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The Amplex Red/horseradish peroxidase assay requires superoxide dismutase to measure hydrogen peroxide in the presence of NAD(P)H

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Abstract

A sensitive fluorescence assay based on Amplex Red (AR) oxidation by horseradish peroxidase (AR/HRP) is described which continuously monitor rates of H₂O₂ production by microsomal enzymes in the presence of relatively high concentrations of NADPH. NADPH and NADH are known to interact with HRP and generate significant quantities of superoxide anion, a radical that spontaneously dismutates to form H₂O₂ which interferes with the AR/HRP assay. Microsomal enzymes generate H₂O₂ as a consequence of electron transfer from NADPH to cytochrome P450 hemoproteins with subsequent oxygen activation. We found that superoxide anion formation via the interaction of NADPH with HRP was inhibited by superoxide dismutase (SOD) without affecting H₂O₂ generation by microsomal enzymes. Using SOD in enzyme assays, we consistently detected rates of H₂O₂ production using microgram quantities of microsomal proteins (2.62 ± 0.20 picomol/min/μg protein for liver microsomes from naïve female rats, 12.27 ± 1.29 for liver microsomes from dexamethasone induced male rats, and 2.17 ± 0.25 picomol/min/μg protein for human liver microsomes). This method can also be applied to quantify rates of H₂O₂ production by oxidases where superoxide anion generation by NADH or NADPH and HRP can interfere with enzyme assays.

Keywords

Amplex Red; horseradish peroxidase; hydrogen peroxide; microsomes; cytochrome P450; NADPH; superoxide dismutase

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Disclosure of Interest

The authors declare that they have no conflicts of interest.

Introduction

Many enzyme complexes utilizing NADPH or NADH as reducing equivalents are sources of reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide (H_2O_2) [1, 2]. One of the best characterized examples of these complexes are found with microsomal enzymes [3, 4]. In a reaction requiring cytochrome P450s, these complexes use NADPH to activate oxygen. Cytochrome P450-oxygen bound intermediates mediate monooxygenase reactions [5, 6], as well as substrate independent NADPH oxidation, leading to the formation of H_2O_2 [7–11]. In earlier studies we showed that H_2O_2 production by these enzymes can be quantified using Amplex Red/horseradish peroxidase (AR/HRP), an assay with high sensitivity under different experimental conditions [12]. However, an important limitation for NAD(P)H-dependent enzymes is that NADPH and NADH react with HRP to directly oxidize phenolic substrates including AR [13–19]. This has restricted the use of AR/HRP to end-point measurements which significantly affects the sensitivity of the assay.

The present studies demonstrate that reactions of NADPH with HRP leading to oxidation of AR are mediated by superoxide anion. In the presence of NADPH and HRP, oxidation of AR can be prevented by catalase and superoxide dismutase (SOD). In contrast, SOD has no effect on enzyme complexes generating H_2O_2 utilizing NADPH or NADH. The addition of SOD to these reactions offers a way to measure H_2O_2 production continuously in NADPH or NADH supported enzyme assays, providing a more sensitive and robust assay for measuring rates of H_2O_2 formation.

Materials and Methods.

I. Chemicals and reagents.

Type VI horseradish peroxidase (HRP, cat. no. P8375), catalase (from *Aspergillus niger*, cat. no. C3515), SOD (from bovine erythrocytes, cat. no. S5395), glucose oxidase (GO, from *Aspergillus niger*, cat. no. G7141), NADPH, D-(+)-glucose, sodium azide, dimethyl sulfoxide (DMSO), diethylenetriaminepentaacetic acid (DETAPAC), 30% H_2O_2 , and all other chemicals were from Sigma-Aldrich (St. Louis, MO). 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red®, AR, cat. No 10010469) was from Cayman Chemicals (Ann Arbor, MI). Human liver microsomes were obtained from BD Gentest (Woburn, MA) and SD rat liver microsomes from Xenotech (Lenexa, KS). The storage, preparation of working suspensions and general characteristics of the microsomal preparations were provided by the manufacturers. Flat bottom black wall 96 well microplates and half area flat bottom transparent 96 well microplates were from Greiner Bio-One (Monroe, NC). Milli-Q ultrapure water (resistance > 18 MΩ) was used in all experiments. A SpectraMax M5 microplate reader equipped with a thermostatic chamber was used for measurements of optical density and fluorescence. Signals were recorded using Softmax Pro, versions 6.2 or 7.0 software.

AR (typically, 5 mg solid) was diluted in oxygen-free DMSO as a 20 mM stock solution and aliquoted into amber vials which were stored in the dark at -20°C until use. For each experiment, AR stock solutions were thawed at room temperature in the dark and used for no longer than 3–4 h. HRP stock solutions (40 Units/ml) were prepared in potassium

phosphate buffer (50 mM, pH 7.7) and stored in 0.4 ml aliquots at -20°C until use. NADPH was dissolved in alkaline water, pH ~ 9.0 , and stored in 0.1 ml aliquots in amber vials at -80°C until use. Solutions of NADPH were thawed on ice just prior to use. Catalase and SOD solutions were prepared in 50 mM potassium phosphate buffer and stored in 0.2–0.4 ml aliquots at -20°C until use.

II. Optimization of the AR/HRP assay for use with NADPH and microsomal enzymes.

Typical reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.7, 200 mM KCl, 0.5 mM sodium azide, and 0.1 mM DETAPAC. Sodium azide was included in reaction mixtures to inhibit endogenous catalase in microsomal preparations. For fluorescence assays, reactions were carried out in 140 μl volumes in 96-well flat bottom black microplates. For recordings of optical density, reactions were carried out in 170 μl volumes in half area transparent flat bottom microplates. Sodium azide was omitted from reactions run in the presence of catalase. AR/HRP was prepared from stock solutions and added to reaction mixes just prior to their additions into microplate wells (10 μM AR, 0.5 units/ml HRP, final concentrations). Reaction mixtures were preincubated for 3 min at 37°C in the microplate reader thermostatic chamber, recordings were then initiated to obtain background values. Unless otherwise indicated, reactions were initiated by the addition of NADPH.

III. H_2O_2 calibration curves.

Since measurements of H_2O_2 by the AR/HRP assay are not direct, relative fluorescence units (RFU) were extrapolated to H_2O_2 calibration standards. For these standards, a 160 mM H_2O_2 solution was prepared using 0.182 ml of 30% H_2O_2 in 10 ml of Milli-Q water. H_2O_2 standards in concentrations ranging from 0–4 μM were prepared from this solution in medium containing 50 mM potassium phosphate buffer, pH 7.7, 200 mM KCl, 0.5 mM sodium azide, and 0.1 mM DETAPAC. All solutions of H_2O_2 were prepared fresh for each experiment. Under our experimental conditions, no significant changes in background values were obtained in the presence or absence of sodium azide, catalase or SOD for at least 60 min (Fig. 1, upper panel). The addition of H_2O_2 to reaction mixes in the absence of catalase generated a marked increase in fluorescence from resorufin formation which persisted for at least 60 min (Fig. 1, upper panel and not shown). Under our AR/HRP assay conditions, 2 min reaction times after the addition of H_2O_2 were sufficient for the development of maximal fluorescence intensity. Catalase inhibited $>95\%$ of resorufin fluorescence. Linear calibrations were obtained for concentrations of H_2O_2 up to 4 μM ($R^2 = 0.98$). Lowest limit of detection (LLD) and lowest limit of quantification (LLQ) were calculated [20] as 30.23 pmol and 100.78 pmol of H_2O_2 per 0.15 ml (final volume of incubation medium for fluorescence) (Fig. 1, lower panel).

Results

The interaction of NADPH and HRP generates H_2O_2 .

The AR-based fluorescence assay used to quantify H_2O_2 requires the presence of peroxidase enzymes. Using HRP, the oxidation of AR by HRP- H_2O_2 complexes leads to the formation of highly fluorescent resorufin. In the absence of exogenous H_2O_2 , the combination of AR and HRP does not oxidize AR (Fig. 2, upper panel). The addition of NADPH to the assay in

the absence of exogenous H_2O_2 causes significant AR oxidation leading to the formation of resorufin (Fig. 2, upper panel). NADPH was also degraded under these conditions, a process that did not require the presence of AR (Fig. 2, lower panel). Rates of resorufin formation and NADPH degradation were concentration-dependent with respect to NADPH and HRP (not shown). These data indicate that NADPH catalyzes HRP-dependent AR oxidation in the absence of exogenous H_2O_2 . The addition of exogenous H_2O_2 to reaction mixes containing NADPH, AR and HRP further increased the formation of resorufin (Fig. 3).

The effects of catalase and SOD on resorufin formation in the AR/HRP assay was next analyzed. As expected, catalase inhibited the formation of resorufin, indicating that the reaction was mediated by H_2O_2 ; in these reactions, catalase did not inhibit degradation of NADPH (Fig. 4, upper panel). However, when SOD was added to assay mixes, the formation of resorufin, as well as degradation of NADPH were strongly inhibited (Fig. 4 lower panel). This suggests that superoxide anion is formed as an intermediary product in the reactions of NADPH and HRP. When SOD was added to mixes containing NADPH, AR/HRP and H_2O_2 , SOD only abolished oxidation of AR due to the formation of superoxide anion generated by the interaction of NADPH and HRP (Fig. 4).

Effects of SOD on enzymatically generated H_2O_2 .

We next determined if SOD could alter enzymatically generated H_2O_2 as detected in the AR/HRP assay. For these studies we used two H_2O_2 generating systems: glucose/glucose oxidase and NADPH/rat liver microsomes. Oxidation of glucose by glucose oxidase proceeds to H_2O_2 via formation of a stable FAD semiquinone superoxide anion intermediate. After protonation this intermediate dissociates forming H_2O_2 and oxidized flavoenzyme. In this enzyme system, no free superoxide anion is formed [21]. The AR/HRP based assay was found to readily estimate H_2O_2 generation during glucose/glucose oxidase activity; this reaction was not affected by SOD (Fig. 5, upper panel). However, in the presence of NADPH, which is not a component of the glucose/glucose oxidase reaction, an increase in H_2O_2 formation was noted; this increase was inhibited by SOD and the rate of H_2O_2 generation was similar as in the absence of NADPH.

In rat liver microsomes, H_2O_2 is largely generated via NADPH-mediated electron transfer to cytochrome P450-oxygen enzyme complexes which undergo autooxidation with intermediate formation of superoxide anion and/or via direct formation of H_2O_2 [21–30]. We found that rat liver microsomes readily generated H_2O_2 in the presence of NADPH, however, the reaction rate was not linear (Fig. 5, lower panel). When reactions were run in the presence of SOD, a linear rate was obtained that was identical to the rate obtained using an end point assay [12]. Thus, increases in H_2O_2 formation with non-linear kinetics were due to NADPH/HRP mediated generation of H_2O_2 , together with microsomal generated H_2O_2 . Linear reaction rates were obtained due to removal of H_2O_2 formed in the assay from the reaction of NADPH with HRP by SOD.

Characteristics of H_2O_2 generated by liver microsomes.

As shown in Fig. 6, the rate of H_2O_2 generation in liver microsomes under reaction conditions that included SOD was linear with respect to time- and protein concentrations

after initiation of electron transport with NADPH. Using this modified AR/HRP assay, the absolute rates of H_2O_2 generation by various liver microsomal preparations were estimated (Table 1). In these studies, H_2O_2 generation by microsomes from naïve male SD rats was significantly higher when compared to microsomes from naïve female SD rats (3.38 ± 0.32 and 2.62 ± 0.20 nmol/min/mg protein, respectively). In addition, the highest rates of H_2O_2 generation were found in microsomes from dexamethasone treated male SD rats (12.27 ± 1.29 nmol/min/mg protein). Less activity was found in microsomes from clofibrate, phenobarbital and isoniazid-treated male SD rats (8.20 ± 0.46 , 7.49 ± 0.54 and 4.07 ± 0.31 nmol/min/mg protein, respectively). H_2O_2 generating activity was also found in human liver microsomes (2.17 ± 0.25 nmol/min/mg protein) which were not as active as liver microsomes from SD rats.

Discussion

The method used in these studies to quantify H_2O_2 based on HRP mediated AR oxidation offers several obvious advantages relative to other commonly used methods: (1) it has a relatively higher sensitivity with the ability to reliably detect as little as 200–500 nM of H_2O_2 ; (2) a large number of samples can be measured using high throughput screening; (3) the chemicals used (AR and HRP) are readily available and stable during storage; and (4) the assay can be carried out in the pH range of 7.5 – 7.8, which permits the continuous monitoring of H_2O_2 production by enzymes under physiological conditions. The chemistry of the HRP-AR H_2O_2 assay is based on a classical peroxidation cycle [31, 32] as indicated below:

In this cycle, the binding of H_2O_2 to peroxidase leads to an intermediate state of HRP, compound I radical {reaction 1}, which can oxidize many phenolic substrates (RH) including AR, leading to the formation of compound II {reaction 2}. This in turn oxidizes a second molecule of RH returning HRP to the resting state {reaction 3}. With AR, the final reaction is expressed as follows:

Thus, two AR radicals undergo dismutation to form one molecule of resorufin and one molecule of AR resulting in an overall 1:1 ratio of H_2O_2 : resorufin [33, 34].

It is well known that in the presence of reduced pyridine nucleotides, NADH and NADPH, HRP-catalyzed reaction pathways become particularly complicated. This complexity is thought to originate, in part, from easy autoxidation of NAD(P)H and/or contamination with traces of H_2O_2 . Following published results [16, 35–41], we speculate that in the presence of

reduced pyridine nucleotides and oxygen, HRP and its reaction intermediates, Compounds I and II, actively form NAD(P)[•] radicals as intermediate products. In turn, these radicals react rapidly with oxygen resulting in the formation of superoxide radicals via “pseudooxidative” or/and “oxidative” cycles [35, 41]. Specifically, HRP intermediates accept electrons from NAD(P)H generating NAD(P)[•] radicals in reactions 5 and 6:

In the presence of oxygen, NAD(P)[•] radicals initiate an oxidative cycle via generation of superoxide radical anion in reaction 7 (the rate constants are in range of $10^7 - 10^9 \text{ M}^{-1}\text{s}^{-1}$) followed by further oxidation of NAD(P)H producing H₂O₂ and recycling NAD(P)[•] radicals in reaction 8, especially at relatively high concentration of NAD(P)H:

*Sum of reactions {7 and 8}:

Reactions 7 and 8 can be considered as a chain reaction cycle or a self-propagating reactions [18, 38, 41]. Thus, in enzyme reactions requiring the presence of NAD(P)H and HRP, the interaction of HRP with NAD(P)H interferes with the classical peroxidative cycle where superoxide anion is not an intermediate product. In our assay, SOD addition will slow down the cycling mechanism {reaction 7 and 8} as a result of eliminating the catalyst (superoxide anion), and inhibiting the corresponding and presumably major pathway of NAD(P)H-dependent H₂O₂ accumulation. In the presence of NADPH, SOD was also effective in the elimination of NADPH interference in the AR/HRP assay where enzymatic oxidation of glucose by glucose oxidase results in the formation of H₂O₂. Under these conditions NADPH is not a participant in the glucose/glucose oxidase system. However, SOD effectively eliminated AR oxidation stimulated only by NADPH.

It is well known that NADPH is the preferential source of electrons mediating the reduction of cytochrome P450 enzymes in eukaryotic cells [42]. In this regard, NADPH is essential for the high activity of cytochrome P450 mediated O₂ activation reactions. As SOD selectively inhibited interference initiated by NADPH/HRP in the AR/HRP assay, we applied this to the measurement of NADPH oxidase/H₂O₂ generation by microsomal enzymes. We found that a) under conditions where HRP and NADPH are continuously present in reaction mixes, SOD does not alter the rate of H₂O₂ generation by microsomal enzymes, b) in the presence of SOD, a linear reaction rate for H₂O₂ generation can be obtained in a continuous assay, and, c) the level of H₂O₂ measured by microsomal enzymes detected in this assay is

equivalent to levels of H_2O_2 measured using an end point AR/HRP assay [12] and other less sensitive assays [23, 24, 43].

The negligible background rates in our continuous assay in the presence of SOD allowed us to record H_2O_2 generation with 2.0 μg of microsomal proteins, a considerable improvement in sensitivity over other assays [22, 24, 27]. For example, we were able to reliably measure NADPH oxidase in microsomes from naïve female rats (2.62 ± 0.20 nmol/min/mg protein), which has the lowest of H_2O_2 generating activity in our studies with rat liver preparations. Of note, as little as 0.5 μg of microsomal protein could be used to analyze H_2O_2 generation rates in microsomes from dexamethasone-treated male rats (12.27 ± 1.29 nmoles/min/mg protein). The high H_2O_2 generation rate in microsomes from dexamethasone-treated male rats implies that rat CYP3A subfamily enzymes may be highly autoxidizable hemoproteins among the cytochrome P450 superfamily proteins.

In earlier studies we demonstrated that the kinetics of H_2O_2 production was distinct for different cytochrome P450 enzymes [44]. Using human recombinant enzymes we demonstrated that activities in different cytochrome P450s ranged from 6.0 nmol H_2O_2 /min/nmol P450 to 0.2 nmol/min/nmol P450. The kinetic parameters (K_m/V_{max}) of H_2O_2 production in microsomes will thus depend on the content of the different cytochrome P450s expressed in the liver. For example, cytochrome P450s in liver microsomes from naïve SD male and female rats are distinct. Thus, microsomes from naïve SD male rats largely express CYP3A2, CYP2C6, and CYP2C11 [45, 46] while female SD rats express CYP2C12, and no or very low amount of CYP3A subfamily proteins [47, 48]. This likely explains the increased H_2O_2 generation in microsomes from naïve SD male rats. Treatment of rats with phenobarbital, dexamethasone, isoniazide or clofibrate is known to dramatically alter the spectrum and content of cytochrome P450 enzymes. In rat liver, phenobarbital is known to induce the CYP2B subfamily [49], while dexamethasone, isoniazide and clofibrate induce the CYP3A subfamily, CYP2E1 and CYP4A subfamily, respectively [50]. These alterations are most likely responsible for the increased rates of microsomal H_2O_2 generation when compared to naïve rats. Human liver microsomes are also distinct in terms of cytochrome P450 expression due to large genetic polymorphisms and environmental variations of exposed individuals. Future experiments are needed to determine the precise contributions of each cytochrome P450 enzyme to the generation of various species of reactive oxygen species including H_2O_2 . Based on the high sensitivity of our assay, we speculate that NADPH oxidase related H_2O_2 generation can be determined in other NAD(P)H requiring enzymes including microsomes from extrahepatic tissues which contain either very low levels of cytochrome P450 enzymes and/or distinct forms of cytochrome P450 enzymes [51, 52].

In summary, the present studies demonstrate that (1) without the addition of exogenous H_2O_2 , NADPH reacts with HRP leading to the oxidation of AR and the formation of resorufin; (2) the rates of resorufin formation varied with NADPH and HRP concentration; (3) the formation of resorufin was prevented by catalase; (4) SOD (200–400 Units/ml) inhibited NADPH/HRP mediated AR oxidation; and (5) the presence of SOD in enzyme assays eliminates the interference of NADPH in the AR/HRP assay allowing us to quantify the NADPH-dependent H_2O_2 generation by rat liver microsomal enzymes from various

sources using microgram quantities of protein. This modification of the AR/HRP based H_2O_2 assay enables the accurate measurement of NADPH-dependent enzyme activities despite the interaction of NADPH and HRP in the assay.

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List of Abbreviations

H_2O_2	hydrogen peroxide
ROS	reactive oxygen species
AR	Amplex Red
HRP	horseradish peroxidase
SOD	superoxide dismutase

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Figure 1. Characteristics of the AR/HRP assay.

Upper panel, real time fluorescence recordings for the formation of resorufin in reaction mixtures without and with H₂O₂. Reactions were run at 37° C in the microplate reader. In the absence of H₂O₂, little or no fluorescence was observed with 0.5 U/ml HRP and 10 µM AR (final concentration) either alone or in combination. There were no significant changes in background fluorescence in reaction mixtures in the presence or absence of 0.5 mM sodium azide, 1000 U/ml catalase or 500 U/ml SOD for at least 60 min. The addition of H₂O₂ (4.0 µM final concentration, shown by the arrow) caused a marked increase in fluorescence. Catalase readily inhibited resorufin formation caused by the addition of H₂O₂ to reaction mixtures. The upper tracing (closed triangles) shows the kinetics of resorufin formation following the addition of H₂O₂ to reactions mixtures with AR/HRP. The lower tracings all overlap and show background fluorescence of reaction mixes containing AR, HRP, AR/HRP, SOD, sodium azide or catalase plus H₂O₂. **Lower panel,** calibration plot using increasing concentrations of H₂O₂ added to reaction mixtures. Each data point represents the mean ± SD. From these data, the lowest limit of detection (LLD, 3.3σ/S) and

lowest limit of quantification (LLQ, $10 \sigma/S$) were calculated as 30.23 pmol and 100.78 pmol of H_2O_2 per final incubation volume of 0.15 ml. In these calculations [20], σ is the standard deviation of blank samples (no H_2O_2 added, $n = 18$), and S is the slope of the calibration curve ($n = 16$).

Figure 2. The interaction of NADPH and HRP generates H_2O_2 .

Upper panel, the addition of NADPH to reaction mixes containing HRP causes significant AR oxidation leading to the formation of resorufin in the absence of exogenous H_2O_2 . Reaction mixes in 140 μ L containing potassium phosphate buffer, 50 mM, pH 7.7, 200 mM KCl, 0.5 mM sodium azide, 0.1 mM DETAPAC and AR/HRP (0.01 mM, and 0.5 units/ml, respectively) were preincubated at 37° C in the microplate reader. After 3 min, NADPH, (final concentrations as indicated, closed circles and squares), or control buffer (closed triangles) was added (shown by the arrow). **Lower panel,** the addition of HRP to reaction mixes causes significant AR oxidation leading to the formation of resorufin in the absence of exogenous H_2O_2 . The reaction mixes were the same as above except that 100 μ M NADPH was present at the start of the reactions and HRP (0.5 U/ml) was added as indicated by

arrows (open circles and triangles). Note that these recordings were done in optical density mode and reactions were carried out in 170 μ l volumes in half area transparent flat bottom microplates. Resorufin formation and NADPH degradation were measured simultaneously by changes in absorbance at 570 nm and 340 nm, respectively. Each data point represents the mean $\pm$ SD (n = 6). Unless shown, error bars fell within the symbol of each data point.

Figure 3. Effects of NADPH on resorufin formation in the AR/HRP assay.

Reaction mixes in 140 μ L contained 50 mM potassium phosphate buffer (pH 7.7), 200 mM KCl, 0.5 mM sodium azide, 0.1 mM DETAPAC and AR/HRP (10 μ M and 0.5 units/ml, respectively). Reactions were run in the absence (closed symbols) and presence of 200 μ M NADPH (open squares). H₂O₂ (1.0 μ M in 10 μ L) was added to the reactions as shown by the arrow (closed and open circles). Each data point represents the mean $\pm$ SD ($n = 6$). Error bars fell within the symbol of each data point.

Figure 4. Effects of catalase and SOD on NADPH utilization and resorufin formation in the AR/HRP assay.

NADPH degradation and resorufin formation were measured simultaneously by changes in absorbance at 340 nm and 570 nm, respectively. **Upper panel**, Reactions mixes in 170 μ L containing 50 mM potassium phosphate buffer, pH 7.7, 200 mM KCl, 0.1 mM DETAPAC and AR/HRP (0.01 mM, and 0.5 units/ml, respectively) were preincubated at 37° C in the microplate reader. After 3 min, NADPH, (200 μ M final concentration) was added (shown by the arrows). Closed squares, metabolism of NADPH in reaction mixes with catalase (1000 Units/ml) added at 0 min; closed circles, metabolism of NADPH in reaction mixes with catalase added at 9 min; open circles, formation of resorufin in reaction mixes with catalase added at 9 min; open squares, formation of resorufin with catalase added at 0 min. **Lower panel**, Reactions were run as described above except that mixes also contained 0.5 mM

sodium azide. Closed squares, metabolism of NADPH in reaction mixes with SOD (600 U/ml) added at 0 min; closed circles, metabolism of NADPH in reaction mixes with SOD added at 10 min; open circles, formation of resorufin in reaction mixes with catalase added at 10 min; open squares, formation of resorufin with SOD added at 0 min. Each data point represents the mean $\pm$ SD ($n = 6$). All error bars fell within the symbol of each data point.

Figure 5. Effects of SOD on the generation of H_2O_2 by glucose/glucose oxidase and rat liver microsomal enzymes.

Upper panel: Glucose/glucose oxidase reactions mixes in 140 μ L containing potassium phosphate buffer, 50 mM, pH 7.7, 200 mM KCl, 25 mM dl-glucose, 0.5 mM sodium azide, 0.1 mM DETAPAC and AR/HRP (0.01 mM, and 0.5 units/ml, respectively) were preincubated at 37° C in the microplate reader. After 3 min, NADPH (0.1 mM final concentration) was added as indicated. After recording background fluorescence, glucose oxidase (1.0 μ L, 10 mU) was added. In some reactions, SOD (500 U/ml) was included in the assay buffer. **Lower panel:** Microsomal reactions were carried out as indicated above except that dl-glucose was omitted and microsomal protein (1.5 μ g in 10 μ L) from dexamethasone induced rats were added to the assay buffer. Background trace (closed triangles), each value represents the mean $\pm$ SD (n = 9)

Figure 6. H_2O_2 generation by liver microsomes from naive male SD rats. Reactions in 140 μL containing potassium phosphate buffer, 50 mM, pH 7.7, 200 mM KCl, 0.5 mM sodium azide, 0.1 mM DETAPAC, 100 U/ml SOD, 0.2 mM NADPH and AR/HRP (0.01 mM, and 0.5 units/ml, respectively) were preincubated for 3 min at 37°C in the microplate reader. Microsomal proteins (in a volume of 10 μL) were then added as indicated by the arrow. Upper panel: Real time fluorescence recordings for the formation of resorufin containing different amounts of microsomal proteins. Lower panel: Effects of increasing microsomal protein on the formation of resorufin. Each data point represents the mean $\pm$ SD ($n = 4$). Error bars fell within the symbol of each data point.

Table 1.

The absolute rates of H₂O₂ generation by liver microsomal preparations.

Liver Microsomes	Rates of H ₂ O ₂ generation (nmol/min/mg of protein) ^I
SD female rats, control	2.62 ± 0.20 (2.93) ^I
SD male rats, control	3.38 ± 0.32 (4.18, 3.73)
SD male rats, phenobarbital induced	7.49 ± 0.54
SD male rats, isoniazide induced	4.07 ± 0.31 (4.27, 3.51)
SD male rats, dexamethasone induced	12.27 ± 1.29 (14.62, 7.62)
SD male rats, clofibrate induced	8.20 ± 0.46 (8.0)
human liver microsomes	2.17 ± 0.25

^I All determinations were performed within the linear range of H₂O₂ formation over time after the addition of NADPH. The background rate of AR oxidation in the absence of microsomal protein was 0.17 nmol of H₂O₂/min (n = 18), which is far below the LLQ (see Fig 1, lower panel). In these assays, 0.5–2 µg of liver microsomal proteins were used to analyze H₂O₂ reaction rates. Reactions were performed under conditions indicated in the legend to Fig. 6. Each value is the mean ± SD, n = 6–12. The values in brackets were found with different lots of liver microsomes from SD rats (mean, n = 2–4).