lower GOX activities. Since oxygen electrodes require permanent perturbation and may destroy the oxygen gradient,
The addition of CAT to GOX decreases the rate of oxygen consumption by 50% (CAT versus no CAT), while the change in CAT activity (1:100,000 versus 1:1,000) has no further effect. Oxygen concentration has been measured using an oxygen electrode.

these measurements could only be performed in large reaction volumes. Therefore, modeling was required according to Fick’s diffusion laws for smaller cell culture systems such as 96-well plates which will be presented in Section 4.3 below. Fig. 9 demonstrates that the presence of catalase decreases the rate of oxygen consumption by 50 percent as compared to GOX alone. However, further variations of catalase activity do not alter oxygen consumption.

The second determinant for control of hypoxia is the medium volume. A large culture volume increases the diffusion distance of oxygen from the medium surface to the culture dish bottom. Fig. 10 shows plots of oxygen concentrations at the bottom of culture vessels as a function of diffusion distance for three different GOX activities. Corresponding volumes of medium for various cultured dishes can be taken from Tab. 3 and 4.

**Table 2.** Various conditions of the GOX/CAT system to obtain steady-state oxygen and H$_2$O$_2$ concentrations at the bottom of a 12-well plate, K$_{GOX}$ = 1.5 x 10$^{-3}$ mol/l s$^{-1}$ and K$_{CAT}$ = 1000 s$^{-1}$. Endogenous K$_{CAT}$ = 0 s$^{-1}$. Enzyme activities and desired [H$_2$O$_2$]ss conditions can be changed in the supplemental Excel Tab. 2S to adapt conditions to other experimental requirements. For completeness, Tab. 2S further provides the actual GOX activities at the bottom of the well that are adjusted to the oxygen concentrations.

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<th>Volume (ml)</th>
<th>GOX Activity (Dilution)</th>
<th>Hypoxia (% oxygen)</th>
<th>CAT Dilution for1 μmol/l H$_2$O$_2$ ss</th>
<th>CAT Dilution for5 μmol/l H$_2$O$_2$ ss</th>
<th>CAT Dilution for10 μmol/l H$_2$O$_2$ ss</th>
<th>24h Glucose consumption (mmol/l)</th>
<th>Oxygen equilibrium time (min)</th>
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<td>466,667</td>
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<td>4.0</td>
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<td>3,500,000</td>
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<tr>
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<td>71,795</td>
<td>358,974</td>
<td>71,794</td>
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<td>87</td>
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<td>100,000</td>
<td>11.0</td>
<td>127,273</td>
<td>636,364</td>
<td>1,272,727</td>
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<tr>
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<td>700,000</td>
<td>3,500,000</td>
<td>7,000,000</td>
<td>1.3</td>
<td>&gt;360</td>
</tr>
</tbody>
</table>

Notes: For sole hypoxia studies, H$_2$O$_2$ should be kept below 0.1 μmol/l. Dependent on the cell line, it is advised to consider the endogenous cellular catalase activity and recalculate the conditions using the Excel spreadsheet provided in Tab. 2S.
4.2. Practical considerations

By adjusting medium volume (diffusion distance) and GOX activity (oxygen depletion rate), the GOX/CAT system allows rapid induction of various stable hypoxic levels between 0.01 and 21% at a defined rate. On the left side of the Tab. 2, various hypoxia conditions can be selected including GOX dilutions and medium volume. The right columns allow identifying appropriate catalase dilutions to independently control H\textsubscript{2}O\textsubscript{2} ss. If the system is designed to only produce hypoxia, H\textsubscript{2}O\textsubscript{2} ss below 0.1 µmol/l should be selected since such concentrations are typical present in most aqueous solutions [13] and do not affect cell regulation and cellular metabolism. The far right part of the table further indicates the amount of glucose consumed over 24h and the time necessary to achieve stable hypoxia (equilibrium). An additional supplemental Tab. 2S is provided as an Excel file that allows to modify experimental conditions and to enter different enzyme activities including cellular catalase activity.

It is important to mention that stirring, and for that matter any movement, may disturb oxygen gradients. Consequently, it is advised to leave the culture plates/dishes undisturbed during incubation. At the time of cell harvest, we typically carefully transfer the cell containers to an ice-water slurry to immediately cool down, before any potential effects due to re-oxygenation might occur. Due to the simplicity and robustness of the GOX/CAT system, duplicate cultures can be setup e.g. for microscopic inspection and they can be discarded thereafter.

Glucose depletion and gluconolactone accumulation are the only other critical limitations of the GOX/CAT system and should be considered in the design of any experiment. As was shown in Fig. 3, GOX activity (expressed in H\textsubscript{2}O\textsubscript{2} production over time) is a function of glucose concentrations. Fig. 3 also demonstrates that depletion of glucose from 25 to 5 mmol/l reduces GOX activity by a factor of two. Tab. 2 approximates glucose consumptions at various settings of the GOX/CAT system. For example, at 25 mmol/l glucose, a GOX activity at a dilution of 1:100,000 will consume about 1.3 mmol/l (or about 5%) of glucose during 24 hour, while a GOX activity at a dilution of 1:10,000 will consume circa 50% glucose. For comparison, 5% equals the 24-hour glucose consumption of about 90 million exponentially growing tumor cells [48].

There are two strategies to minimize substrate depletion. First, large volumes of medium can be used which, in turn, will increase the time to reach stable hypoxia. Second and most often employed by us, medium can be replaced with a fresh GOX/CAT system by 12 to 24 hours. If the accumulation of growth factors is critical for the experimental outcome, we use conditioned medium for the fresh system. Third, culture media enriched with glucose can be used. Most cells tolerate high 25 mmol/l glucose media, however, we recommend to carefully check the impact of higher concentrations on the

---

**Table 3. Culture media volumes for various diffusion distances in different culture dishes.**

<table>
<thead>
<tr>
<th>Cell culturedish type</th>
<th>Area (mm(^2))</th>
<th>Medium volume (µl) for various diffusion distances</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.32 mm</td>
<td>2.63 mm</td>
</tr>
<tr>
<td>6 well plate</td>
<td>960</td>
<td>1,262.4</td>
</tr>
<tr>
<td>12 well plate</td>
<td>380</td>
<td>499.7</td>
</tr>
<tr>
<td>24 well plate</td>
<td>190</td>
<td>249.9</td>
</tr>
<tr>
<td>96 well plate</td>
<td>32</td>
<td>42.1</td>
</tr>
<tr>
<td>75 cm(^2) flask</td>
<td>7,500</td>
<td>9,862.5</td>
</tr>
<tr>
<td>4 chamber slide</td>
<td>160</td>
<td>210.4</td>
</tr>
<tr>
<td>8 chamber slide</td>
<td>64</td>
<td>84.2</td>
</tr>
<tr>
<td>6.5 cm dish</td>
<td>2,100</td>
<td>2,761.5</td>
</tr>
<tr>
<td>10 cm dish</td>
<td>7,850</td>
<td>10,322.8</td>
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</table>

**Table 4. Diffusion distances for commonly used culture media volumes in different culture dishes.**

<table>
<thead>
<tr>
<th>Cell culturedish type</th>
<th>Area (mm(^2))</th>
<th>Medium volume (ml)</th>
<th>Diffusion distance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 well plate</td>
<td>960</td>
<td>2</td>
<td>2.08</td>
</tr>
<tr>
<td>12 well plate</td>
<td>380</td>
<td>1</td>
<td>2.63</td>
</tr>
<tr>
<td>24 well plate</td>
<td>190</td>
<td>0.5</td>
<td>2.63</td>
</tr>
<tr>
<td>96 well plate</td>
<td>32</td>
<td>0.1</td>
<td>3.13</td>
</tr>
<tr>
<td>75 cm(^2) flask</td>
<td>7,500</td>
<td>20</td>
<td>2.67</td>
</tr>
<tr>
<td>4 chamber slide</td>
<td>160</td>
<td>0.5</td>
<td>3.13</td>
</tr>
<tr>
<td>8 chamber slide</td>
<td>64</td>
<td>0.2</td>
<td>3.13</td>
</tr>
<tr>
<td>6.5 cm dish</td>
<td>2,100</td>
<td>5</td>
<td>2.38</td>
</tr>
<tr>
<td>10 cm dish</td>
<td>7,850</td>
<td>20</td>
<td>2.55</td>
</tr>
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</table>
Finally, accumulation of gluconolactone/gluconic acid leads to acidification of the medium which affects the GOX/CAT system in a complex manner. Thus, GOX activity will be increased at lower pH, but decreased with accumulating gluconolactone. In general, acidification causes a slow re-oxygenation. Our studies showed that gluconolactone does not significantly inhibit activities of GOX and catalase below 10 mmol/l and no effects on cell regulation such as HIF1 induction (see also Section 5) were seen under such conditions. Varying buffer capacity of the buffering systems should also be taken into account. For instance, the buffer capacity of RPMI is lower compared to the one of DMEM but can be increased by addition of HEPES buffer.

4.3. Simulation of the GOX/CAT system (modeling)

The GOX/CAT system has been modeled using Excel (see Table 3S) and Matlab. (A) Modeled data for generation of hypoxia are in good agreement with measured data shown in Fig. 7. (B) Modeling now easily allows determining the best experimental conditions. In a well of a 12-well plate, stable hypoxia is reached by 20 minutes at a medium height of 2 mm (thin solid lines) compared to about 50 minutes with 5.6 mm liquid height (thick solid line). Dissolved oxygen of 220 \( \mu \text{mol/l} \) \( \text{O}_2 \) corresponds to 21% \( \text{O}_2 \) in air.

Figure 11. Simulated oxygen rates with varying GOX activity and diffusion distance.

Hepatoma Huh7 cells remove \( \text{H}_2\text{O}_2 \) from the cultured medium three times faster than hepatic stellate cells CSFC-2G. Such differences have to be considered to adequately use the GOX/CAT system. For practical use it is sufficient to consider cellular catalase but not GPO and peroxiredoxins.

catalase activities and in any setting (culture vessels, liquid height, incubation time). The Tab. 3S requires entering of the diffusion distance in mmol/l and the final GOX activity of the applied dilution in \( \text{mol/l}\times\text{s} \). Diffusion distances and their corresponding medium volumes are provided in Tab. 3 and 4 for several commercially available culture dishes including microplates. Other parameters that could be entered are initial oxygen levels (in \( \mu \text{mol/l} \)) and the oxygen steps in which the iterations are performed (0.1 \( \mu \text{mol/l} \) is set as standard).

Such simulation studies also allow to demonstrate the behaviour of oxygen under various conditions. Fig. 11A shows the modeled oxygen gradient developing over time at the bottom of a well using the same conditions as described for Fig. 8 (12-well culture plate, liquid height of 5.6 mmol/l, GOX 1: 50,000 = 5x10^(-8) \( \text{mol/l}\times\text{s} \)). These modeled data are in excellent agreement with the actual measurements. Fig. 11B demonstrates that establishment of steady-state hypoxia can be accelerated by changing the height of the fluid. In a well of a 12-well plate, stable hypoxia is reached within 10 minutes at a medium height of 2 mmol/l (thin solid lines) compared to 40 minutes with 5.6 mmol/l liquid height (thick solid line). Consequently, the 2 mmol/l setting would be chosen for the design of experiments with fast hypoxia/ re-oxygenation cycles, while the 5.6 mmol/l setting would be of better use for long-term experiments, aimed at minimizing GOX substrate depletion and product accumulation. For comparison, oxygen concentrations are given as dissolved oxygen with 220 \( \mu \text{mol/l} \) \( \text{O}_2 \) corresponding to 21% \( \text{O}_2 \).

Figure 12. Degradation of \( \text{H}_2\text{O}_2 \) in cultured cells by cellular catalase.
5. Applications

5.1. Biological validation of the GOX/CAT system by measuring H$_2$O$_2$-cytotoxicity:

The actual H$_2$O$_2$ concentration in the normoxic H$_2$O$_2$ producing system by the luminol/NaOCl-assay as described in Appendix B. This is not the case for the hypoxia/H$_2$O$_2$ system. A major reason is that self-forming oxygen- and H$_2$O$_2$-gradients gradually decrease GOX activity from top to bottom. Cells are exposed only to the very bottom of the gradients (e.g. 2% oxygen and 4 µmol/l H$_2$O$_2$) while upper layers of the gradient are irrelevant. Any attempt to measure the bottom concentration by the luminol/NaOCl assay destroys the gradients and averages H$_2$O$_2$-concentration over the whole well. In addition, oxygen electrodes require stirring and again destroy the gradient while ‘hypoxia-dyes’ such as pimonidazole (an imidazole derivative that forms adducts under hypoxic conditions) are non-quantitative [19]. For this reason, we started simulating H$_2$O$_2$-levels and oxygen concentrations in an Excel-based program. The resulting H$_2$O$_2$ ss levels under hypoxic conditions were modeled based on H$_2$O$_2$ measurements at normoxia and Fick’s law (see supplemental Tab. 2S and 3S). Several calculated H$_2$O$_2$ –concentrations generated by different GOX/CAT combinations and at different oxygen-concentrations were then selected for a cross-validation experiment based on an H$_2$O$_2$ cytotoxicity assay. Earlier studies had shown that only H$_2$O$_2$ levels but not other confounding factors such as glucose depletion or hypoxia determine cell toxicity of a GOX/CAT system (not shown). Thus, we assumed that identical [H$_2$O$_2$] ss levels would cause identical degrees of cytotoxicity independent of oxygen-levels and enzyme mixtures.

In the example shown here we compare two cell lines known to have different capacities to degrade and tolerate H$_2$O$_2$. Huh7 hepatoma cells are relatively resistant to H$_2$O$_2$ with an IC$_{50}$ of ca. 20 µmol/l while CSFC-2G cells (hepatic stellate cells, a cell type related to myofibroblasts) are sensitive to H$_2$O$_2$ with an IC$_{50}$ of ca. 5 µmol/l. Both cell types were exposed to varying conditions of the GOX/CAT system.

Fig. 12 shows the individual H$_2$O$_2$ degrading capacity of the two cell lines. Huh7 cells degrade H$_2$O$_2$ almost three times faster than CSFC-2G cells. It has to be mentioned that the degradation of H$_2$O$_2$ in cultured cells is more complex, but we focused on the predominant, exponential, catalase-dependent H$_2$O$_2$ decay as a raw estimate and ignored potential different contributions by GPO or peroxiredoxins at lower H$_2$O$_2$ concentrations [45].

After establishing the individual H$_2$O$_2$-tolerance of Huh7 and CSFC-2G cells, we proceeded to the actual validation experiment (protocol 2 in Appendix A). Cells were seeded into 96-well plates and exposed to different GOX/CAT combinations (i.e. oxygen and H$_2$O$_2$ levels) for 24h. At the end of the experiment, cell viability was determined semiquantitatively by trypan blue exclusion test. Fig. 13 (upper panel) shows the experimental conditions (GOX dilution, CAT dilution, medium volume, oxygen level) creating increasing [H$_2$O$_2$]ss levels from left to right. Cytotoxicity is represented by gray shades in each of the tested conditions. We then simulated the resulting [H$_2$O$_2$]ss concentrations by taking into account GOX activity, total CAT activity (cellular CAT activity + exogenous CAT from the GOX/CAT-system in the medium) and oxygen levels. The resulting [H$_2$O$_2$]ss levels are shown in the lower panels of Fig. 13 for both cell lines. These results are striking for several reasons:

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**Figure 13. Biological validation of the GOX/CAT system using H$_2$O$_2$ mediated cytotoxicity as read-out.**

- **CSFC 2G cells**
  - GOX dilution (µl) 1000: 0.9, 1.0, 1.1
  - CAT dilution (µl) 1000: 0.1, 0.6, 1.0
  - Estimated H$_2$O$_2$ ss (µmol/l) 0.1, 0.5, 1.0
- **Huh7 cells**
  - GOX dilution (µl) 1000: 0.9, 1.0, 1.1
  - CAT dilution (µl) 1000: 0.1, 0.6, 1.0
  - Estimated H$_2$O$_2$ ss (µmol/l) 0.1, 0.5, 1.0

$^*$ K$_{cat}$ endog ene 100U/l and K$_{cat}$ endog ene 5.3E-04 is

---

H$_2$O$_2$ determines cellular toxicity of a GOX/CAT system independent of hypoxia. Cell cytotoxicity of Huh7 and CSFC-2G cells has been determined by trypan blue dye exclusion under various oxygen and H$_2$O$_2$ concentrations. The GOX and CAT dilutions are shown in the upper two panels. The results, indicated by the gray value in the upper two panels, are in good agreement with the modeled data. The predicted steady-state H$_2$O$_2$ levels are given in the lower two panels. For details see text 5.1.
Remarkable differences in viability are observed between CSFC-2G and Huh7 cells when exposed to the same GOX/CAT conditions (Fig. 13, upper panels). This fits well to their different endogenous catalase activity (Fig. 12).

No toxicity in Huh7 and less toxicity in GCSF-2G cells are observed under hypoxic conditions (i.e. GOX-dilution 1:10,000, Fig. 13, upper panels row 1 and 4). This clearly demonstrates formation of hypoxia that protects from \( \text{H}_{2}\text{O}_2 \) toxicity. Since GOX activity will decrease from top to bottom of the well, less \( [\text{H}_2\text{O}_2] \) levels are formed at the bottom of the well. Interestingly, however, toxic \( \text{H}_2\text{O}_2 \) levels are still obtained despite hypoxia (Fig. 13, left upper panel).

The scheme explains, apart from other potential mechanisms, how an oxidant such as \( \text{H}_2\text{O}_2 \) could directly induce HIF1 despite hypoxia.

1. Remarkable differences in viability are observed between CSFC-2G and Huh7 cells when exposed to the same GOX/CAT conditions (Fig. 13, upper panels). This fits well to their different endogenous catalase activity (Fig. 12).

5.2. Rapid induction of HIF1 by enzymatic hypoxia

HIF1 (hypoxia inducible factor 1 alpha) is a transcription factor that orchestrates tissue response to hypoxia by up-regulating genes that are important for hypoxic adaptation (e.g. VEGF for angiogenesis, erythropoietin for increased red blood cell mass, and enzymes involved in anaerobic metabolism) [49]. Under low oxygen tension, HIF1 is hydroxylated at a proline residue by so-called prolyl-hydroxylases (PHD) following ubiquitination and proteasomal degradation [50]. PHDs are instrumental for the oxygen sensing mechanism since they require oxygen but also Fe(II), ascorbic acid and α-ketoglutarate as cofactors. In the presence of oxygen, HIF1 is completely degraded. Under hypoxia, HIF1 dimerizes with the constitutively expressed HIF1-beta-subunit, migrates to the nucleus and induces genes that contain so-called hypoxia responsive elements in their promoter.

Although there has been an enormous progress in understanding the regulation of HIF1 in the last decade, it has remained poorly understood why mammalian tissues do not show expression of HIF1 despite physiologically low oxygen levels around 1-2%. It has also remained obscure.
The GOX/CAT system: A novel enzymatic method to independently control hydrogen peroxide and hypoxia in cell culture

why PHD2 and PHD3 are themselves target genes of HIF1 [51], leading to a decreasing HIF-upregulation over time. In order to investigate the feedback mechanism between HIF1 and PHD2, we exposed Huh7 hepatoma cells to enzymatic hypoxia of 3% by use of the GOX/CAT hypoxia system as described in protocol 3 in Appendix A. Results were compared to experiments performed in a conventional hypoxia chamber flushed with an identical 3% oxygen gas mixture.

Fig. 14 shows the response of Huh7 cells to hypoxia. Rows 1 and 2 represent the experiment using the GOX/CAT system; Row 3 represents results from the hypoxia chamber. Surprisingly, HIF1 disappears despite persisting hypoxia. We have recently shown that degradation of HIF1 under sustained enzymatic hypoxia requires hydroxylation of the oxygen dependent degradation domain especially by PHD2 [19]. As shown in Fig. 15, PHD2 is indeed dramatically upregulated, ultimately resulting in complete HIF1 degradation despite hypoxia. As soon as stable hypoxic conditions are formed, PHD upregulation again leads to complete abrogation of HIF1 levels [19]. In contrast, HIF1 expression differs markedly using the hypoxia chamber (lower panel) since onset of hypoxia is much slower under such conditions requiring many hours [9].

Fast enzymatic hypoxia by the GOX/CAT system has been very useful to demonstrate the dynamics of the tight feedback loop between HIF and PHD. This loop explains why in any tissue under stable (even very low) oxygen conditions HIF1 is virtually absent but at the same time is inducible upon further decrease of oxygen concentration. Moreover, the HIF1/PHD loop is probably designed to compensate for tissue fluctuations of oxygen (21% O2 for bronchial epithelium, 16% O2 in arteries, 1-2% in parenchymatous organs such as liver or spleen) and to stay tuned for other oxygen-independent signals. Based on these studies we would like to suggest that HIF1/PHD loop is not primarily functioning to sense hypoxia but could be a fundamental metabolic control. Since decreasing oxygen typically indicates increased metabolic turnover under physiological conditions, the response of HIF1 towards falling oxygen could also simply be part of this metabolic control. This would also explain why many genes are controlled by HIF1 that are not directly related to hypoxia but metabolism. A scheme of the oxygen-dependent and –independent regulation of HIF1/PHD is shown in Fig. 16. More aspects will be discussed in the next paragraph.

5.3 Induction of HIF1 by H2O2 under hypoxia

Similar to many other proteins, HIF1 does not only respond to hypoxia but to many other stimuli including H2O2 [52, 53]. These observations have caused some confusion and seem to be paradox. How can both hypoxia and H2O2 induce HIF1? As discussed above, we have recently presented a concept that could explain the HIF1 response to oxygen and H2O2 without the need of additional signaling pathways [19]. Thus, HIF1 and PHDs form a close intracellular posttranslational-transcriptional feed back loop that always results in degradation of HIF1. Hypoxia only transiently decreases PHD activity since HIF1 ultimately induces de novo synthesis of PHDs. The only way to continuously upregulate HIF1 can be achieved by a complete disruption of the loop (Fig. 16). This could be either blockade of PHDs (e.g. by cobalt chloride or iron chelators) or at any other place within the loop. PHDs seem to be an interesting target for such a loop disruption since they contain soluble Fe(II) in the reactive center and iron chelating agents and cobalt chloride inhibit PHDs by binding or competitively replacing Fe(II). Since H2O2 can easily oxidize Fe(II) to Fe(III), this could be the underlying mechanism by which H2O2 induces

---

**Figure 17. HIF1 is rapidly upregulated by H2O2.**

<table>
<thead>
<tr>
<th>H2O2 bolus</th>
<th>Untreated</th>
<th>5 μmol/l</th>
<th>10 μmol/l</th>
<th>20 μmol/l</th>
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</thead>
<tbody>
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<td>HIF1α</td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Figure 18. Upregulation of HIF1 by H2O2 under hypoxia.**

Complete degradation of HIF1 under 12 h of 2% hypoxia can be prevented by H2O2 using different activities of catalase at constant GOX activities. Thus, all lanes represent identical hypoxia but varying H2O2 levels as indicated.
HIF1. First preliminary studies on HIF1 regulation using the GOX/CAT system strongly support this concept. Using either H2O2 bolus (Fig. 17A) or GOX alone at very high dilution to generate low levels of H2O2 under normoxia, HIF1 could be induced without hypoxia (Fig. 17B). Using an experimental set-up with a GOX/CAT system tuned in a way that it produces hypoxia and increased levels of H2O2 at the same time (2% oxygen and 1 to 10 µmol/l H2O2, respectively) we can disrupt the downregulation of HIF1 (Fig. 18) that normally occurs after approx. 12h (Fig. 14 and 15) and HIF1 will remain permanently upregulated despite persisting low oxygen concentrations. This experiment impressingly underlines the potentials of the GOX/CAT system to independently study redox-sensitive factors even under hypoxia thus mimicking (patho)physiology under in vitro conditions.

CONCLUSIONS

The GOX/CAT system is a novel approach to independently control hypoxia and hydrogen peroxide in cell culture. It offers the unique opportunity to study redox-sensitive cellular functions by H2O2 under low oxygen concentrations and much closer mimics in vivo conditions as compared to conventional methods. In comparison to hypoxia chambers, enzymatic hypoxia can be induced much faster and in a controlled manner. Since the GOX/CAT system is a simple and rather inexpensive method, not requiring special technical equipment, its broad usage in biomedical research is anticipated.

ACKNOWLEDGMENTS

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REFERENCES


