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

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ABSTRACT

The increasing demand in studying cellular functions in cultured cells under various levels of oxygen and hydrogen peroxide (H_2O_2) is only partly fulfilled by conventional approaches such as hypoxia chambers, bolus additions of H_2O_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 H_2O_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, H_2O_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 H_2O_2 levels under hypoxic conditions truly simulating H_2O_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 H_2O_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 H_2O_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 H_2O_2. In addition to their role in cell metabolism and survival, H_2O_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 H_2O_2 and hypoxia under several experimental conditions is only partly fulfilled by conventional approaches such as hypoxia chambers and H_2O_2 bolus additions. Hypoxia chambers show a slow onset of hypoxia [9] and prevent the co-exposure to H_2O_2 during hypoxia. H_2O_2 boli (usually between 50 and 500 μmol/l) expose cells to artificially high concentrations of H_2O_2 as compared to (patho) physiological H_2O_2 levels e.g. the release by inflammatory cells below 10 μmol/l [10]. Another drawback of H_2O_2 boli is the short half-life time of approximately 10 min under cell culture conditions [11] which prevents long term exposure to H_2O_2 for hours to days.

These insufficiencies have led us to develop a completely different approach in studying the effects of hypoxia and H_2O_2 alone or in combination. The oxygen depleting/H_2O_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 H_2O_2 in cultured cells. It has first been successfully used to study signaling functions of H_2O_2 in iron homeostasis [12-18] and, recently, it has been extended to induce hypoxia [19]. We aim here at introducing the GOX/CAT system to a broad readership.

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 H_2O_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 H_2O_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 H_2O_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 *in vivo* conditions such as less than atmospheric oxygen pressures and physiological H_2O_2 levels.

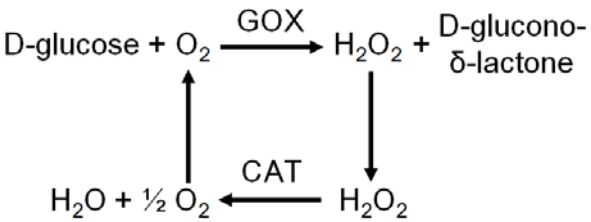
Due to a large size of the full article its Supplemental Tables and Appendices are available only online at: http://dx.doi.org/10.2478/v10039-009-0042-3.

REVIEW

1. Important notice

In this review, we report dilutions of glucose oxidase (GOX) and catalase (CAT) stock solutions instead of enzymatic rates. This allows for easier reading and comparison between experiments. We determined the initial enzymatic rate of GOX stock solution (G0543, Sigma-Aldrich, 50,000 units per 8.3ml) as K_{GOX} = 1.5 mmol/l s^{-1}, the enzymatic rate of catalase stock solution (C3155 Sigma-Aldrich) as K_{CAT} = 1000 s^{-1}. Thus, GOX 1:100.000 and CAT 1:100.000 mean that the final solutions have an activity of K_{GOX} = 1.5 x10^{-8} M s^{-1} and K_{CAT} = 0.01 s^{-1}, respectively. In our experiences variations in enzyme rates between batches of commercially available stock solutions have been minimal. However, it has to be noted that they can substantially vary under different experimental conditions, which also accounts for slightly different values in some of our previous publications.

Figure 1. **Stoichiometry of the GOX/CAT system.**



GOX converts 1 mol oxygen and glucose to 1 mol gluconolactone and H_2O_2, while catalase catalyzes the dismutation of 1 mol H_2O_2 into ½ mol oxygen and water. Thus, the system allows both the induction of hypoxia and the control of H_2O_2.

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 H_2O_2 by consuming oxygen while catalase degrades H_2O_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 H_2O_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 H_2O_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 H_2O_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 *Aspergillus niger* and the bacterium *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 H_2O_2 as an oxidant. Commercially available GOX preparations are purified from *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 H_2O_2. 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 H_2O_2 since it cannot be saturated by H_2O_2. In other words, turnover rates solely and linearly depend on the concentration of H_2O_2 up to molar concentrations. In mammalian cells, catalase is normally localized in peroxisomes rendering it less efficient in removing extraperoxisomal H_2O_2 [31]. In the food industry, catalase is used to remove H_2O_2 from milk prior to cheese production.

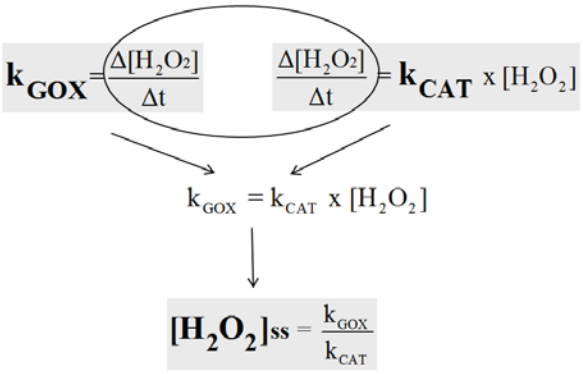
The first combined applications for GOX and catalase have been used to avoid accumulation of H_2O_2, to prevent oxidation reactions, to develop H_2O_2 sensors [32] and to induce hypoxia in cultured bacteria [33]. In addition, GOX has been used as oxidative stress model for *in vitro* and *in vivo* studies [34-38]. Based on a previously developed ultra-sensitive real time assay for H_2O_2 [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 functions of H_2O_2 mostly related to iron metabolism such as iron regulatory protein 1 (IRP1) [12-18]. Only recently, a modified GOX/CAT system allowed us to study hypoxia and the hypoxia inducible factor 1alpha (HIF1) in cell culture [18,19]. Depending on the experimental design, the GOX/CAT system can be supplemented with additional enzymes. Thus, addition of myeloperoxidase allows the continuous release of hypochlorous acid while maintaining a constant homeostasis of H_2O_2 in order to mimic the oxygen burst by neutrophils [24]. In addition, the parallel set up of a xanthine oxidase/catalase system has enabled us to discriminate H_2O_2-from superoxide-dependent processes [17].

Meanwhile, the unique and powerful research tool of combining GOX and catalase has received attention from other research groups [39, 40]. Similar combinations of enzymes to produce H_2O_2 such as the use of xanthine oxidase, catalase and superoxide dismutase by Dringen et al. [41] have been reported but the involvement of three enzymes makes such systems more complex and less robust in its application as compared to the GOX/CAT system.

2.3. Oxygen-consuming GOX as H_2O_2 source

GOX (EC 1.1.3.4) is the oxygen consuming and H_2O_2 generating part within the GOX/CAT system. The enzyme and its reactions have been well characterized [21,25,42]. GOX catalyzes the molar conversion of D-glucose to D-glucono-1,5-lactone while reducing molecular oxygen to H_2O_2 (*Fig.1*).

Figure 2. **Formation of steady-state H_2O_2 concentrations in a GOX/CAT system under saturating glucose conditions.**



H_2O_2 generation by GOX follows a zero order kinetics, while catalase removes H_2O_2 by first order kinetics. H_2O_2 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 H_2O_2 source for cell culture applications:

a) In contrast to other oxidases such as xanthine oxidase, GOX exclusively converts oxygen to H_2O_2 in a stochiometrically 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 (K_M 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.

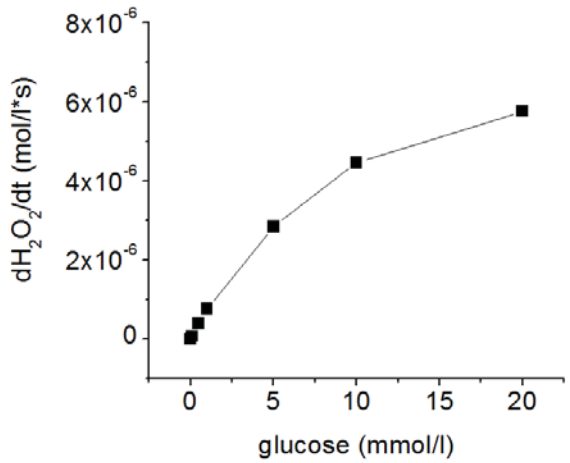
The activity of GOX also leads to the production of D-gluconolactone which may cause acidification of culture media. In water, gluconolactone slowly, within minutes to hours, hydrolyses to gluconic acid/gluconate with a pKa of 3.5 to 3.8. In *Aspergillus niger*, the acidification is avoided by the presence of gluconolactoses [44]. Accumulation of gluconolactone and concomitant acidification of the medium are not relevant as long as the GOX/CAT system is solely used for H_2O_2 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.).

Table 1. **GOX and CAT dilutions from stock to obtain 1, 5 and 10 µmol/l $[H_2O_2]ss$ at the bottom of a 12-well plate, filled with 1 ml medium. K_{GOX} = 1.5 x 10^{-3} mol/l s^{-1} and K_{CAT} = 1000 s^{-1}. Endogenous cellular is K_{CAT} = 5 x 10^{-4} s^{-1}. Enzyme activities and desired $[H_2O_2]ss$ conditions can be changed in the supplemental Excel Tab. 1S to adapt conditions to other experimental requirements.**

H_2O_2ss (µmol/l)	GOX dilution*	Oxygen (%)	Catalase dilution**	24h Glucose consumption (mmol/l)	Time for $[H_2O_2]ss$ (min)
1	100,000	17.1	81,871	1.1	4
	200,000	18.8	148,936	0.6	7
	500,000	20.0	350,000	0.2	15
	1,000,000	20.5	682,927	0.1	20
5	100,000	17.1	409,357	1.1	20
	200,000	18.8	744,681	0.6	35
	500,000	20.0	1,750,000	0.2	75
	1,000,000	20.5	3,414,634	0.1	100
10	100,000	17.1	818,713	1.1	40
	200,000	18.8	1,489,362	0.6	70
	500,000	20.0	3,500,000	0.2	150
	1,000,000	20.5	6,829,268	0.1	200

* 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_2O_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%.

2.4. Control of H_2O_2 by catalase

Catalase (E.C. 1.11.1.6) is added to the system to control H_2O_2 levels for several reasons. It decomposes H_2O_2 to water and oxygen without requiring further substrates. Second, it is not saturable even at molar H_2O_2 concentrations. This results in a typical exponential decomposition kinetics of H_2O_2 as is shown in *Fig. 20*. This is also the reason why H_2O_2 degradation by catalase directly depends on the concentration of H_2O_2 (*Fig. 2*). This kinetic property of catalase is an important prerequisite to generate hydrogen peroxide steady-state, $[H_2O_2]ss$ levels in combination with GOX. Although other H_2O_2-degrading enzymes exist, they are less suitable as enzymatic partners for GOX in an H_2O_2-producing system. Thus, glutathione peroxidase (GPO) becomes saturated at concentrations of H_2O_2 higher than 1 µmol/l. In addition, H_2O_2 affects cellular glutathione levels preventing GPO to form stable $[H_2O_2]ss$ in combination with GOX [45]. Although the H_2O_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_2O_2 system

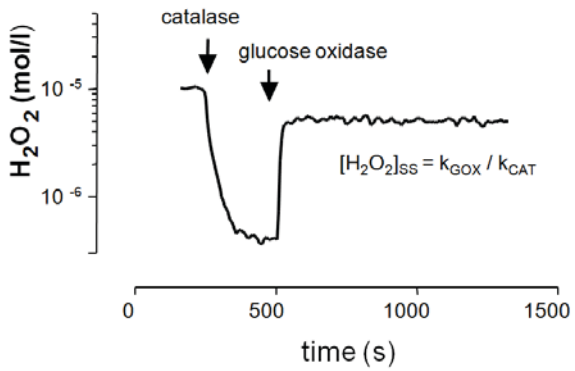
In general, the GOX/CAT system can be used in three ways. First, it can be used to expose cells physiologically relevant and sustained H_2O_2 concentrations which will will be discussed in more details in the next two sections 3.1 and 3.2, respectively. Second, the system can be used to induce hypoxia, and, third, combinations between various degrees of hypoxia and H_2O_2 concentrations are possible which will be discussed in section 4.

3.1. Control of H_2O_2

Fortunately, generation of H_2O_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_2O_2 are reached as soon as the H_2O_2 degradation rate of catalase equals the H_2O_2 production by GOX (*Fig. 2*). *Fig. 4* shows in real time the formation of a H_2O_2 steady state after addition of GOX to a solution containing catalase and glucose. It can be seen that $[H_2O_2]ss$ forms within seconds. *Fig. 6* demonstrates that such sustained H_2O_2 levels can be maintained for many hours.

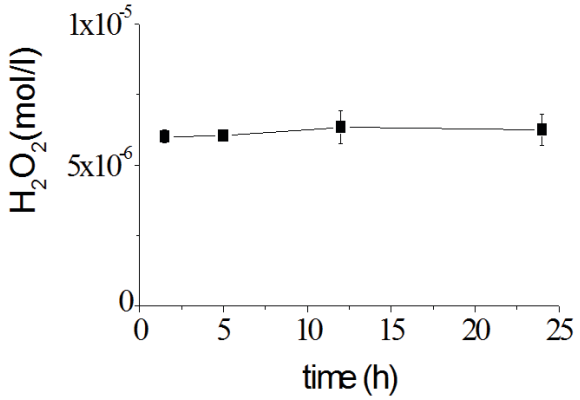
The ratio of GOX and catalase activity determines the concentration of H_2O_2. Under equilibrium conditions, H_2O_2 steady state concentrations can be directly calculated as $[H_2O_2]ss = K_{GOX}/K_{CAT}$. Since $[H_2O_2]ss$ concentrations are

Figure 4. **Generation of H_2O_2 steady-state concentrations by the GOX/CAT system.**



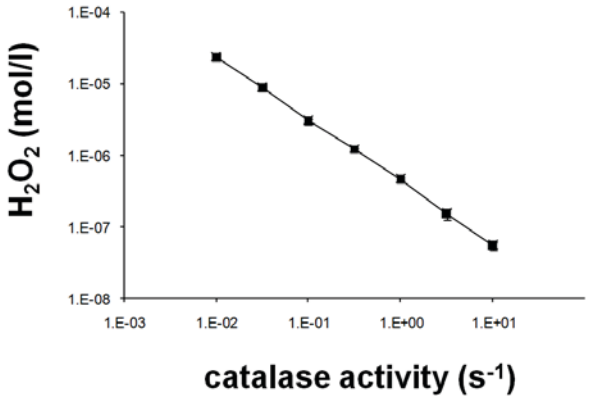
Using the luminol/hypochlorite assay, the formation of H_2O_2 ss by catalase and GOX is shown in real time. The H_2O_2 level can be calculated by the ratio of GOX and CAT activities.

Figure 5. **Long-term maintenance of the steady-state H_2O_2 concentration in a GOX/CAT system.**



H_2O_2 steady-state level of the GOX/CAT system can be maintained for 24 hours in culture medium DMEM with 25 mmol/l glucose. K_{GOX} = 3 x 10^{-8} M s^{-1}, K_{CAT} = 4.8 x 10^{-3} s^{-1}. The luminol/hypochlorite assay was used to measure H_2O_2.

Figure 6. **Effect of catalase on $[H_2O_2]ss$ in a GOX/CAT system.**



The activity of catalase reciprocally controls the steady-state H_2O_2 concentration at any given GOX activity. H_2O_2 was measured using the luminol/hypochlorite assay (see Appendix B).

described as a mere ratio between GOX and catalase activity, the same $[H_2O_2]ss$ can be achieved at various absolute enzyme concentrations Thus, 1 µmol/l H_2O_2 will be achieved when combining a K_{GOX} of 1.5x10^{-6} M s^{-1} with a K_{CAT} of 1.5 s^{-1} but also when combining K_{GOX} of 5x10^{-6} M s^{-1} and K_{CAT} of 5 s^{-1}. *Fig. 6* demonstrates that the resulting $[H_2O_2]ss$ 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 $[H_2O_2]ss$ level and the time needed until a stable H_2O_2 concentration is reached.

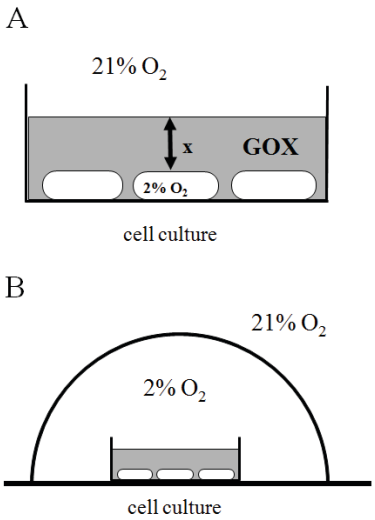
Especially in culture media with 25 mmol/l, glucose can be provided in such excess that V_{max} is maintained over 24 hours. As a rule of thumb, depletion of glucose from 25 to 5 mmol/l lowers GOX activity by about 50% which needs to be considered when calculating H_2O_2 ss conditions (*Tab. 1*). On 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_2O_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_2O_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 $[H_2O_2]ss$ concentrations. Detailed protocols are presented in Appendix B. Alternative assays e.g. by Dringen et al., [46] can be used to confirm H_2O_2 ss levels. However, they do not allow real time measurements which can become a limiting factor in case of fast changes of H_2O_2. GOX and catalase activities can also be determined by oxygen electrodes although no H_2O_2 ss can be validated with such methods [47]. Finally, it should be noted that the time of equilibrium formation of $[H_2O_2]ss$ 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_2O_2 source. In addition, the glucose consumed over 24 h and the time necessary to achieve the $[H_2O_2]ss$ are indicated. The supplemental *Tab. 1S* allows to enter cellular catalase activity as e.g. determined in *Fig. 17*. Although other H_2O_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 $[H_2O_2]ss$ 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 $[H_2O_2]ss$ within one experiment.

Figure 7. **Generation of hypoxia by (A) an enzymatic GOX/CAT system in comparison to (B) a hypoxia chamber.**

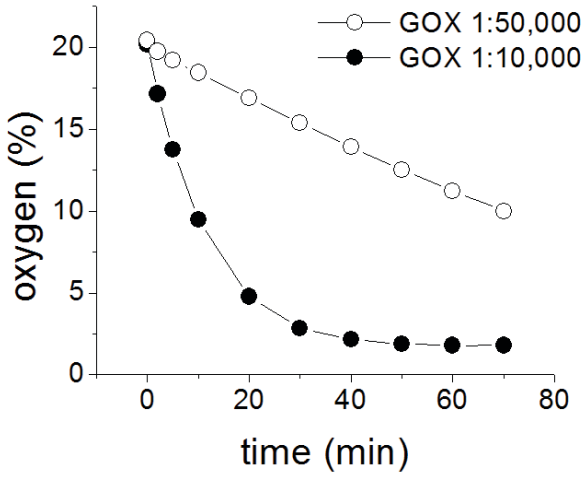


The presence of GOX generates hypoxia within minutes at the bottom of a culture dish, dependent on the medium height x above the cells and the GOX activity. In contrast, hours are required in conventional hypoxia chambers that are based on hypoxia equilibration between the gaseous and liquid phase.

We have earlier shown that the cellular toxicity in a GOX/CAT system is mainly determined by the level of H_2O_2 and not other factors such as hypoxia, glucose depletion etc. Therefore, it is advisable to know exactly the H_2O_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 $[H_2O_2]$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_2O_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^{2+} can interfere with the system. Such potential interferences should be tested, for instance by using the luminol/hypochlorite assay.

Figure 8. **The effect of GOX on oxygen levels in a GOX/CAT hypoxia system.**



Generation of hypoxia depends on the rate of oxygen consumption by GOX and steady-state is reached faster at higher GOX concentrations. Oxygen concentration has been measured using an oxygen electrode.

4. GOX/CAT as steady-state hypoxia system

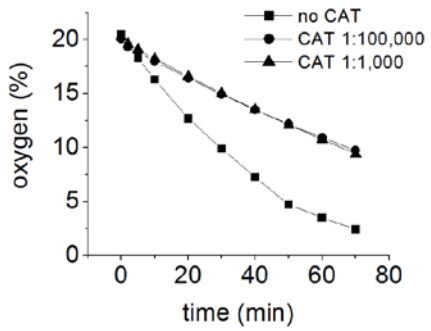
We will now mainly focus on the conditions of the GOX/CAT system to induce hypoxia in cultured cells. In general, as shown in *Fig. 1*, the GOX/CAT system is an oxygen consuming system since the catalase reaction recycles only 50% of oxygen. Hypoxia develops under conditions where oxygen consumption 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 $[H_2O_2]$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

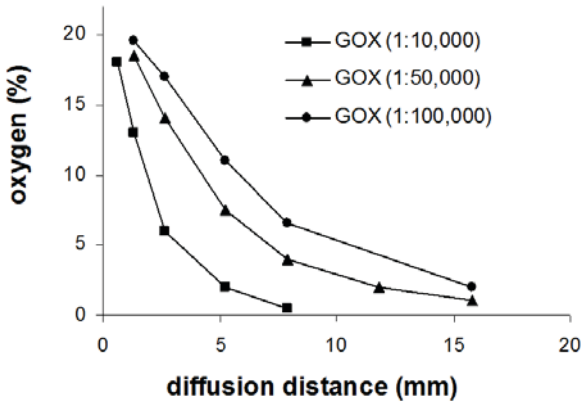
GOX activity and medium volume which determines the diffusion distance of oxygen from the surface to the bottom of the culture dish are the two major factors that affect hypoxia in a GOX/CAT system. *Fig. 8* shows that a high activity of GOX (1:10,000 dilution) causes a more rapid and pronounced hypoxia of about 1% oxygen as compared to lower GOX activities. Since oxygen electrodes require permanent perturbation and may destroy the oxygen gradient,

Figure 9. **The effect of catalase on oxygen consumption in a GOX/CAT hypoxia system.**



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.

Figure 10. **Various hypoxia conditions as function of medium volume and GOX activity using a GOX/CAT system.**



The height of the liquid above the cells determines the diffusion distance for oxygen from air to replenish the oxygen consumed by GOX. The final steady-state oxygen concentration is hence a function of the GOX activity and the diffusion distance. 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_2O_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 $[H_2O_2]$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.**

Volume (ml)	GOX Activity (Dilution)	Hypoxia (% oxygen)	CAT Dilution for1 µmol/l H_2O_2 ss	CAT Dilution for5 µmol/l H_2O_2 ss	CAT Dilution for10 µmol/l H_2O_2 ss	24h Glucose consumption (mmol/l)	Oxygen equilibrium time (min)
0.25	10,000	18.0	7778	38,889	77,778	13.0	6
0.5	10,000	13.0	10,769	53,846	107,692	13.0	17
1.0	10,000	6.0	23,333	116,667	233,333	13.0	64
2.0	10,000	2.0	70,000	350,000	700,000	13.0	153
3.0	10,000	0.5	280,000	1,400,000	2,800,000	13.0	164
0.5	50,000	18.5	37,838	1,891,89	378,378	2.6	23
1.0	50,000	14.0	50,000	250,000	500,000	2.6	65
2.0	50,000	7.5	93,333	466,667	933,333	2.6	240
3.0	50,000	4.0	175,000	875,000	1,750,000	2.6	321
4.5	50,000	2.0	350,000	1,750,000	3,500,000	2.6	343
6.0	50,000	1.0	700,000	3,500,000	7,000,000	2.6	346
0.5	100,000	19.5	71,795	358,974	71,7949	1.3	23
1.0	100,000	17.0	82,353	411,765	823,529	1.3	87
2.0	100,000	11.0	127,273	636,364	1,272,727	1.3	255
3.0	100,000	6.5	215,385	1,076,923	2,153,846	1.3	336
6.0	100,000	2.0	700,000	3,500,000	7,000,000	1.3	>360

Notes: For sole hypoxia studies, H_2O_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.

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

Cell culturedish type	Area (mm^2)	Medium volume (µl) for various diffusion distances		
		1.32 mm	2.63 mm	5.26 mm
6 well plate	960	1,262.4	2,524.8	5,049.6
12 well plate	380	499.7	999.4	1,998.8
24 well plate	190	249.9	499.7	999.4
96 well plate	32	42.1	84.2	168.3
75 cm^2 flask	7,500	9,862.5	19,725.0	39,450.0
4 chamber slide	160	210.4	420.8	841.6
8 chamber slide	64	84.2	168.3	336.6
6.5 cm dish	2,100	2,761.5	5,523.0	11,046.0
10 cm dish	7,850	10,322.8	20,645.5	41,291.0

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

Cell culturedish type	Area (mm^2)	Medium volume (ml)	Diffusion distance (mm)
6 well plate	960	2	2.08
12 well plate	380	1	2.63
24 well plate	190	0.5	2.63
96 well plate	32	0.1	3.13
75 cm^2 flask	7,500	20	2.67
4 chamber slide	160	0.5	3.13
8 chamber slide	64	0.2	3.13
6.5 cm dish	2,100	5	2.38
10 cm dish	7,850	20	2.55

4.2. Practical considerations

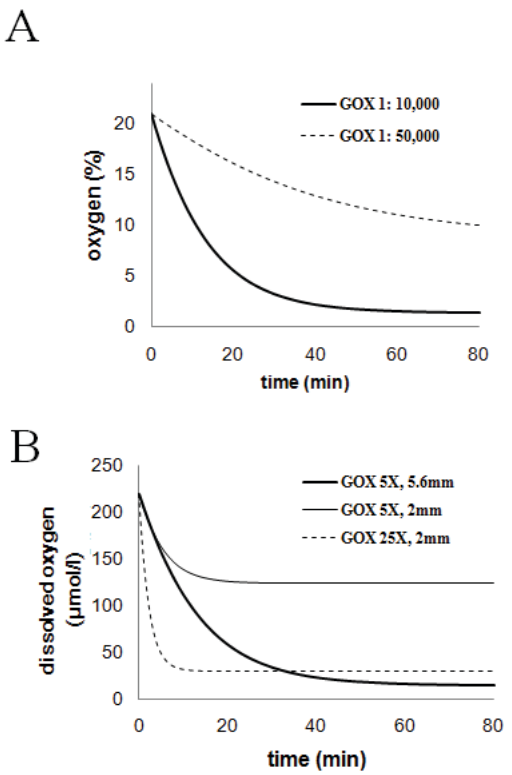
By adjusting medium volume (diffusion distance) and GOX activity (oxygen depletion rate), the GOX/CAT system allows rapid induction of various stable hypoxic levels between 0.01 and 21% at a defined rate. On the left side of the *Tab. 2*, various hypoxia conditions can be selected including GOX dilutions and medium volume. The right columns allow identifying appropriate catalase dilutions to independently control H_2O_2 ss. If the system is designed to only produce hypoxia, H_2O_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_2O_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

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



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 µmol/l O_2 corresponds to 21% O_2 in air.

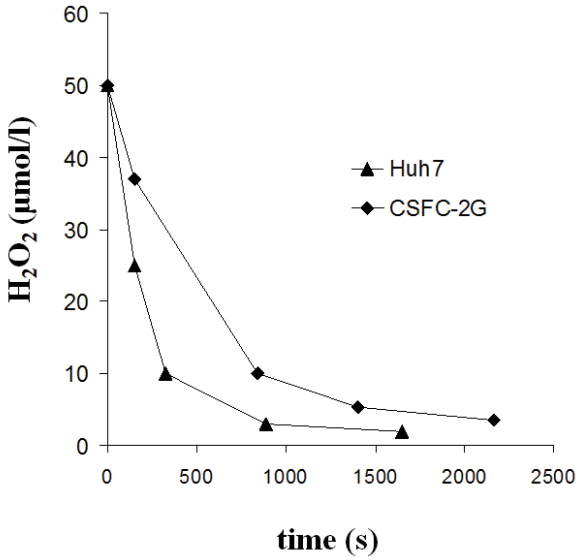
individual cell model.

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)

Hypoxia formation by GOX/CAT has been found in excellent agreement with simulation studies according to Fick's laws of diffusion. An Excel spreadsheet is provided as supplemental *Tab. S3* that allows predicting oxygen and H_2O_2 concentrations within culture vessels at any combination of GOX and

Figure 12. **Degradation of H_2O_2 in cultured cells by cellular catalase.**

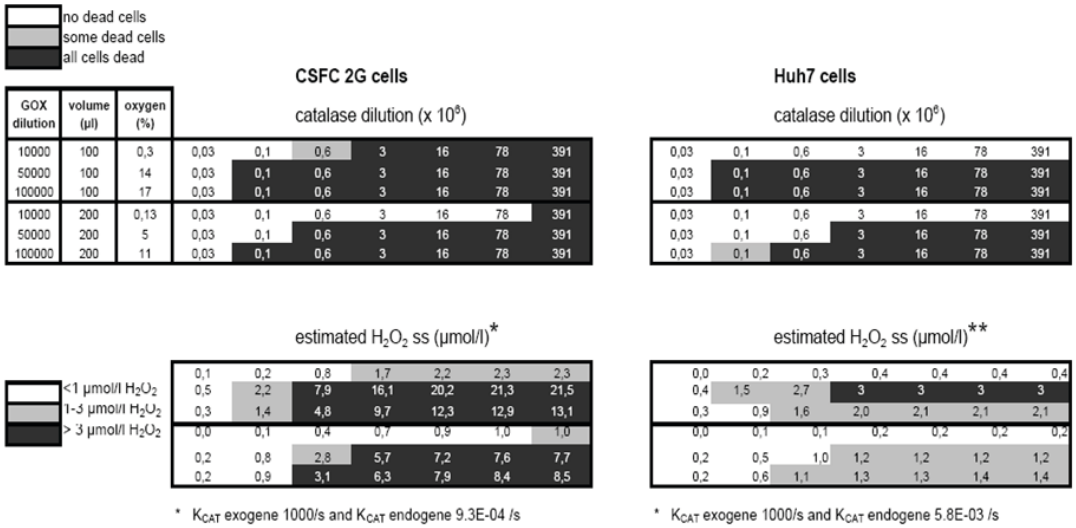


Hepatoma Huh7 cells remove H_2O_2 from the cultured medium three times faster than hepatic stellate cells CSFC-2G. Such differences have to be considered to adequately use the GOX/CAT system. For practical use it is sufficient to consider cellular catalase but not GPO and peroxiredoxins.

catalase activities and in any setting (culture vessels, liquid height, incubation time). The *Tab. 3S* requires entering of the diffusion distance in µmol/l and the final GOX activity of the applied dilution in mol/l*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 µmol/l) and the oxygen steps in which the iterations are performed (0.1 µmol/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 condtions as described for *Fig. 8* (12-well culture plate, liquid height of 5.6 mmol/l, GOX 1: 50,000 = $5x10^{-8}$ mol/l*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 µmol/l O_2 corresponding to 21% O_2.

Figure 13. **Biological validation of the GOX/CAT system using H_2O_2 mediated cytotoxicity as read-out.**



H_2O_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_2O_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_2O_2 levels are given in the lower two panels. For details see text 5.1.

5. Applications

5.1. Biological validation of the GOX/CAT system by measuring H_2O_2-cytotoxicity:

The actual H_2O_2 concentration in the GOX/CAT system can be measured accurately in the normoxic H_2O_2 producing system by the luminol/NaOCl-assay as described in Appendix B. This is not the case for the hypoxia/H_2O_2 system. A major reason is that self-forming oxygen- and H_2O_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_2O_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_2O_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_2O_2-levels and oxygen concentrations in an Excel-based program. The resulting H_2O_2 ss levels under hypoxic conditions were modeled based on H_2O_2 measurements at normoxia and Fick`s law (see supplemental *Tab. 2S and 3S*). Several calculated H_2O_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_2O_2 cytotoxicity assay. Earlier studies had shown that only H_2O_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 $[H_2O_2]$ ss levels would cause identical degrees of cytotoxicity independent of oxygen-levels and enzyme mixtures.

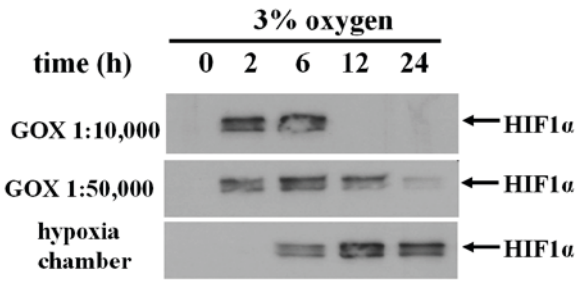
In the example shown here we compare two cell lines known to have different capacities to degrade and tolerate H_2O_2. Huh7 hepatoma cells are relatively resistant to H_2O_2 with an IC_{50} of ca. 20 µmol/l while CSFC-2G cells (hepatic stellate cells, a cell type related to myofibroblasts) are sensitive to H_2O_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_2O_2 degrading capacity of the two cell lines. Huh7 cells degrade H_2O_2 almost three times faster than CFSC-2G cells. It has to be mentioned that the degradation of H_2O_2 in cultured cells is more complex, but we focused on the predominant, exponential, catalase-dependent H_2O_2 decay as a raw estimate and ignored potential different contributions by GPO or peroxiredoxins at lower H_2O_2 concentrations [45].

After establishing the individual H_2O_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_2O_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 $[H_2O_2]$ss levels from left to right. Cytotoxicity is represented by gray shades in each of the tested conditions.

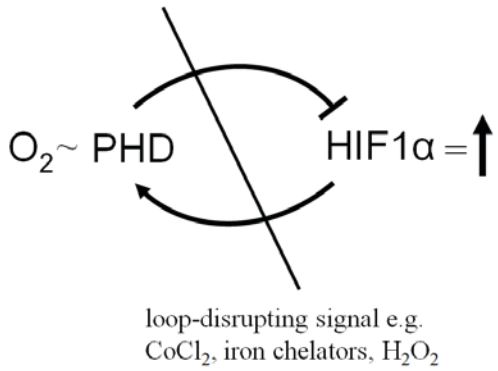
We then simulated the resulting $[H_2O_2]$ss concentrations by taking into account GOX activity, total CAT activity (cellular CAT activity + exogenous CAT from the GOX/CAT-system in the medium) and oxygen levels. The resulting $[H_2O_2]$ss levels are shown in the lower panels of **Fig.** *13* for both cell lines. These results are striking for several reasons:

Figure 14. **Induction of hypoxia inducible factor 1 (HIF1) depends on the onset but not the degree of hypoxia.**



Rapid induction of hypoxia by the GOX/CAT system (upper two lanes) shows different HIF1 expression as compared to a slow inducing hypoxia chamber (third lane). Huh7 cells were used an exposed to similar degrees of hypoxia (3% oxygen). For details, see text 5.2. HIF1 has been determined by Western blotting.

Figure 16. **Scheme of the posttranslational-transcriptional HIF1/PHD feedback loop.**



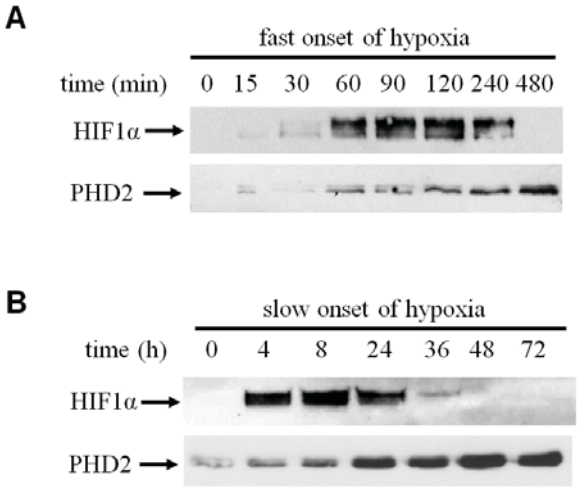
HIF1 only transiently responds to decreasing oxygen, but is completely degraded under constant and sustained hypoxia due to transcriptional HIF1-mediated induction of PHD. The GOX/CAT system has been instrumental in demonstrating the rapid HIF1/PHD feed back loop. Only disruption of the loop leads to a sustained upregulation of HIF1 which is always oxygen-independent. Such loop disrupting agents are inhibitors of PHD's such as cobalt chloride and iron chelators but also H_2O_2. The scheme explains, apart from other potential mechanisms, how an oxidant such as H_2O_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*).

(2) No toxicity in Huh7 and less toxicity in GCSF-2G cells are observed under hypoxic conditions (i.e.GOX-dilution1:10,000, *Fig. 13*, upper panels row 1 and 4). This clearly demonstrates formation of hypoxia that protects from H_2O_2 toxicity. Since GOX activity will decrease from top to bottom of the well, less $[H_2O_2]$ss levels are formed at the bottom of the well. Interestingly, however, toxic H_2O_2 levels are still obtained despite hypoxia (*Fig. 13*, left upper panel).

(3) The impact of cellular H_2O_2 degradation capacity becomes overt when comparing the patterns of upper and lower panel. In the shown experimental set-up for both cell types, cytotoxic effects start from $[H_2O_2]$ss levels between 1

Figure 15. **Transient induction of HIF1 under sustained hypoxia is due to HIF1-mediated PHD2 expression.**



(A) Rapid induction of hypoxia by the GOX/CAT system leads to transient upregulation of HIF1 following its rapid degradation and concomitant PHD2 induction. (B) Slow induction of hypoxia using a hypoxia chamber shows similar results over a much longer time interval. These effects occur despite persistent hypoxia. For details, see text 5.2. HIF1 has been determined by Western blotting.

and 3 µmol/l and cells are dead at $[H_2O_2]$ss levels exceeding 3µmol/l. Some inter-experimental variability is usually observed and discussed in section 3.2. The bottom line in *Fig. 13* shows the cellular catalase activity used for calculation of $[H_2O_2]$ss levels.

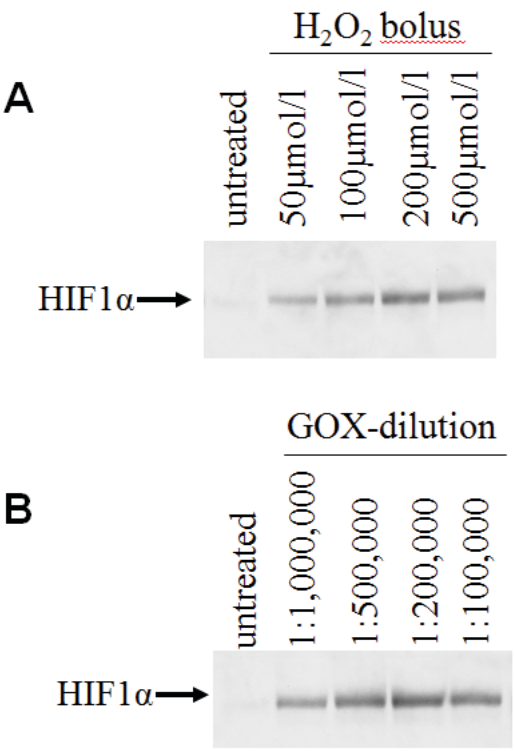
Taken together, these toxicity studies with varying GOX/CAT conditions very convincingly demonstrate (1) the independent control of H_2O_2 and oxygen in the GOX/CAT system and (2) corroborate the simulated $[H_2O_2]$ss levels using different combinations of GOX and CAT.

5.2. Rapid induction of HIF1 by enzymatic hypoxia

HIF1 (hypoxia inducible factor 1alpha) 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 promotor.

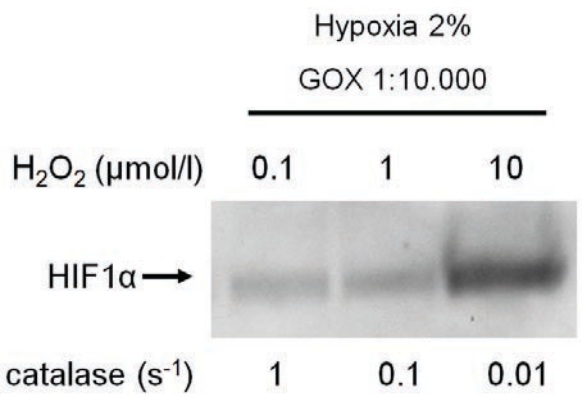
Although there has been an enormous progress in understanding the regulation of HIF1 in the last decade, it has remained poorly understood why mammalian tissues do not show expression of HIF1 despite physiologically low oxygen levels around 1-2%. It has also remained obscure

Figure 17. **HIF1 is rapidly upregulated by H_2O_2.**



HIF1 is induced both by bolus (A) and steady-state (B) H_2O_2.

Figure 18. **Upregulation of HIF1 by H_2O_2 under hypoxia.**



Complete degradation of HIF1 under 12 h of 2% hypoxia can be prevented by H_2O_2 using different activities of catalase at constant GOX activities. Thus, all lanes represent identical hypoxia but varying H_2O_2 levels as indicated.

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% O_2 for bronchial epithelium, 16% O_2 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 H_2O_2 under hypoxia

Similar to many other proteins, HIF1 does not only respond to hypoxia but to many other stimuli including H_2O_2 [52, 53]. These observations have caused some confusion and seem to be paradox. How can both hypoxia and H_2O_2 induce HIF1? As discussed above, we have recently presented a concept that could explain the HIF1 response to oxygen and H_2O_2 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 H_2O_2 can easily oxidize Fe(II) to Fe(III), this could be the underlying mechanism by which H_2O_2 induces

HIF1. First preliminary studies on HIF1 regulation using the GOX/CAT system strongly support this concept. Using either H_2O_2 boli (*Fig. 17A*) or GOX alone at very high dilution to generate low levels of H_2O_2 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 H_2O_2 at the same time (2% oxygen and 1 to 10 µmol/l H_2O_2, 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 H_2O_2 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.

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