A chance insight into the mechanism of phosgene toxicity

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Abstract

It has long been known that phosgene, a deadly war gas and industrial reagent, causes intense oxidative stress, but how it does so remains unclear. Here we report an accidental discovery: electron spin resonance spectroscopy (ESR) of live fruit flies reveals that phosgene exposure results in a distinctive manganese (II) hyperfine spectrum. The Mn(II) signal correlates with mitochondrial SOD2 expression. We suggest that phosgene acylates a highly conserved SOD2 active site tyrosine. This prevents the Mn redox cycling between ESR-silent Mn(III) and ESR-active Mn(II) that is required for superoxide dismutation. We propose that mitochondrial SOD2 inactivation is responsible for phosgene toxicity.

Introduction

While studying the effect of general anesthetics on the electron spin content of Drosophila, we encountered an unexpected phenomenon. Exposing the flies to vapor from an aged batch of chloroform resulted in a striking hyperfine six-line electron spin resonance (ESR) signal, as opposed to the single carbon-centered radical resonance typically observed[1,2]. The flies never recovered from the exposure and exhibited signs of poisoning, among which a phase of spasmodic proboscis movements preceding death. We were unfamiliar with the new ESR signal, but a search for "ESR six lines" revealed it to be diagnostic of manganese in the Mn(II) state[3]. Because chloroform gradually degrades to phosgene over time[4,5], we suspected that the Mn(II) signal was caused by phosgene.. Here we show that the presence of phosgene-induced Mn(II) spin signal depends on the expression of mitochondrial SOD2. Our results suggest that the previously unclear mechanism of phosgene toxicity may be inactivation of SOD2, a critical cellular defense mechanism against reduced oxygen species.

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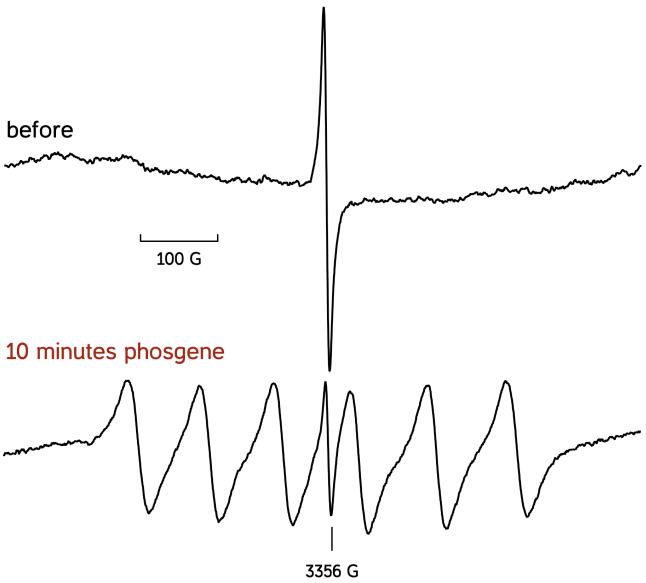

Figure 1 Two X-band scans of 30 fruit flies ten minutes apart. The top scan is the control, showing a typical carbon-centered radical line at 3356 G, approximately 10 G in width. The bottom scan shows the same flies after a 10-minute exposure to phosgene vapor. The carbon-radical line is reduced in amplitude, and six new equally spaced lines have appeared, diagnostic of Mn(II). The traces have been linearly detrended and low-pass filtered for clarity.

Results

Figure 1 depicts our findings. The top trace shows the ESR spectrum of 30 live flies (approx. 30 mg, see methods). The central resonance is caused by stable free radicals found in fruit flies, specifically eumelanin, pheomelanin, and sclerotins, henceforth "melanins" [6]). The bottom trace depicts the same flies following a 10-minute exposure to phosgene diluted in toluene (see methods). The central melanin resonance is significantly reduced, while six prominent hyperfine lines have appeared that overlap with it. The six-line pattern identifies Mn(II) ions in solution [7]. A control with only toluene shows no hyperfine lines (figure SM1).

Since there is no possibility of Mn itself being introduced by phosgene exposure, the Mn[II] signal must arise from the reduction of ESR-silent Mn[III] present in the flies, somehow caused by phosgene. Manganese is a relatively unusual metal in biochemistry [8]. Several crucial enzymes contain Mn, notably mitochondrial pyruvate carboxylase and superoxide dismutase (SOD2). SOD2 is a highly conserved enzyme that uses manganese as both an electron acceptor and electron donor in two different reactions, both of which turn the superoxide anion into less reactive species, respectively oxygen and hydrogen peroxide. The manganese cycles between Mn(III) (ESR silent) and Mn(II) (ESR active) in reactions (1) and (2) below. We suggest that phosgene prevents reaction (2) and traps the manganese in its divalent state.

superoxide dismutase

(1) $Mn^{3+} + O_2^{-}\bullet \longleftrightarrow Mn^{2+} + O_2$

(2) $Mn^{2+} + O_2^{-}\bullet + 2H^{+} \longleftrightarrow Mn^{3+} + H_2O_2$

phosgene

(3) $ROH + COCl_2 \longrightarrow ROCOCl + HCl$

(4) $ROCOCl \longrightarrow RCl + CO_2$

Phosgene (carbonyl dichloride) is an acylating agent that reacts with hydroxyl groups and other nucleophiles to form chloroformates[5]. The reaction with phenols is of primary interest here, because phenols are both abundant in melanins and present in proteins as tyrosine side-chains. Furthermore, a highly conserved tyrosine (tyr34) is located near the SOD2 active site and is necessary for SOD function[9]. We therefore hypothesize that the Mn(II) signal is caused by chloroacylation of Tyr 34 in SOD (equation 3), which prevents the Mn ion from cycling between the ESR-silent Mn(III) and ESR-active Mn(II) states, a key part of its catalytic mechanism (equations 1 and 2) [10]. We cannot, of course, rule out the possibility that acylation occurs on another amino acid, for example nearby serine 131, also conserved, and that the effect of acylation on Mn redox cycling is caused by a change in conformation.. The reduction of the melanin resonance can also be accounted for by chloroacylation of phenols. Most of the radical content of melanins and sclerotins is due to their semiquinone and quinone radicals, both derived from phenols. Phosgene reacts with phenols to produce chloroformates, which are then unable to form quinones.

Remarkably, the effect of phosgene on both features of the ESR spectrum is partially reversible. The flies typically do not immediately die after phosgene exposure, and their Mn[II] and melanin content remains unaltered for at least 1 hour, after which the Mn[II] signal gradually disappears while the melanin signal is slowly restored to close to its original amplitude, albeit with a small up-field change of the turnover point of the central resonance (Figure 2). The recovery may arise from hydrolysis of the ester produced in reaction 3 to regenerate the phenol[11]. It is less likely to be caused by reaction (4) above, which would require 4-chlorotyrosine to allow SOD2 to function, and chlorophenols to form quinone radicals.

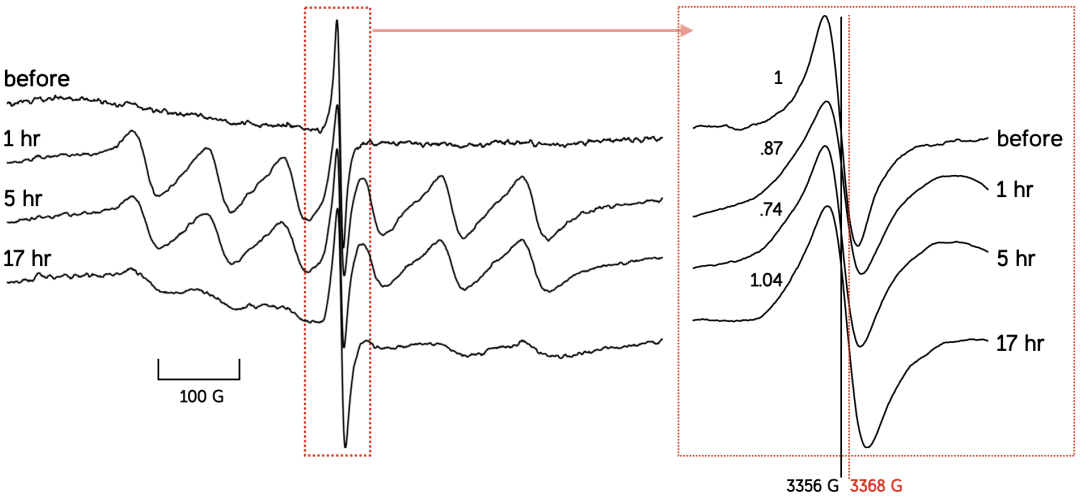

Figure 2 The Mn(II) and melanin peaks as a function of time after a brief phosgene exposure. **Left**: ESR traces taken at different times after a single 10 minute exposure to phosgene vapor. The Mn(II) signal appears rapidly, then decreases slowly over time. **Right**: size and position of the melanin peak in the traces at left. Relative sizes referred to the "before" peak are indicated. The melanin peak recovers completely from its decrease over 17 hours. Traces are low-pass filtered for clarity.

The effect of phosgene on the Mn signal requires metabolism. Recently dead flies show no Mn[II] signal when exposed to phosgene, whereas the melanin signal still decreases (Figure SM2). This is consistent with the notion that the electron that reduces Mn[III] to Mn[II] comes from superoxide itself, and superoxide will not be produced when oxidative metabolism has stopped. By contrast, the effect of phosgene on melanin persists in recently dead flies. The traces have been low-pass filtered.

A direct test of the involvement of SOD2 in the Mn[II] signal is provided by SOD2 deletion mutants (Figure 3). Two of these, ($Sod2^{\Delta 02}$) and ($Sod2^{\Delta 12}$) are available, in which the coding region of one allele of the SOD2 gene has been deleted with P-element activity leading to complete loss of function. This mutation is lethal when homozygous, underlining the importance of SOD2 to survival. Heterozygous flies grow more slowly than wild type but survive to adulthood. The mutants are in a yellow-white (yw) background, and therefore controls must be done in the same background for comparison. Measurements were made in 1 day old and 7 days old flies. The results show that there is practically no detectable Mn(II) at 1 day, and a definite, though smaller Mn(II) signal in the SOD deletion mutant at day 7. The difference between peak amplitudes in controls and SOD mutants is statistically significant both at day 1 and day 7 ($p<10^{-3}$).

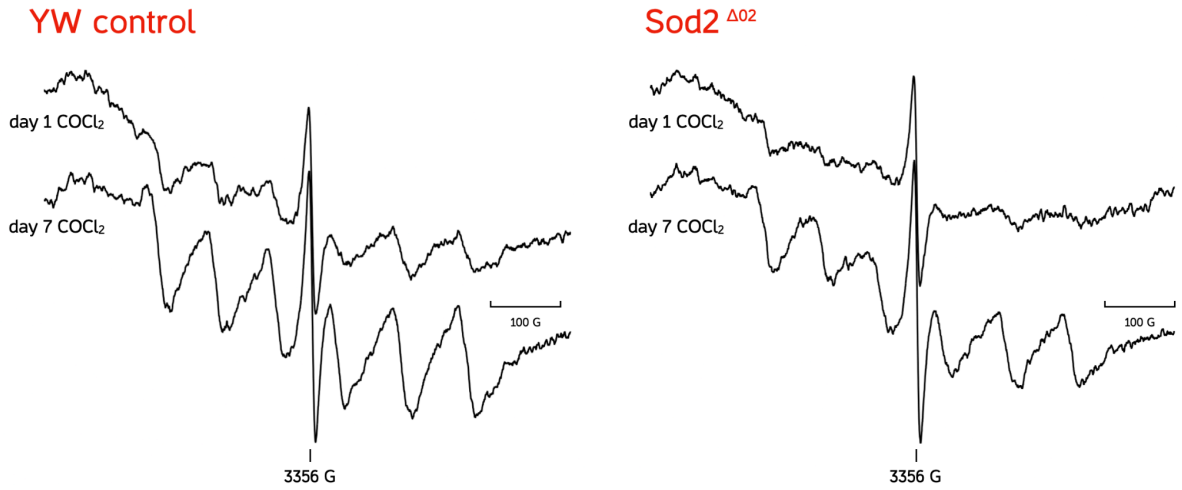

Figure 3 Size of the Mn(II) signal in control yellow-white flies (**left**) and in flies where one of the two copies of the Sod2 gene has been inactivated (**right**). 1-day old flies (upper traces) show a clear Mn(II) signal in the controls, and a much weaker one in the $Sod^{\Delta 02}$ mutant. After seven days, the Mn(II) signal is much larger in the controls than in the mutant. Each trace is an average of three different experiments, and three successive scans were averaged in each measurement. Traces are low-pass filtered for clarity.

To evaluate the likelihood of reaction of phosgene with SOD enzyme, we performed a molecular mechanics energy minimisation on the all-atoms human SOD structure (pdb 5vf9) in water (Figure 4). The tyrosine (yellow) is exposed to the solvent, right next to the three histidines (in blue) coordinating the Mn ion. Chloroformylation of the tyrosine followed by energy minimisation causes only a small outward movement of the tyrosine, shown at right in human and bacterial SOD2, whose structures are highly conserved. It therefore seems plausible that chloroformylation of SOD can occur, and likely disrupt SOD function like other tyr34 alterations [9].

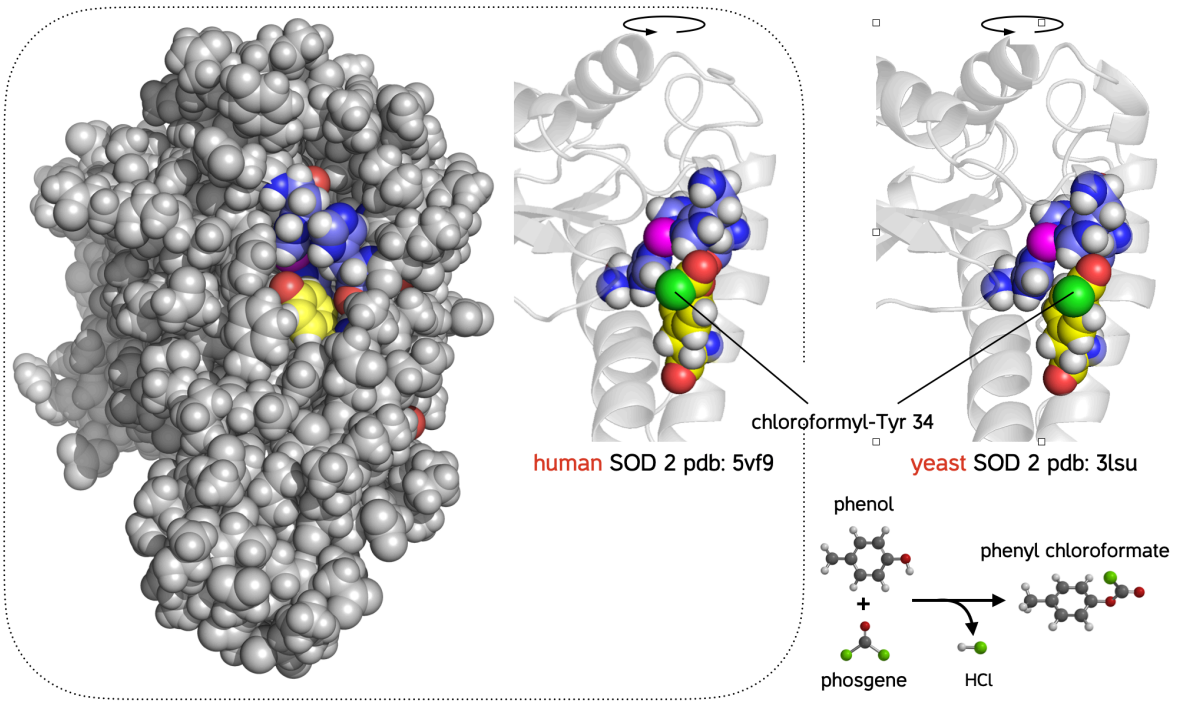

Figure 4 Left: All-atoms model of human mitochondrial superoxide dismutase 2. The pdb model (5vf9) was imported into a molecular mechanics code (see Methods), hydrogens and solvent water (not shown) were added and the structure energy-minimised. The tyrosine 134 ring is shown in yellow, with phenolic oxygen visible, the histidines surrounding the manganese ion in blue and the manganese ion itself in purple. **Inset, middle:** The tyrosine was chloroformylated and the resulting structure energy-minimised again. The chloroformyl group fits in the active site. **Inset, top right:** The active site in *Saccharomyces cerevisiae* SOD 2, pdb 3lsu after energy minimisation. The structure of the two enzymes are very similar.

Discussion

Phosgene is an infamous Great War gas, thought to have killed tens of thousands of soldiers[12–15]. The effects of exposure to high concentrations of phosgene typically follow three phases. The first is an immediate, transient choking reaction, likely due to the release of hydrochloric acid on contact with water and lung tissue. The second is a symptomless delay phase, of length inversely proportional to the exposure dose of gas, typically lasting hours. The third phase begins when the deleterious effects of phosgene begin to manifest themselves. Fluid leaks into the lungs, breathing becomes impaired and death can follow rapidly.

Phosgene is an exemplar of Haber's rule[16] which relates the *dose* of a noxious chemical to its effects. In Haber's analysis, dose means (concentration) x (duration). Remarkably, Haber's rule applies even to very long exposures at very low concentrations. This raises the possibility that even the low exposure levels in industrial uses of phosgene, commonly

used in production chemistry to replace hydroxyls with chlorine[17], may nevertheless result in lung damage over time[15,18].

The adherence of phosgene to Haber's rule, and the nature of phosgene as an acylation reagent has long been taken as an indication that the toxicity of phosgene is attributable to the acylation of one or more protein components of lung tissue. If (a) the reaction is irreversible, (b) the damage done is proportional to the fraction acylated, and (c) the concentration of phosgene is the rate-limiting step in the acylation reaction, then Haber's rule follows.

Phosgene acylates nucleophilic amino acid side-chains, among which hydroxyls (ser, thr, and tyr), amines (lys) and thiols (cys). Most proteins contain all these amino acids, so this does not usefully narrow down the range of possible targets for phosgene action. Accordingly the action of phosgene has been variously attributed to its effects on glutathione levels, lipid peroxidation and cellular ATP levels [19–22] .

There is, however, an aspect of phosgene action which may help narrow the range of possible targets. While acylation will proceed in all parts of the body exposed to phosgene, the damage done is concentrated in the lungs. They are, of course, exposed to the highest concentration of inhaled phosgene but also to the highest concentration of oxygen of any tissue of the body. The relation between free radical production, and in particular reduced oxygen species, and phosgene toxicity has been noted many times[23]. Phosgene exposure depletes cellular pathways involved in the cellular response to free radicals[24]. Further, the toxic effects of the inhalation of pure oxygen, which also follow Haber's rule, are similar to those of phosgene inhalation[25,26], pointing directly to a role for reduced oxygen species in both cases.

Our accidental discovery of the effect of phosgene on superoxide dismutase 2 suggestively brings these threads together: SOD2 is the primary defense mechanism against ROS created during oxidative phosphorylation[27,28]. SOD 2 contains a conserved tyrosine hydroxyl close to the active site[29]. That tyrosine has been shown to be crucial to SOD function[9]. The Mn(II) ESR signal tells us that SOD is unable to cycle, and therefore function, after phosgene exposure. The lung damage caused by phosgene can therefore be ascribed to the coincidence between inactivation of SOD and high oxygen levels in the lungs.

Given the likely large number of cellular components potentially acylated by phosgene, our identification of SOD as its target cannot be considered definitive. Biochemical studies are needed to confirm acylation of SOD at Tyr34. It would be interesting to know whether Tyr34 is unusually reactive. Treatment of tissues with phosgene could yield a rapid and quantitative test for SOD2. If confirmed by further studies, the mechanism we propose may

eventually lead to a rapid diagnostic test of phosgene poisoning, provided the Mn(II) ESR signal due to SOD acylation can be detected in other tissues of the body, e.g. blood or skin. We suggest Drosophila could be a useful animal model for the development of both tests and therapeutics of phosgene toxicology.

Author's contributions: ED and LT devised the project, ED performed experiments, ED and LT analysed data, LT wrote paper, ED and LT revised paper.

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Methods

ESR spectroscopy on live flies has been described before[1]. In our protocol, the control ESR trace was obtained from 30 male fruit flies inside a PTFE tube.

Phosgene exposure

The flies were placed in a 1.1 ml cylindrical centrifuge polypropylene test tube, The tube walls are perforated with a needle to allow gas exchange. The tube bottom is cut off and plugged with a shortened pipette tip wrapped with a 225µ nylon mesh. A snug fit is obtained, and excess mesh trimmed. The flies then sit a few mm above the tube bottom.

The tube containing the flies was placed into glass bottles containing an aliquot of phosgene-toluene solution. The bottom of the glass bottle has a dimple that can hold approximately 26µl of phosgene solution. After a timed exposure, the flies were removed from the polypropylene tube, tipped into the ESR PTFE tube and a post exposure ESR spectrum was taken.

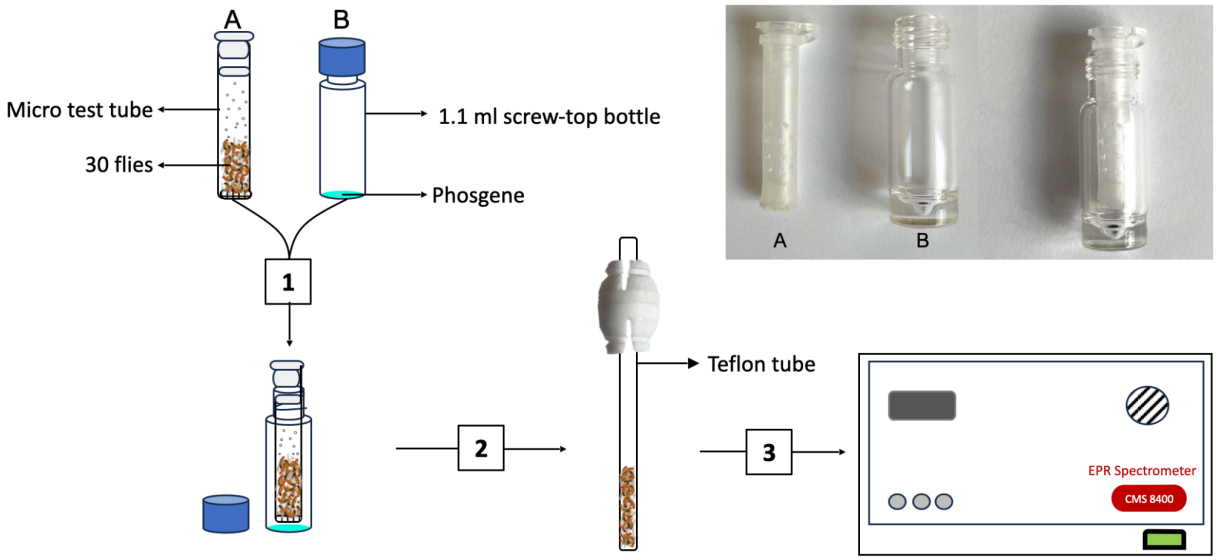

Figure 1: Experimental steps are depicted in the figure.

Phosgene Aliquots Preparation

Phosgene solution was purchased from Sigma-Aldrich. The aliquots of 25 µl, 50 µl, and 100 µl were pipetted into a 1.1 ml glass screw-top autosampler bottle. All aliquots were kept at 4C to reduce evaporation of phosgene.

Drosophila melanogaster

Flies are cultured using an instant drosophila mix obtained from Advanced Husbandry. This mixture was supplemented with propionic acid and nipagin in order to postpone spoilage and protect the medium from unwanted organisms. The ambient temperature was 18-25C with a humidity range of 40-80%. The natural light cycle of the Midlands United Kingdom was followed. Flies used in the experiments were purchased from. We used y^1w^1 for control (Bloomington Drosophila Stock Center #1495), and two heterozygote mutants with deletion of SOD2 coding region, $SOD2^{\Delta 2}$ and $SOD2^{\Delta 12}$ (Bloomington Drosophila Stock Center # 27643, # 27644)

ESR protocol

CW (continuous wave) ESR was used for the spin measurements. All the parameters were identical for control and mutant strains. The central field was set to 3370 gauss with 800 gauss width. Ambient gas inside ESR was a constant flow of nitrogen gas to keep flies immobile. Drosophila melanogaster is known to survive anoxia for up to 12 hours (Campbell, Werkhoven, and Harrison 2019). The experiments were conducted at room temperature.

Statistical Analysis

Experiments with mutations are conducted three times with three distinct fly populations. The significance was determined using Welch's t-test in R.

Results

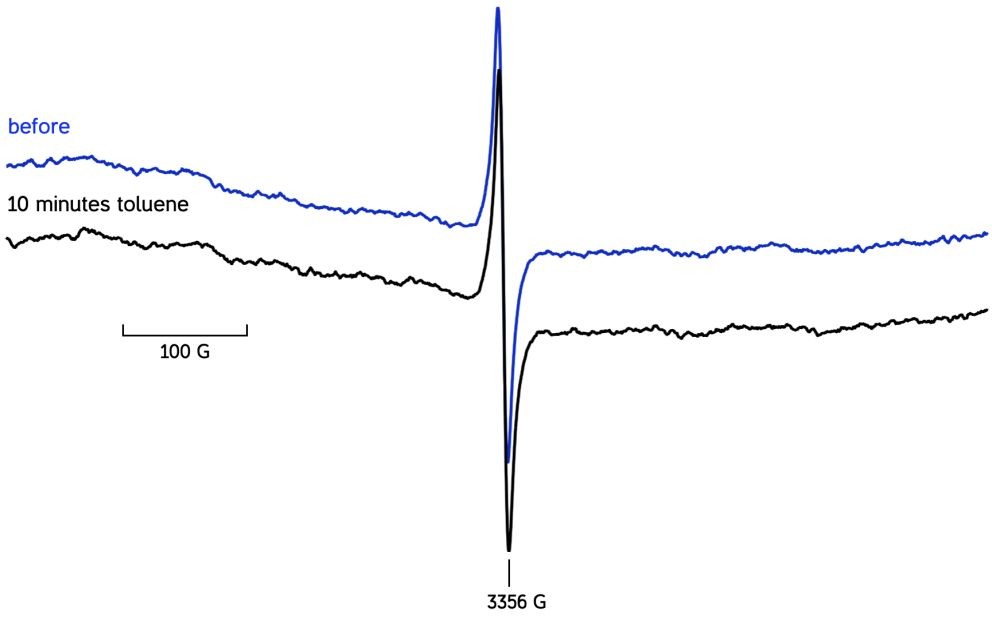

Figure SM1: ESR spectra of live flies before and after exposure to toluene alone.

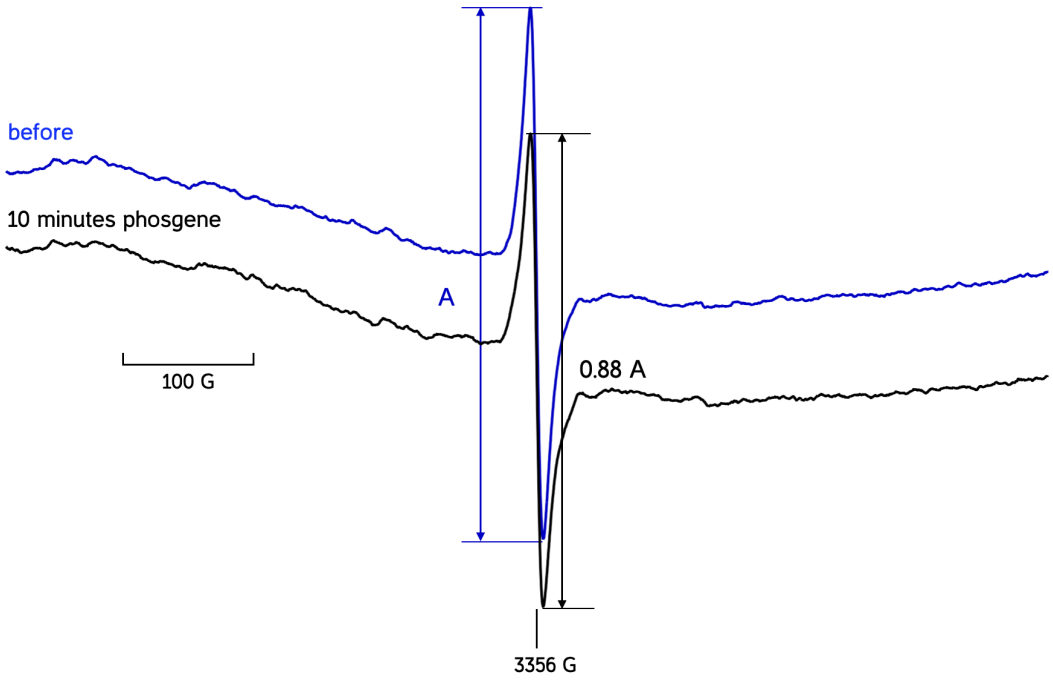

Figure SM2: ESR spectra of dead flies before and after exposure to phosgene

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