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Large Differences in Erythrocyte Stability Between Species Reflect Different Antioxidative Defense Mechanisms

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We have developed a screening assay for erythrocyte stability, which is rapid, easy, inexpensive, robust, and suitable for handling a large number of samples in parallel. Erythrocytes are incubated overnight in 96-well microtiter plates in absence or presence of various oxidants, intact cells are pelleted by centrifugation, and lysis is determined by release of intracellular constituents into the supernatant as either activity of lactate dehydrogenase (LDH) or absorbance of hemoglobin at 406 nm. There is good correlation between the methods. A number of advantages by the present method are that only small amounts of blood is needed, washing is optional, erythrocytes may be stored for at least one day before assay, and large numbers of samples can be handled in parallel. Using this set-up, we have compared erythrocyte stability from several different animal species. We find that erythrocyte susceptibility towards lysis induced by H_2O_2 and 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) is highly species dependent. The different susceptibility between species is due to cellular components, since swapping of plasma between species has little or no effect. As a novel observation, we find that erythrocytes from chicken are the most sensitive of the species tested towards lysis by H_2O_2 and are almost four orders of magnitude more sensitive than erythrocytes from man. This is due to a much lower content of catalase in erythrocytes from chicken. A more narrow range is observed for susceptibility towards AAPH and the ranking between the species is different. Thus, chicken erythrocytes are more resistant towards AAPH than some mammals by up to two orders of magnitude. This differential stability towards different oxidative stressors is likely due to evolution/selection of different defense mechanisms.

Keywords: Erythrocyte; Membrane stability; Oxidative stress; Catalase; Species

INTRODUCTION

Oxidative damage to cellular components is an important marker in diagnosis of various pathological conditions,[1] and sensitive biomarkers of oxidative damage in living organisms have been subject of extensive research.[2] Many biomarkers of oxidative alterations are determined as single oxidation products,[2,3] e.g. lipid oxidation is often assessed as malonedialdehyde[4] and protein oxidation as either carbonyls,[5,6] or specific reaction products, e.g. 2-amino-adipic semialdehyde[4,7] and γ-glutamyl semialdehyde.[8,9] However, lysis of erythrocytes, as a more complex model system, has also been investigated[3,10,11] and shown to be dependent on vitamin E content,[11,12] membrane lipid composition,[13] and intracellular glutathione concentrations.[14–16] Several aspects make erythrocyte stability interesting and applicable as a biomarker of oxidative stress: (1) highly polyunsaturated lipid composition of the cell membrane (increased oxidative sensitivity), (2) high concentration of hemoglobin (pro-oxidative), (3) very efficient antioxidative systems, e.g. catalase, and (4) accessibility and availability (sampling). The first objective of the present work was to set up a rapid, inexpensive, and robust screening assay for erythrocyte stability as a measure of oxidative status in animals.

Free radicals and other oxidants are generated constantly in living organisms, and effective antioxidative defense mechanisms are critical in order

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to avoid deleterious oxidative modifications of lipids, proteins and DNA. Endogenous protection against oxidative stress include antioxidative enzymes like catalase, superoxide dismutase and glutathion peroxidase as well as low molecular weight antioxidants.[17] Under normal physiological conditions the integrated defense system is very delicately balanced for each species. The relative importance of each defense mechanism may, due to evolution/selection,[18] differ between species[19–22] and may end up compensating for each other.[20,23] In studying oxidative damage in living organisms, we have observed dramatic differences between species in the stability of erythrocytes. The second objective of the present work was to study these differences in more detail and increase our understanding of some of the mechanisms behind these differences.

MATERIALS AND METHODS

Blood from different species: chicken ($n = 4$), pheasant ($n = 1$), rat ($n = 1$), cat ($n = 1$), rabbits ($n = 4$), pigs ($n = 8$), cow ($n = 1$) and humans ($n = 3$) was sampled into EDTA-coated tubes, diluted 50 times into phosphate buffered saline (PBS: 10 mM sodium phosphate, pH 7.4, 150 mM NaCl), and poured into disposable plastic trays. Erythrocytes were evenly suspended by gentle, manual or magnetic stirring, while 100 μl aliquots were pipetted with a multichannel pipette into 96 well round-bottom microtiter plates (Nunc, Roskilde, Denmark). An equal volume of PBS containing oxidant at various concentrations was added to each well, giving a final erythrocyte suspension of 0.3–0.4% (100-fold dilution of whole blood). Controls received PBS without oxidants. Additionally, total lysis was determined for each blood sample by addition of Triton X-100 (final concentration: 1%) to three replicate wells. Oxidants at identical concentrations were also added to cells lyzed by Triton X-100 to allow correction for the effect of oxidant on absorbance of hemoglobin and activity of lactate dehydrogenase (LDH)—see also "Results" section. Microtiter plates were incubated for 20 h on a Delfia microplate shaker (Wallac, Allerød, Denmark) at ~1000 rpm. Residual cells were pelleted by centrifugation at 900*g* for 3 min.

Determination of cell lysis through release of intracellular constituents was performed by sampling 50 μl supernatant into a flat-bottom microtiterplate and addition of 200 μl LDH reagent (20 mM imidazole, pH 7.0, 1 mM pyruvate, 175 μM NADH, 0.02% bovine serum albumin) and (i) monitoring the rate of NADH oxidation at 340 nm in a Powerwave X microplate reader (Bio-Tek Instruments, Winooski, Vermont), followed by (ii) determination of absorbance at 406 nm (Soret band absorption of hemoglobin). Results are expressed as percentage of lysis based on determination of absorbance at 406 nm, unless otherwise specified. The concentration of oxidant at which 50% of the erythrocytes are lyzed (ED_{50}) is calculated from a fit of data to a sigmoid curve: $\text{lysis}(\%) = 100/(1 + e^{-(X-ED_{50})/b})$, where X is concentration of oxidant, and b is an empirical parameter (steepness of the curve), using Sigmaplot software (SPSS Science, Chicago, IL). For washing experiments, cells were pelleted by centrifugation at 300*g* for 15 min and plasma removed. The buffy coat consisting of white blood cells was left with the erythrocytes. PBS was added back to the original whole blood volume, and processed as for whole blood.

Plasma swap experiments were carried out with EDTA-treated blood from chicken and pigs. The blood was centrifuged, plasma removed, erythrocytes washed twice in PBS, and aliquots of erythrocytes resuspended in either their native plasma or that from the other species. The whole blood samples were then diluted 100 times and assayed for oxidative stability.

Catalase activity was determined in erythrocyte lysates after appropriate dilution into PBS by rate of removal of hydrogenperoxide using the FOX1 assay.[24] Lysates of erythrocyte suspensions (0.4%) were prepared by addition of Triton-X100 to 1% final concentration, and pipetted vigorously. Three-fold serial dilutions of these lysates were made in PBS, and 50 μl was added to 200 μl H_2O_2 (22 μM in PBS) and incubated at room temperature for up to 20 h. Fifty microliters of these samples were added to two hundred microliters FOX1 reagent, allowed to develop for 30 min and absorbance was determined at 560 nm, or alternatively, optical density was determined on an HP desktop computer scanner and quantified by densitometric analysis (QuantityOne, BioRad, CA).

2,2′-Azobis (2-amidinopropane) hydrochloride (AAPH) was obtained from PolySciences (Warrington, Pennsylvania), and all other reagents of analytical grade were from Sigma (St. Louis, MO).

RESULTS

Correlation Between Assay Methods

Lysis of erythrocytes results in release of intracellular contents and measurement of any intracellular component in the supernatant should reveal degree of lysis. Figure 1 shows that there is a linear correlation between leakage of LDH, as measured by rate of NADH oxidation in presence of pyruvate, and leakage of hemoglobin from erythrocytes for all species tested (coefficients of correlation are 0.96 [rabbit], 0.98 [man], 0.84 [cow]; not shown: 0.76

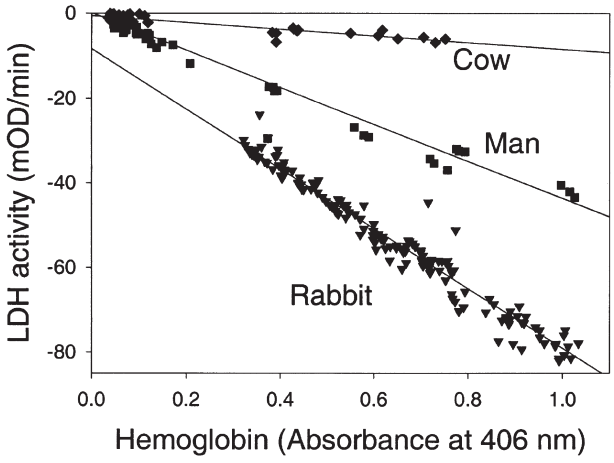

FIGURE 1 Correlation between activity of LDH and absorbance at 406 nm of hemoglobin leaked from erythrocytes from rabbit (four animals total of 192 individual points), man (three persons total of 144 individual points), and cow (one animal total of 48 individual points). Other species (pig, chicken, rat, cat, and pheasant) fall between rabbit and cow and have been left out for clarity. Activity of LDH and absorbance of hemoglobin from chicken, rabbit, man and cow were determined in parallel, and those from pig, cat, rat and pheasant were obtained in a separate experiment.

[chicken], 0.97 [pig], 0.97 [rat], 0.87 [cat], 0.66 [pheasant]). Thus, activity of LDH relative to the amount of hemoglobin released from erythrocytes varies up to 10-fold as determined from the slope of the regression lines in Fig. 1: −71 (rabbit), −44 (man), −7.9 (cow). Not shown: −33 (chicken), −47 (pheasant), −46 (cat), −47 (rat), and −25 (pig). Thus, erythrocytes from different species display considerable diversity in the amount of intracellular LDH and hemoglobin.

Correction Factors

Oxidants not only lyse erythrocytes but also affect released cellular constituents used for determination of degree of lysis, i.e. bleaching of hemoglobin or inactivation of LDH. Parallel determination of direct effects of oxidants on either absorbance of hemoglobin or activity of LDH was made using completely detergent-lyzed erythrocytes and allows correction for this effect. Figure 2 shows the influence of AAPH and H_2O_2 on activity of LDH and absorbance of hemoglobin at 406 nm for two species (chicken

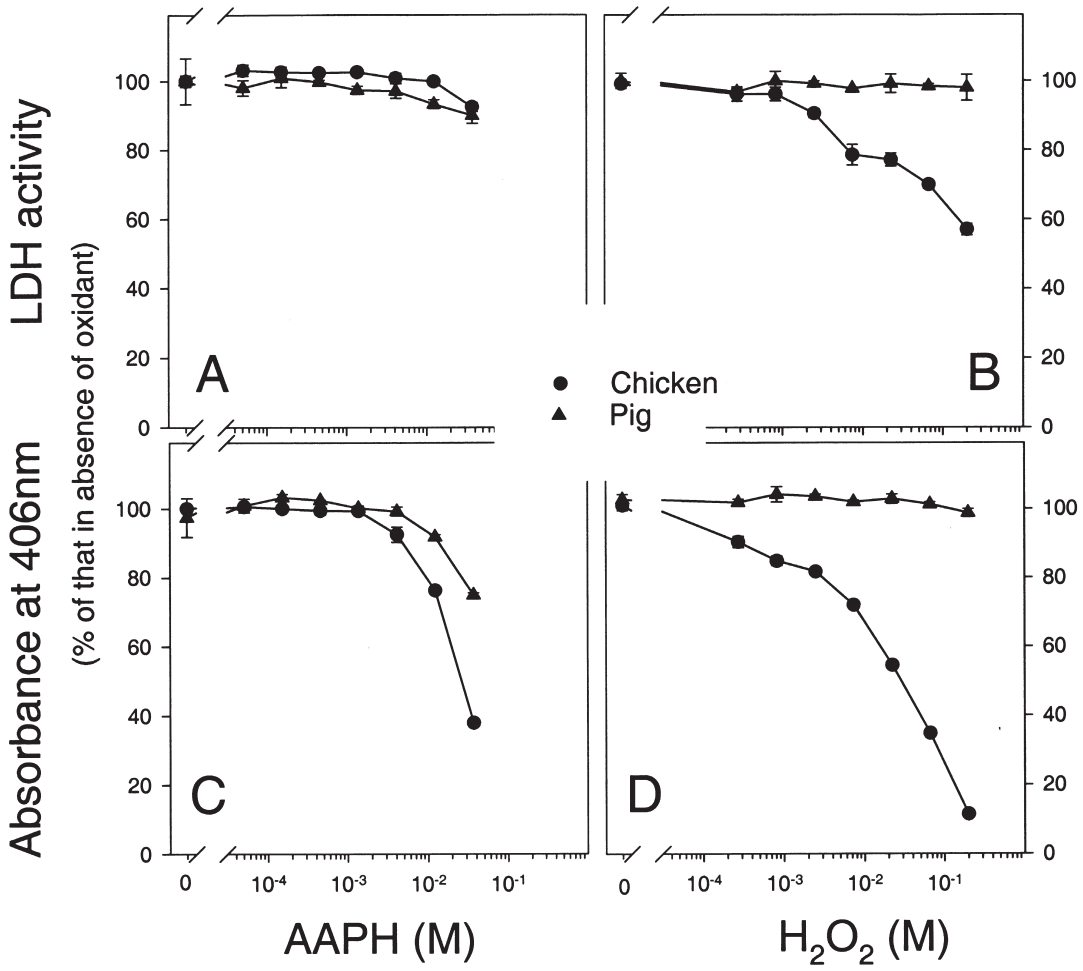

FIGURE 2 Effect of AAPH and H_2O_2 on LDH activity and absorbance of hemoglobin at 406 nm in erythrocyte lysates after 20 h at room temperature. Values are mean (±SEM) of four subjects, each performed in triplicate. Only values for lysates from chicken and pig erythrocytes are shown, since values for the other species fall between those for chicken and pig and have been left out for clarity.

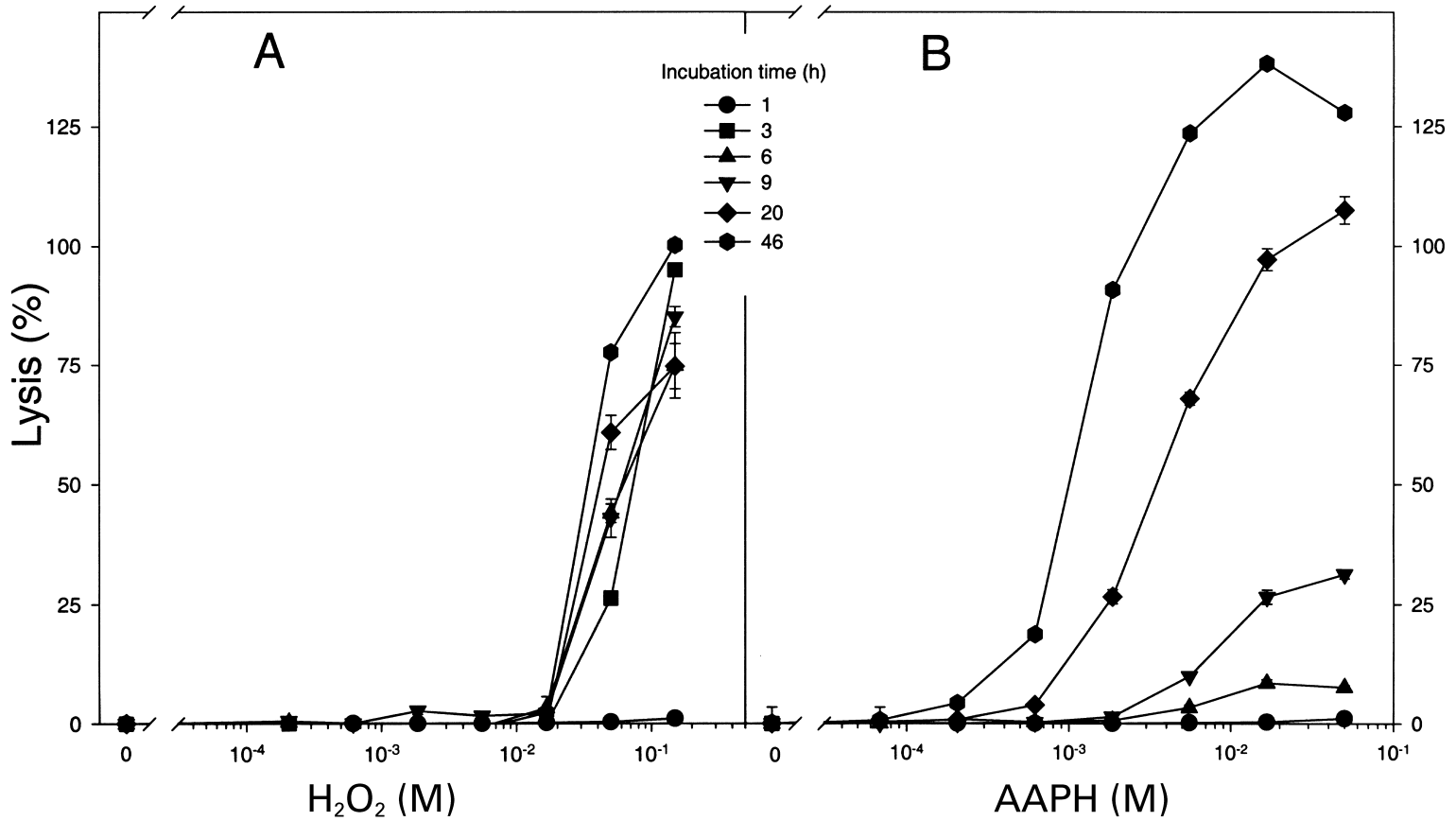

FIGURE 3 Kinetics of lysis of porcine erythrocytes by H_2O_2 (A) and AAPH (B). Mean (±SEM) of triplicate determinations from one pig, representative of results obtained for total of three pigs.

and pig). LDH activity is almost unaffected by AAPH (Fig. 2A), and only LDH activity from lyzed chicken erythrocytes was reduced considerably (~40%) in chicken at 0.2 M H_2O_2 (Fig. 2B). AAPH caused substantial bleaching of hemoglobin as measured by absorbance at 406 nm for both pig (~15%) and chicken (~60%) at the highest concentration of AAPH (Fig. 2C), whereas H_2O_2 at 0.2 M caused extensive decrease in absorbance of hemoglobin at 406 nm only for chicken (88%) as seen in Fig. 2D. Corrections due to these direct effects of oxidants are only approximate and therefore values above 100% may be obtained particularly when bleaching is extensive. All results of induced lysis displayed in the following have been corrected accordingly.

Time Course

Our standard assay conditions, i.e. incubation at room temperature for 20 h, result in low basal lysis (~5–8%), which increased to approximately double of that after 46 h of incubation (data not shown). Figure 3A shows that induction of lysis occurs only after incubation for more than 1 h at concentrations of H_2O_2 above 0.02 M. Time course for effect of AAPH (Fig. 3B) show a more gradual lysis with time. Lysis continues to increase up to the longest incubation time tested (46 h), where concentrations as low as 2×10^{-4} M AAPH induce lysis above that of basal lysis.

Effect of Washing

Next, we tested the effect of washing erythrocytes in PBS before incubation with oxidants. Figure 4A show that basal lysis increases with washing (unwashed: ~5%, washed: ~20%), though repeated washing has no further effect. There is no effect of washing on ED_{50} for AAPH-induced lysis, but at the highest concentration of H_2O_2 (0.15 M), unwashed erythrocytes are more stable than those washed once, twice or thrice, but less stable than those washed four times.

Effect of Storage

Normally, we assay stability of the erythrocytes the same day the blood is drawn. A storage experiment (Fig. 4B) shows that basal lysis is lowest when erythrocytes are assayed the same day the blood is drawn, but the slight increase in basal lysis observed after storage for one day does not increase further with prolonged storage in PBS. However, ED_{50} for AAPH-induced lysis decreases after two days of storage and further after three days compared to lysis of erythrocytes assayed immediately or stored for one day, indicating that erythrocytes become progressively more fragile. There is no apparent effect of storage on ED_{50} for H_2O_2-induced lysis, although lysis at 50 mM H_2O_2 indicates a gradual increase in resistance towards H_2O_2.

Effect of Erythrocyte Density and Addition of Plasma

Differences in final density of erythrocytes due to variation in hematocrit values, i.e. the volume percentage of erythrocytes in whole blood, between individuals or due to sampling, e.g. partial clotting, could possibly affect sensitivity towards induction of lysis. However, we observed only small variations in hematocrit values (CV% 5–10 for blood samples from eight pigs), or due to handling (CV% < 5 for repeated pipetting of the same blood sample) and these variations are therefore unlikely to affect the results. Our assay conditions specify a final density of ~0.4% erythrocytes in PBS. Basal lysis increases slightly with decreasing density of erythrocytes (Fig. 4C), and erythrocytes diluted to ~0.3% show increased sensitivity towards AAPH-induced lysis relative to those at densities of 0.4 and 0.8%. When oxidation is induced by H_2O_2, lysis decreases with increasing density of erythrocytes.

Plasma could possibly influence sensitivity towards lysis. The diluted erythrocyte suspension (0.4%) contains 0.6% plasma. Addition of plasma to washed erythrocytes shows that stability towards AAPH is not significantly increased by addition of plasma up to 1%, but increases after addition of 2 and 4% plasma (Fig. 4D). Erythrocyte stability following H_2O_2 exposure did not increase significantly by plasma addition up to 2%, and the increased stability after addition of 4% plasma was modest.

Erythrocyte Stability in Different Species

Figure 5 shows lysis of erythrocytes from the eight species when exposed to either AAPH or H_2O_2, and the effective dose (as added) of either oxidant resulting in lysis of 50% of the cells (ED_{50}) is shown in Table I for each species. Particularly, H_2O_2-induced lysis differed by almost four orders of magnitude among the species. The most sensitive species towards lysis by H_2O_2 is chicken which is between 100 and 4000 times more sensitive than erythrocytes from other species. The ranking between the different species is different when erythrocytes are challenged with AAPH (Fig. 5, insert) and tends to be inversely related, although without statistical significance ($r^2 = 0.21$). Rabbit erythrocytes are the most sensitive towards lysis by AAPH, being up to 50 times more sensitive than erythrocytes from other species.

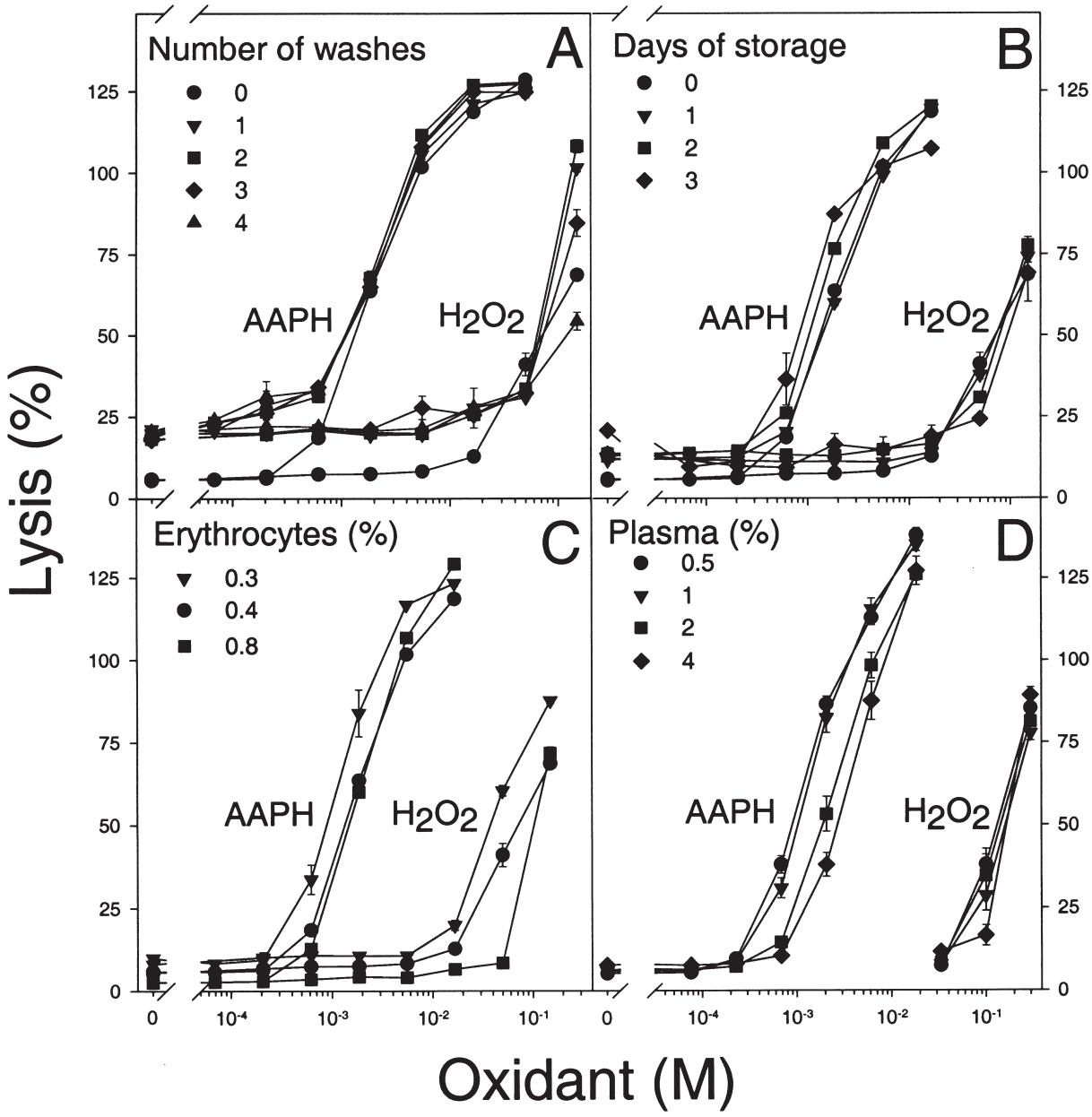

FIGURE 4 Effect of wash (A), storage (B), amount of erythrocytes (volume percentage) (C) and plasma (D) on AAPH and H_2O_2-induced lysis of porcine erythrocytes. Mean (±SEM) of three pigs, each determined in triplicate is shown.

Plasma Swap Between Species

The large differences in oxidative stability between species, e.g. chicken and pig, could be due to properties of either the erythrocytes or the plasma. Swapping plasma between species allows us to distinguish between these two possibilities, as shown in Fig. 6. Erythrocyte stability is largely independent of which plasma is added, however chicken erythrocytes seems to be more stable towards H_2O_2 when resuspended in pig plasma relative to that of chicken plasma.

Effect of Sodium Azide Addition

Oxidation by H_2O_2 is likely to be affected by the amount or activity of intracellular catalase. To test this hypothesis, we assessed erythrocyte stability in the absence or presence of inhibitors of catalase (Fig. 7). Thus, addition of 10 mM sodium azide decreases stability of all the species tested by 1–3 orders of magnitude. Interestingly, in the presence of sodium azide erythrocytes from different species display comparable sensitivity with an ED_{50} of approximately 1×10^{-4} M H_2O_2.

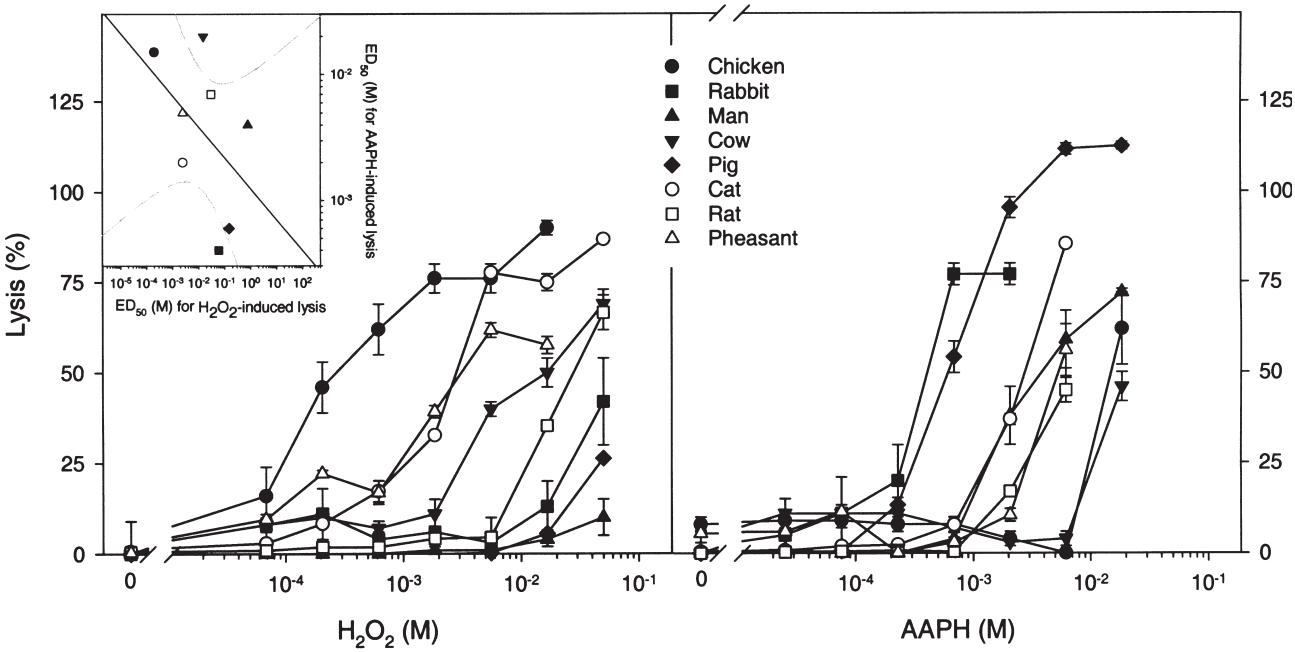

FIGURE 5 AAPH and H_2O_2-induced lysis of erythrocytes from different species. Triplicate determinations from chicken ($n = 4$), pheasant ($n = 1$), rat ($n = 1$), cat ($n = 1$), rabbits ($n = 4$), pigs ($n = 8$), cow ($n = 1$) and humans ($n = 3$) (mean ± SEM) are shown. Erythrocytes from chicken, rabbit, man and cow were analyzed in parallel, and pig, cat, rat, pheasant and man was analyzed separately. The inset is a plot of ED_{50} for AAPH and H_2O_2-induced lysis, and the regression line is shown with 95% confidence intervals.

Catalase Activity

We next measured catalase activity in lysates of erythrocytes for the various species tested in the same experiment. Figure 8A shows removal of H_2O_2 by serial dilutions of lysate, and it is apparent that catalase activity is very different between species, with at least 100-fold lower catalase activity in chicken and pheasant erythrocytes compared to the other species. Removal of H_2O_2 by the lysates could be completely inhibited by the specific catalase inhibitor 3-amino-1, 2, 4-triazole or by NaN_3 (data not shown). Figure 8B is a plot of the observed erythrocyte stability (ED_{50}) towards oxidation by H_2O_2 versus titer of lysate that degrades completely the added H_2O_2. Coefficient of correlation for the linear regression is 0.79, which is significant ($P < 0.01$).

TABLE I Stability of erythrocytes expressed as concentration of oxidant (as added) at which 50% of erythrocytes are lyzed (ED_{50}). Erythrocytes from chicken, rabbit, man and cow were analyzed in parallel, and erythrocytes from pig, cat, rat, pheasant and man were analyzed in parallel (data from Fig. 5). Some ED_{50} values for H_2O_2-induced lysis (rabbit, pig, and man) and AAPH-induced lysis (cow) are approximate values due to extrapolation to 50% lysis

Species	H_2O_2-induced lysis ($ED_{50} \times 10^{-3}$ M)	AAPH-induced lysis ($ED_{50} \times 10^{-3}$ M)
Chicken	0.2	15
Pheasant	2	5
Cat	2	2
Cow	12	~20
Rat	30	7
Rabbit	~60	0.4
Pig	~150	0.6
Man	~800	4

DISCUSSION

We have focused on setting up a screening assay for the oxidative status of animals using the stability of erythrocytes as target and AAPH and H_2O_2 as oxidants. These oxidants attack the erythrocyte membrane differently and therefore provide information on different defense mechanisms. Oxidative induction of lysis or erythrocytes has been studied previously as a biomarker of oxidative status,[10,11] using many different oxidants.[14,15,25] Our assay conditions allow several different oxidants to be tested in parallel, since it is simple, easy, and robust. Our objectives were to avoid washing of the erythrocytes, and adjust the density of the cells to obtain a maximally diluted sample with minimal interference from plasma, yet allow sufficient signal for measurement of lysis, i.e. release of intracellular LDH or hemoglobin. Microtiter plates ease handling through use of multichannel pipettes and reading of either LDH activity or absorbance of hemoglobin on a microtiter plate reader. Thus, full lysis of 0.4% (vol/vol) erythrocytes results in an activity of LDH corresponding to a rate of change in absorbance of ~0.1 unit per minute at 360 nm or an absorbance of ~1 unit at 406 nm from the content of hemoglobin. Incubation conditions, i.e. time and temperature,

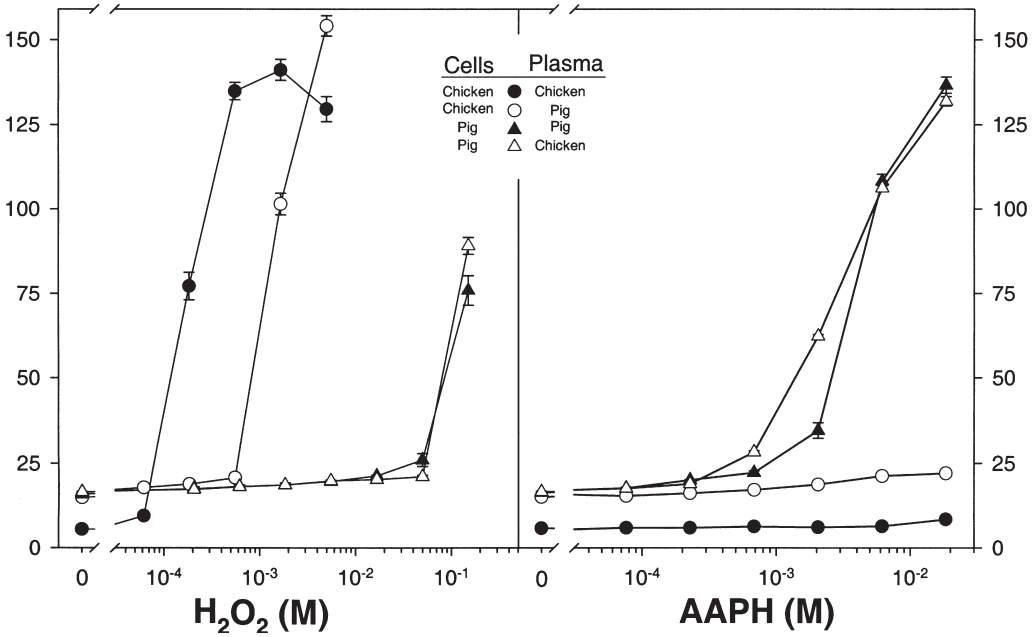

FIGURE 6 Effect of swapping plasma on lysis of erythrocytes from chicken and pig by AAPH or H_2O_2. Each point is mean (±SEM) of three replicates determinations from one experiment.

were chosen as 20 h and room temperature, respectively, to allow manipulation of a large number of samples by one person in one day and also assures sufficient radical generation by AAPH at suboptimal temperature.

The density of erythrocytes in the assay is critical, since stability increases with increasing erythrocyte density (Fig. 4A), particularly when exposed to H_2O_2. This is likely due to the increased amount of intracellular catalase in the sample, which effectively degrades H_2O_2. Indeed, added H_2O_2 is completely degraded after incubation for 20 h (data not shown), and probably much sooner.

Erythrocyte stability is often assayed after three washes,[11,26] but handling of many blood samples is incompatible with washing, since this is a tedious and time-consuming step. Washing is, however, necessary if plasma has an effect on erythrocyte stability and if the contribution of plasma is unwanted in the assay. Thus, we have compared

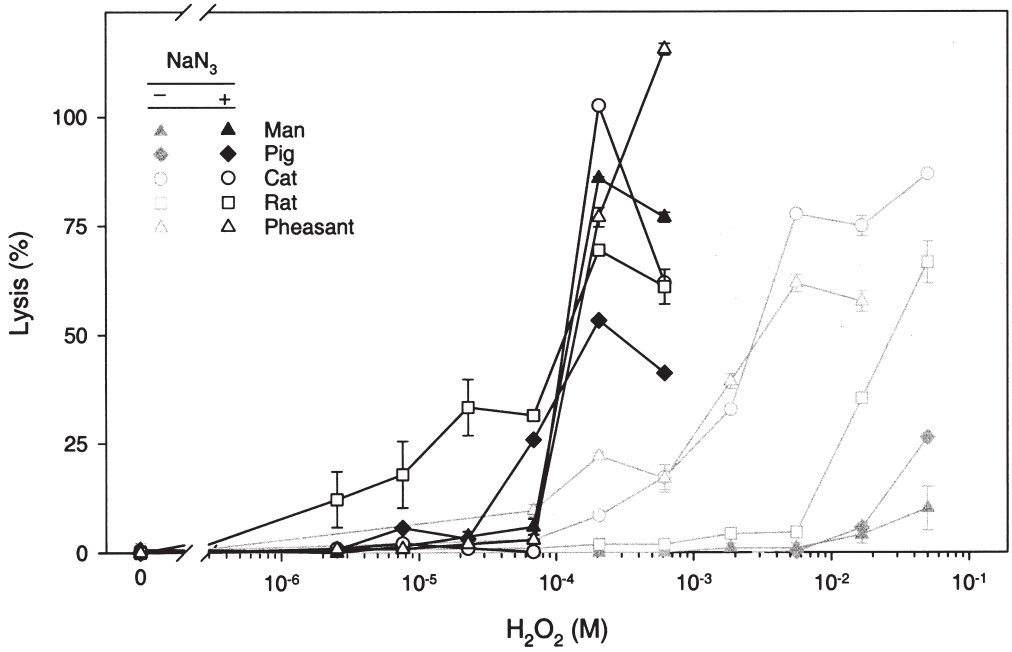

FIGURE 7 H_2O_2-induced lysis of erythrocytes in absence or presence of 10 mM sodium azide. Values are mean (±SEM) from one experiment, with three replicates per point.

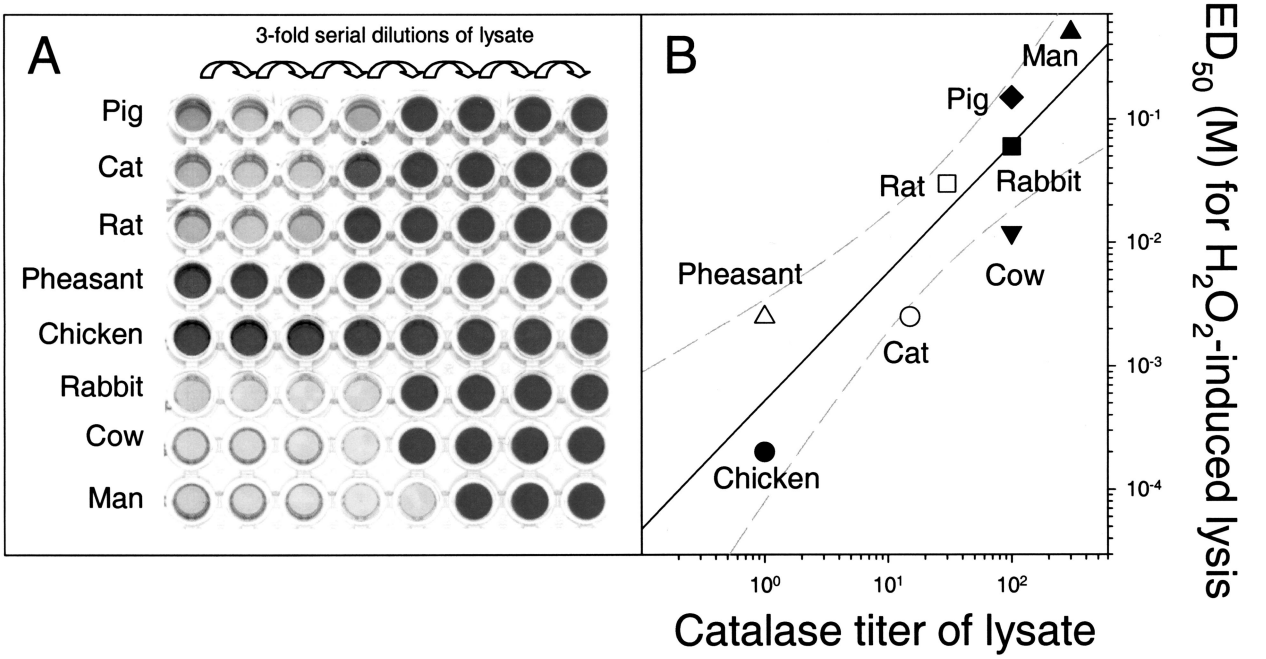

FIGURE 8 (A) Catalase activity of erythrocyte lysates determined as removal of hydrogen peroxide by FOX1 assay. Presence of H_2O_2 results in dark wells due to formation of Fe(III)/xylenol orange complexes, hence catalase activity is detectable as absence of stain. (B) Titer of catalase in lysates is shown against ED_{50} values obtained from Table I. Regression line is shown with 95% confidence intervals.

stability towards oxidants between unwashed and repeatedly washed cells. We find that unwashed erythrocytes do have an increased stability in absence of oxidants, which possibly could be ascribed to the more gentle treatment compared to erythrocytes that have been washed. There was no effect of repeated washing on AAPH-induced lysis, but mechanical disruption of erythrocytes during washing and resuspension would release intracellular catalase and thereby cause an apparent increase in resistance towards H_2O_2 (Fig. 4C).

Erythrocyte stability towards either oxidant is not affected by addition of up to 1% plasma (Fig. 4B), indicating that the amount of plasma (~0.6%) in the erythrocyte samples does not interfere significantly. We always perform the erythrocyte stability assay on the day of sampling, but storing the erythrocytes for one day in PBS increases only basal lysis slightly and does not change sensitivity towards lysis induced by either oxidant (Fig. 4D). Storage time could possibly be extended if erythrocytes are left in plasma.

Hemoglobin is oxidized by H_2O_2[12] and AAPH, and LDH activity is also affected by oxidants. Data has to be corrected for this bleaching/inactivation, and we therefore include lyzed erythrocytes exposed to oxidants in parallel, for each experiment and for each species. This correction is, however, only approximate, e.g. APPH does not pass the membrane and is thus not able to oxidise hemoglobin/LDH until lysis has occurred. H_2O_2 diffuses freely into erythrocytes, but the intracellular concentration of H_2O_2 will be maintained at a very low level because of the high concentration of catalase inside the erythrocytes, preventing substantial bleaching/inactivation. Both oxidants oxidize hemoglobin/LDH leaked from erythrocytes, and the kinetics of lysis is therefore critical for the applicability of a correction factor estimated from a 20 h incubation of oxidant in lysate. H_2O_2-induced lysis has almost reached the endpoint after 3 h (Fig. 3A), whereas AAPH-induced lysis progresses throughout the assaying time of 20 h (Fig. 3B). The correction factor is therefore more applicable for H_2O_2-induced lysis than for AAPH-induced lysis. Because of this, results presented as % lysis may exceed 100, when AAPH is used as oxidant. However, this approximation does not invalidate the results, since all comparable samples are analyzed in the same run.

Both absorption of hemoglobin[10,27] and activity of LDH[11] have been used to assay hemolysis. In this study, acceptable correlation between activity of LDH and absorbance of hemoglobin at 406 nm were obtained (Fig. 1), although activity of LDH is less sensitive to errors due to correction for inactivation, except for H_2O_2-induced lysis of chicken erythrocytes. In conclusion, both methods are easy and relatively inexpensive, but the applicability needs to be tested for each species.

The use of microtiter plates vastly increases the capacity of the erythrocyte stability assay, which allows extensive titration of oxidants, and therefore readily enables observations of differences in

erythrocyte stability of several orders of magnitude as illustrated in Fig. 5.

Erythrocyte susceptibility towards lysis induced by H_2O_2 and AAPH is highly species dependent (Fig. 5 and Table I). Interestingly, chicken erythrocytes exhibit extreme sensitivity towards H_2O_2 but high resistance towards AAPH. This apparently compensatory property is displayed by other species, as illustrated by the plot of ED_{50} for resistance towards lysis by H_2O_2 and AAPH (inset in Fig. 5). This differential stability towards various oxidative stressors is probably due to evolution/selection of different defense mechanisms.[18] Various species differ in defense mechanisms, for example in erythrocyte catalase[19–21] and glutathion reductase[22] activities, and compensatory mechanisms between species have been reported between erythrocyte catalase and glutathion peroxidase activities.[20,23] The individual genetic background within species also influence for example catalase activity as reported for blood levels from normal and acatalasemic mice[21,28] and humans.[29] Polymorphism in the promotor region of the human catalase gene has been shown to cause significant differences in blood catalase levels.[30] Furthermore, the life span of erythrocytes has been shown to be dependent on radical formation and efficiency of intrinsic antioxidant systems.[31]

The differences in erythrocyte stability between species as detected in our assay could be due to either antioxidative compounds/systems in plasma or properties of the erythrocyte itself. This was tested by mixing plasma from one species with erythrocytes from another species (Fig. 6). Chicken and pig were used for this experiment, since erythrocytes from these species are very different in stability towards H_2O_2 and AAPH. It is apparent from Fig. 6 that oxidative stability is an attribute of the erythrocytes, since the difference between the two species is largely independent of which plasma is added. Chicken erythrocytes appear to be more stable towards oxidation by H_2O_2 when resuspended in pig plasma relative to that of chicken plasma. However, considering the very high content of catalase in pig erythrocytes (see Fig. 8), just 1% lysis during sampling would leak sufficient catalase to explain this effect of porcine plasma. In the present work we have tested two water-soluble oxidants, which are quite different in radical generation and site of action. AAPH generates very reactive peroxyl radicals at a constant rate[32] and these radicals react with any molecule in proximity to the site of generation. H_2O_2, on the other hand, easily passes the erythrocyte membrane, and may react with hemoglobin and myoglobin, producing reactive higher oxidation states of the heme iron.[12] Furthermore, excess molar concentrations of hydrogen peroxide is believed to cause heme degradation and release of iron, which can then react with hydrogen peroxide in the Fenton reaction.[33]

H_2O_2-induced radical formation thus occurs inside the erythrocytes, where antioxidative defense mechanisms are present at high concentrations. The intracellular concentrations of enzymes and possibly low molecular antioxidants may therefore have a larger impact on lysis induced by hydrogen peroxide compared to lysis induced by AAPH. Both catalase and glutathion peroxidase from erythrocytes can remove H_2O_2, and the relative importance of these enzymes differ between species, i.e. for humans the removal rate of catalase has been reported to be approximately 125 times that of glutathion peroxidase, whereas the rate for catalase was only approximately 50% higher than that of glutathion peroxidase for mouse.[23] Although erythrocyte stability differ substantially between species, addition of 10 mM sodium azide, which inhibit catalase but not glutathion peroxidase activity, decreases stability of all the species tested to similar low stability (Fig. 7), indicating that catalase is the predominant factor determining the H_2O_2-induced lysis of erythrocytes. In accordance with this, the catalase activity of chicken erythrocytes is at least 100-fold lower than that of erythrocytes from other species (Fig. 8), and chicken erythrocytes are two to three orders of magnitude more sensitive to H_2O_2-induced lysis relative to that of erythrocytes from other species (Fig. 5). Our results confirm those of Van den Berg *et al.* (1992), who showed that human erythrocytes lyzed only after extensive oxidation of membrane fatty acids.[12] They did not find any lipid peroxidation or lysis at millimolar concentrations of hydrogen peroxide, but by inactivating catalase with 1 mM sodium azide, they could initiate peroxidation by 100 μM hydrogen peroxide.

Although differences in catalase activity appear to account for differences in erythrocyte stability towards H_2O_2, we have observed differences of up to one order of magnitude in H_2O_2 sensitivity in chicken erythrocytes with comparable catalase activities (data not shown). Factors like vitamin E content,[11,12] incorporation of polyunsaturated fatty acids into the erythrocytes,[13] and intracellular glutathion concentrations[14–16] affect the erythrocyte stability when challenged with various oxidants. Some of these other factors may play a role in the possible differences in antioxidative defense mechanisms between the species.

In conclusion, very large species differences in defense mechanisms towards oxidative stressors have been demonstrated for erythrocyte resistance towards lysis by H_2O_2 and AAPH. Furthermore, species differences in H_2O_2-induced lysis of erythrocytes are mainly attributable to differences in catalase activities.

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