

Intricacies of Redoxome Function Demonstrated with a Simple *In vitro* Chemiluminescence Method, with Special Reference to Vitamin B12 as Antioxidant

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Abstract

The homeostatic control of the redox system (the redoxome) in mammalian cells depends upon a large number of interacting molecules, which tend to buffer the electronegativity of cells against oxidants or reductants. Some of these components kill – at high concentration – microbes and by-stander normal cells, elaborated by professional phagocytes. We examined whether a simple, *in vitro* chemiluminescence set-up, utilizing redox components from human polymorphonuclear neutrophils (PMN) and red blood cells (RBC), could clarify some unexplained workings of the redoxome. PMN or purified myeloperoxidase (MPO) triggers formation of reactive oxygen species (ROS), quantified by light emission from oxidized luminol. Both PMN and RBC can generate abundant amounts of ROS, necessitating the presence of a high-capacity redoxome to keep the cellular electronegativity within physiological limits. We obtained proof-of-principle evidence that our assay could assess redox effects, but also demonstrated the intricacies of redox reactions. Simple dose–responses were found, as for the PMN proteins S100A9 (A9) and S100A8 (A8), and the system also revealed the reducing capacity of vitamin B12 (Cbl) and lutein. However, increased concentrations of oxidants in the assay mixture could decrease the chemiluminescence. Even more remarkable, A9 and NaOCl together stimulated the MPO response, but alone they inhibited MPO chemiluminescence. Biphasic responses were also recorded for some dose–response set-ups and are tentatively explained by a ‘balance hypothesis’ for the redoxome.

Introduction

Homeostasis of the redox potential of cells and tissues is essential for their vitality [1]. The redox potential is determined by an interacting system of redox enzymes and sulfhydryl proteins, as well as their substrates. These are smaller molecules, such as NADPH/NADP, superoxide, hydrogen peroxide, thioredoxin and glutathione (GSH/GSSG) – all together constitute the redoxome. The redoxome can be affected by a number of oxidants and reductants (antioxidants), and its ‘buffering’ of the cells’ electrochemical (redox) potential is apparently as important as the buffering of pH for the function of enzymes and other cellular constituents [2, 3].

As there is intimate connection between the various component, reversible reactions within the redoxome, like in a system of interacting cogwheels, it may be sufficient to analyse one or a few of these components (like the glutathione system) to assess the state of the whole redoxome [1].

We have earlier characterized a very simple, *in vitro* chemiluminescence assay for the redoxome. Chemiluminescence was initiated by hydrogen peroxide (H₂O₂) plus myeloperoxidase (MPO) or calprotectin (S100A8/A9), which is the predominant protein in neutrophilic granulocytes (PMN). We tested the influence of various substances – with or without redox potential – on this assay, used as both a cell-free and a cellular system [4]. First of all, we here extended our study of the calprotectin peptides – shorthand: A8 and A9 – with and without combination with hypochlorite. A8 and A9 are certainly pleiotropic, and numerous functions have been ascribed to them [5–7], but rarely also antioxidant defence [8, 9], and some findings need to be clarified.

One new substance now examined was vitamin B12 (hydroxycobalamin, Cbl). Cbl is needed for growth and development of many organ systems and is particularly important for maintaining normal haematopoiesis and the integrity of the nervous system [10]. Cbl deficiency may

affect body organs in different ways. Megaloblastic anaemia reflects impaired thymidylc acid synthesis [11] and thereby disturbed DNA synthesis. Cbl normally acts as a cofactor in the conversion of homocysteine to methionine [12, 13]. However, it may also have a very different effect. In a recent work, it was shown that Cbl may be antioxidative [14], as it reduced the cell killing effect of H₂O₂ on a sensitive cell line. Interestingly, co-administration of Cbl and vitamin E improved nerve function following sciatic nerve injury in rats [15]. Our *in vitro* findings of antioxidant properties of Cbl may be relevant to these observations.

Erythrocytes (RBC) have a well-developed redoxome, to protect them from the continuous auto-oxidation that takes place spontaneously in air, involving haemoglobin (Hb) and O₂ to produce O₂⁻ and H₂O₂, other ROS and methaemoglobin. RBC and PMN are well equipped with antioxidant proteins [16, 17]. In the present study, we have examined chemiluminescence induced by both PMN and RBC proteins and whether it is inhibited by Cbl, which would confirm its possible antioxidant effect.

We also included ox brain-derived S100B – related to S100A8/A9 – as a test molecule, because it has been involved in proliferative as well as degenerative processes in the central nervous system and has been implicated in learning and memory processes as well. S100B has been found in astrocytes, microglia, Schwann cells and many other cell types [6, 7, 18, 19] and has been insufficiently characterized.

Finally, we examined the antioxidative actions of lutein, with Cbl as a control. The carotenoids lutein and zeaxanthin, normal components of the retina, act as antioxidants in the eye and are used in the treatment of age-related macular disease (AMD). At present, the general view is that they may help decrease the rate, if not the progression, of AMD [20].

Materials and methods

Chemicals. Hydrogen peroxide (30%), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), myeloperoxidase (MPO), S100B protein, phorbol 12-myristate 13-acetate (PMA) and Dulbecco's phosphate-buffered saline (D 8662) were purchased from Sigma (St Louis, MO, USA), and Hepes buffer from BioWhittaker (Walkersville, MD, USA), as reported previously [4]. Lutein was obtained from Extrasynthese (Genay Cedex, France). Dextran 500 (Pharmacia, Uppsala, Sweden) was dissolved in 0.9% NaCl and used as a 3 or 6% solution. Sodium hypochlorite (NaOCl, 0.5 M in 0.1 M NaOH), purchased from BDH (Dorset, UK), was diluted with water. Cbl (1 mg/ml) was obtained from GE, Oslo Norway. Luminol, PMA and Lutein were dissolved in dimethylsulphoxide (Sigma) and further diluted in water or a buffered salt solution. Phosphate buffers of different pH were made by mixing

appropriate portions of 0.2 M NaH₂PO₄ and 0.2 M Na₂HPO₄.

Purification of recombinant S100A8 (A8) and S100A9 (A9). Recombinant A8 (MW-10635 d) and A9 (MW-13242 d) proteins were produced as described previously [4].

Extract from red blood cells (RBC extract). The extract was provided by two different procedures. (i) Following haemoglobin (Hb) measurement, EDTA blood was diluted with water to approximate dilutions of 1/5000, 1/50 000 and 1/250 000, corresponding to final concentrations of 20, 2 and 0.4 µg Hb/ml. (ii) The blood was washed three times with 0.9% saline. Then, the cells were counted and diluted with water as in procedure (i). The protein concentration was determined, and the RBC extract and Sigma-Hb (20, 2 and 0.4 µg/ml) were tested in parallel.

Protein determination. Protein determination was carried out with the Bio-Rad or Ultima assay, and gel electrophoresis with the Bio-Rad-Mini-PROTEAN Precast Gel (Bio-Rad Laboratories AB, Oslo, Norway).

Chemiluminescence. Chemiluminescence exerted by intact granulocytes was examined in a luminol-dependent system with either PMA or H₂O₂ as stimulators. Otherwise, a cell-free system was used as model of the respiratory burst in PMN: H₂O₂ + MPO + chloride + luminol. In some experiments, MPO was replaced by A8, A9 or S100B. The luminol-enhanced chemiluminescence was measured as relative light units in a FLx800 luminometer (Biotek Instruments, Inc. Winooski, VT, USA), using 96-well, white culture plates (Nunc, Roskilde, Denmark). Unless otherwise indicated, the recording was carried out at room temperature (22–25 °C). Each well contained 25 µl H₂O₂ solution (final concentrations as indicated), 5–10 µl MPO (final concentration 0.05–3.0 µg/ml), 50 µl luminol (0.1 mM), 10–20 µl test substance or cell suspension, and buffer (140–170 µl) to yield a total volume of 250 µl. MPO (10–40 µg) was dissolved in 1 ml water and stored at 4 °C. The activity was stable for some weeks, but upon further dilution (0.05–3.0 µg/ml), its activity was often strongly reduced after a couple of days. The chemiluminescence was recorded (4–8 wells per group) at 1- to 2-min intervals for 30 min, and the integral of the response curve [area under the curve (AUC)] was calculated.

Significant responses with MPO, calprotectin, A8, A9, S100B or cells were observed with 8–16 µM H₂O₂ as stimulator [4]. However, consistent results were best obtained at higher concentration (30–100 µM). Without NaOCl in the system, a high (250–500 µM) H₂O₂-concentration was needed to obtain an appreciable chemiluminescence with A8 and A9. On the other hand, together with NaOCl, the sensitivity may be greatly enhanced [4]. The chemiluminescence of control buffer, or buffer plus NaOCl (or other reactants), was measured to assess background values. With NaOCl, we sometimes found unacceptably large variability at the left edges of the plates, and it became an established routine to use these column

wells for buffer controls only. The plates were tested with control buffers to measure variability in chemiluminescence readings. Tris-buffers were avoided because they trigger substantial chemiluminescence when combined with NaOCl.

Cell separation. Human blood from healthy colleagues was collected in vacutainers containing EDTA. The granulocytes were separated, as described previously [21], and remaining erythrocytes were removed by NH_4Cl lysis. Finally, the granulocytes (>95% purity) were suspended in Dulbecco's buffer (pH 7.4) with 5 mM glucose.

Statistics. The Student's *t*-test was used for testing the difference between two groups. Two-sided *P* values <0.05 were considered statistically significant. The results are given as means with their standard error (SEM).

Results

Simple dose–response phenomena

In previous work, we have shown that recombinant A8 and A9 elicited chemiluminescence in response to the presence of H_2O_2 , but it was virtually absent if A8 or A9 had been exposed to reducing substances (mercaptoethanol, dithiothreitol) during their production. However, the activity could be restored and even significantly enhanced when they were combined with NaOCl [4]. To further characterize our assay, we extended these dose–response studies over a larger concentration range (Fig. 1A). Near linear chemiluminescence dose dependency was found for the 0–240 $\mu\text{g}/\text{ml}$ range for both A8 and A9 (Fig. 1B).

Below 20 $\mu\text{g}/\text{ml}$, the luminol-dependent response to H_2O_2 (Fig. 1B) was lower for A8 than for A9 ($P < 0.01$), even without corrections for molecular weight, confirming previous results [4]. At higher concentrations, chemiluminescence intensities differed relatively less and increased less abruptly at concentrations above 120 $\mu\text{g}/\text{ml}$. H_2O_2 up to 500 μM gave good light emission, although 16 μM proved sufficient for significant stimulation.

Complex dose–response phenomena

NaOCl enhanced the chemiluminescence induced by A8 and A9 (Fig. 2A), but very differently for the two. With A8, the response was close to linear in the 0.5 to 8 $\mu\text{g}/\text{ml}$ range, but there was a further increase up to 16 $\mu\text{g}/\text{ml}$ ($P < 0.01$). With A9, the initial response was much stronger than with A8, but the chemiluminescence diminished markedly above 8 $\mu\text{g}/\text{ml}$ ($P < 0.001$). For routine use, 2–4 $\mu\text{g}/\text{ml}$ of A8 or A9, 20 μM NaOCl and H_2O_2 in the 16–100 μM range were chosen. Preferably, the $\text{H}_2\text{O}_2/\text{NaOCl}$ molar ratio should be in the 1.6–3.0 range [4]. A higher ratio may increase variability.

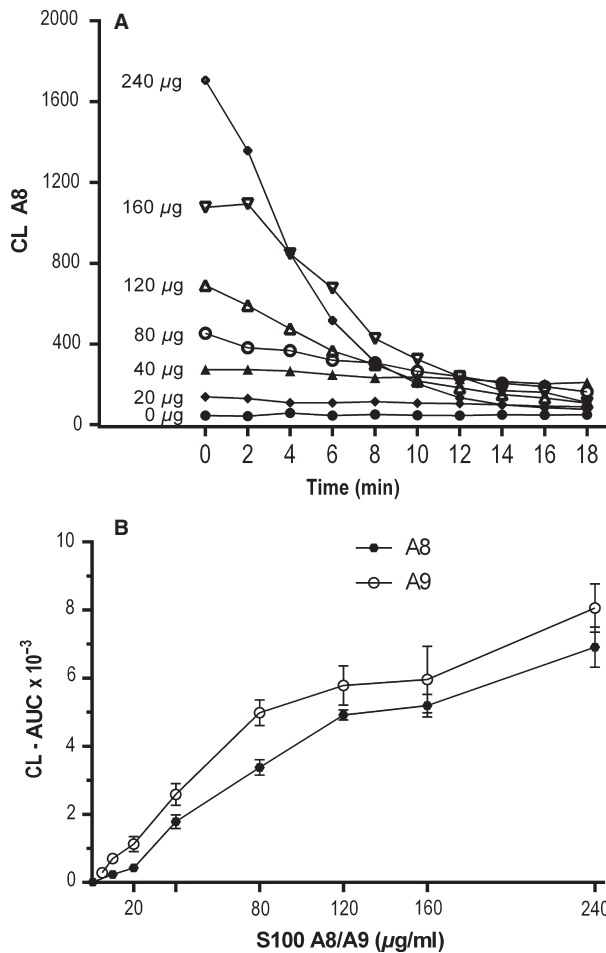


Figure 1 (A) A representative dose–response picture of chemiluminescence (CL) induced by recombinant S100A8 (=A8), as measured in arbitrary light units. Time response pattern was recorded with a mixture of 165 μl phosphate buffer (pH 8.0) with 60 mM NaCl, 10 μl A8, 50 μl luminol (0.1 mM final conc.) and 25 μl H_2O_2 (500 μM final conc.) per well. Mean of eight values per point. (B) Dose–response curves for recombinant A8 and A9. Each well contained buffer and chemicals as described above. The results are presented as CL-area under the curves (CL-AUC) for each protein concentration. The values were scored in quadruplicate in two experiments. Mean values \pm SEM.

S100B, isolated from ox brain, did not alone appreciably induce chemiluminescence in response to luminol and H_2O_2 , probably due to exposure to dithiothreitol in the separation process. However, a strong stimulation was noted when NaOCl was added (Fig. 2B). The response pattern was similar to that observed for A9, and again chemiluminescence was markedly reduced at a S100B concentration above 16 $\mu\text{g}/\text{ml}$ ($P < 0.001$).

Gel electrophoresis revealed dimerization of recombinant A8 and A9 (Fig. 3A), in some batches not found for A8. ROS induction depended upon the degree of dimer formation. Only when NaOCl was added to S100B native protein, dimerization was obtained (Fig. 3B).

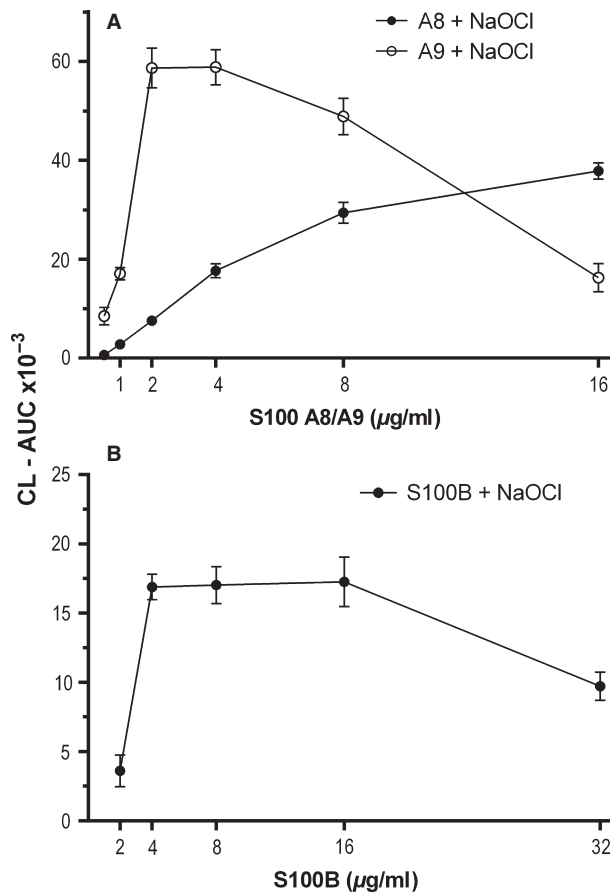


Figure 2 (A) Dose–response for A8 and A9, both plus NaOCl. The phosphate-saline buffer (see legend to Fig. 1) was used, but 10 µl of the buffer was replaced by 10 µl of 500 µM NaOCl (final concentration of 20 µM). The plates were then incubated at room temperature for 17 min before luminol and H₂O₂ (63 µM) was added to initiate ROS formation. Mean values from four experiments, each scored in quadruplicate. (B) Dose–response for S100B protein purified from ox brain. Phosphate-saline buffer pH 8.0. After incubation at room temperature for 17 min, H₂O₂ was added (50 µM). Mean values from two quadruplicate experiments. Corrections have been made for background CL.

Modification of the chemiluminescence response by some intracellular components

Chemiluminescence stimulation may also be obtained with erythrocyte lysate (Fig. 4). It is probably caused by its haemoglobin and was enhanced 2- to 3-fold by 20 µM NaOCl (not shown, $P < 0.01$). The chemiluminescence was much stronger with purified haemoglobin (Sigma) (Fig. 4), presumably due to an effect of methaemoglobin, and, noticeably, there was no additional effect of NaOCl.

The chemiluminescence induced by MPO was inhibited by recombinant A9 and brain-derived S100B (Fig. 5). NaOCl itself strongly decreased the MPO activity, and 10 µM NaOCl was sufficient to cause 85% inhibition of chemiluminescence (Fig. 6, insert). This inhibition was counteracted in a dose-dependent manner by A9 (Fig. 6), which itself (4 µg/ml) exerted approximately 40% inhibi-

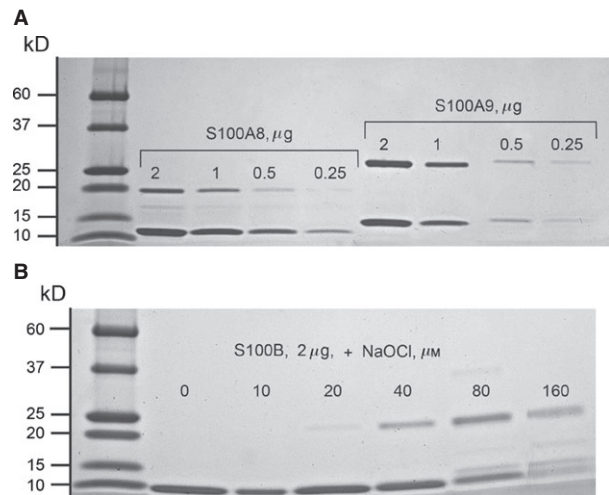


Figure 3 Gel electrophoresis revealing spontaneous dimerization of A8 and A9 (A) and of S100B only in the presence of an oxidant (B), at different concentrations. Two microgram protein/well.

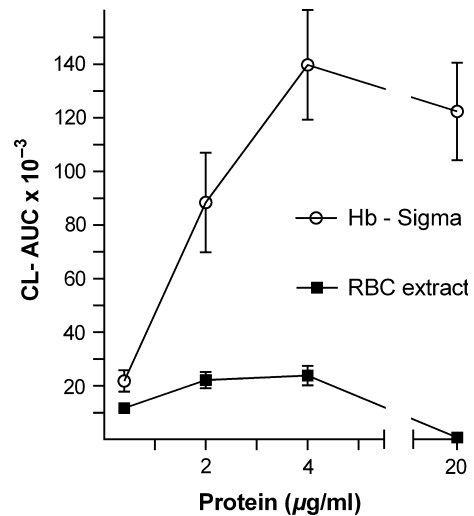


Figure 4 Biphasic dose–response of erythrocyte (RBC) extract and haemoglobin (Hb). RBC extract from two individuals (see Materials and methods) were compared (quadruplicate measurements) with corresponding concentrations of purified Hb (Sigma).

tion of the MPO activity (Fig. 6). It is noteworthy that two regulators, NaOCl and A9, which when added alone had an inhibitory effect on MPO-generated chemiluminescence, stimulated light emission when combined (Fig. 6) ($P < 0.05$). Altogether, we have found considerable variability in the A9 effect on MPO chemiluminescence, and mostly so that strong inhibition correlated positively with low chemiluminescence values in the MPO control.

Antioxidant effect of vitamin B12 (Cbl) and lutein

The chemiluminescence obtained with cells (PMN) stimulated with H₂O₂ or PMA (Fig. 7A) was significantly

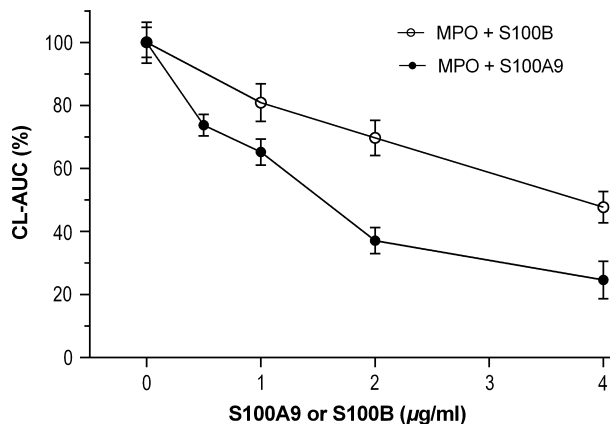


Figure 5 The effect of A9 and S100B on MPO (0.1 µg/ml) chemiluminescence. Dulbeccos medium pH 7.4, H₂O₂ 100 µM; recombinant A9; S100B (Sigma) purified from rat brain. Mean values ± SEM from two experiments in per cent of control. The values were corrected for background buffer CL values.

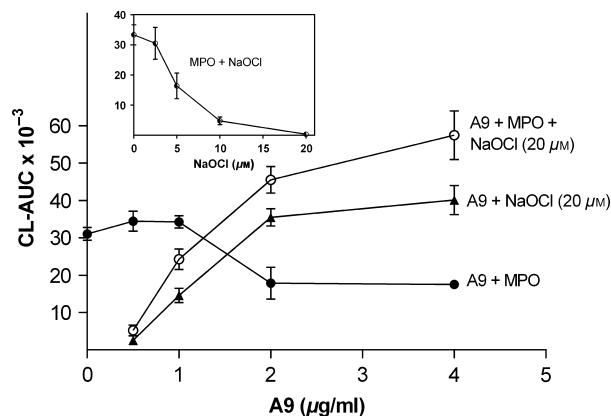


Figure 6 Dose–response of A9 in combination with another granulocyte component, myeloperoxidase (MPO) and also with NaOCl included. The effect of NaOCl on MPO was tested separately (three experiments, in insert). The following combinations were tested in parallel (three experiments), (i): A9 (0.5–4 µg/ml) + MPO (0.1–0.3 µg/ml), (ii): A9 + NaOCl, (iii): A9 + MPO + NaOCl.

reduced ($P < 0.01$) by Cbl concentrations of 5 µM or more. It is of note that a moderate stimulation (30%) was observed at 0.5 µM Cbl ($n = 40$, $P < 0.01$). A similar biphasic response possibly occurred with A9 (Fig. 7B) and a mixture of S100B and NaOCl (Fig. 7C), whereas the inhibitions effected by higher concentrations of Cbl were all statistically significant (for MPO, 0 versus 20 µM, $P < 0.001$; A9, 0 versus 5 µM, $P < 0.01$; S100B plus NaOCl, 0 versus 20 µM $P < 0.0001$). The chemiluminescence induced by haemoglobin (Fig. 4) was also significantly inhibited by Cbl in the 5–20 µM range, with and without NaOCl (Fig. 7D). Cbl itself did not affect chemiluminescence (five experiments with 500 µM H₂O₂) and apparently did not change the degree of dimerization of A9.

Lutein inhibited chemiluminescence induced by RBC extract (2 µg/ml), and a particular strong inhibition was observed with A9 + NaOCl as stimulators (Fig. 8).

Discussion

In the present work, we have studied the redox system (redoxome) *in vitro*, with a chemiluminescence assay, to examine the interplay of different redox proteins (MPO and S100 proteins) from PMN and brain tissue. The reactions of intact cells, PMN, were also assessed.

Calprotectin (S100A8/A9) and its component peptides A8 and A9 make up a surprisingly high concentration in PMN. Several functions have been ascribed to these proteins [6, 7], but rarely redox functions [8, 9, 22, 23] – and then as antioxidants. The same applies to the brain-derived S100B, which we have also included in our studies.

The MPO redox chain reactions are well established and constitute the foundation of our *in vitro* assay. The combination of MPO, H₂O₂ and chloride leads to the formation of HOCl/NaOCl, a strong oxidant that in the presence of metal ions also participates in the generation of OH radicals (which may be the predominant chemiluminescence-eliciting ROS in our assay) [4]. *In vitro* 0.1–0.3 µg/ml of MPO is sufficient to initiate this process, as measured by chemiluminescence. S100 proteins trigger chemiluminescence at a higher concentration (>15 µg/ml) than MPO, but do not need chloride ions. For both monomers (A8 and A9), there was a steady increase of chemiluminescence in the tested concentration range up to 240 µg/ml (Fig. 1).

Some of our findings were surprising. When the strong oxidant NaOCl (20 µM) was added to A8 and A9, a large increase of chemiluminescence was seen, more than tenfold at low concentrations of the peptide (1–4 µg/ml), where combination with A9 showed the strongest response. However, at higher concentrations of A9, chemiluminescence became much weaker (Fig. 2), as also shown previously [4]. Such decline was also the case when A8 or A9 at increasing concentration supplemented the basic MPO system (Fig. 5). S100B + NaOCl had a similar, biphasic reaction pattern (Fig. 2), but S100B did not elicit light emission alone, without NaOCl. S100B dimerized only in the presence of an oxidant (NaOCl) in our experiments (Fig. 3). On the other hand, A8 and A9 dimerized spontaneously in solution (Fig. 3). Extra oxidants, as well as the dimerizations (by removing available sulfhydryl (-SH) groups), might tend to increase the oxidant power of the assay.

Our explanatory hypothesis for the findings summarized above includes the presumption that the assay's overall reduction potential must be kept within a certain range for chemiluminescence to occur. Too much of either oxidant or antioxidant potency will inhibit chemiluminescence, by destroying the balance between the component redox

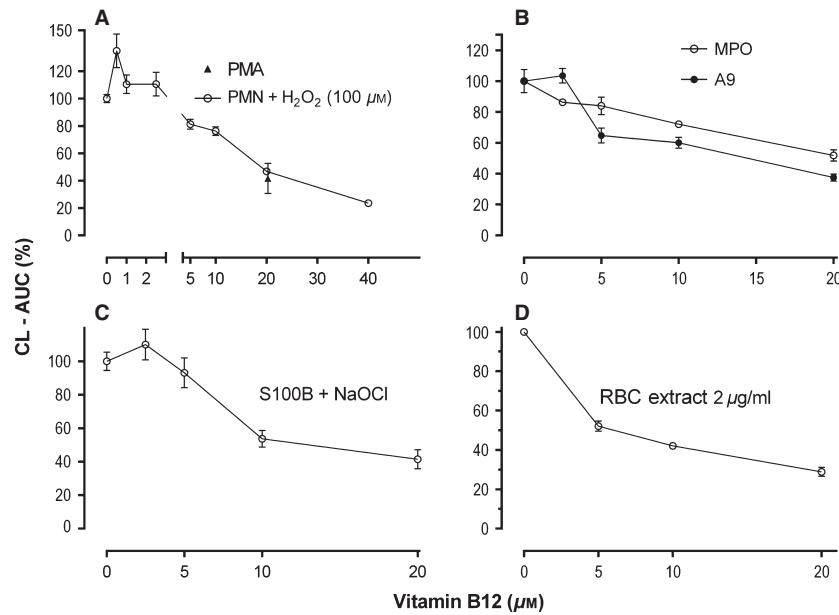


Figure 7 (A) The inhibition obtained by vitamin B12 (Cbl) on respiratory burst (chemiluminescence) elicited by 10⁵ human PMN during 30 min. The cells were suspended in Dulbeccos medium (pH 7.4) and were stimulated by 100 μM H₂O₂ (at 37 °C) or PMA (10⁻⁷ M) at room temperature). The figure shows mean values of six experiments with 6–8 determinations per point. Mean CL of control without Cbl was 3750. The PMA stimulation is given as mean ± SEM of four experiments (20 μM vit B12; mean CL of control was 3470). (B) Dose–response of the inhibitory effect of Cbl on MPO (0.2 μg/ml) and A9 (20 μg/ml), ran in parallel in three experiments, but with a possible stimulation of A9 at a low Cbl concentration. MPO was tested in Dulbeccos medium with pH 7.4, 100 μM H₂O₂ and A9 in phosphate-buffered saline (pH 8.0), with 500 μM H₂O₂ as initiator of chemiluminescence. (C) As in panel (B) for S100B protein (4 μg/ml). Phosphate-buffered saline pH 8.0, NaOCl 20 μM, H₂O₂ 32 μM. Mean values ± SEM from 15 to 16 determinations in two experiments. (D) Dose–response of Cbl on chemiluminescence induced by RBC extract (2 μg/ml). 100 μM H₂O₂; two experiments.

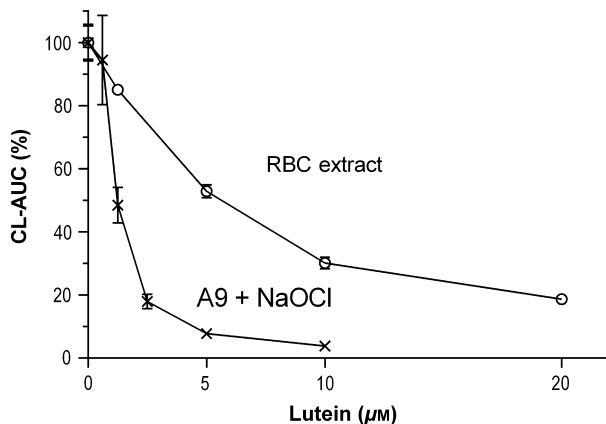


Figure 8 Dose–response of Lutein on chemiluminescence induced by RBC extract (two experiments) and A9 + NaOCl (three experiments).

reactants. As a corollary, the addition of one species of antioxidant – such as vitamins C or E to humans – might skew the redoxome so that the intended favourable effect, for example reduction of mutation rate or sparing by-stander cells in an inflamed tissue, will not occur.

As shown in Fig. 6, NaOCl completely abrogated the chemiluminescence by MPO, in agreement with previous findings [24]. Hypochlorous acid could also inhibit some other redox enzymes [25]. In general, NaOCl has

a tendency to inactivate many kinds of protein, in particular those with haem groups (catalase, superoxide dismutase, glutathione peroxidase, etc.) [25, 26]. NaOCl may release iron from such proteins, which presumably also would inactivate them. Furthermore, it is noteworthy that superoxide radicals can synergize with hypochlorite to damage proteins [27], so that high concentration of oxidants in our assay might inactivate vital components. However, A9, at 2 μg/ml, totally prevented the complete chemiluminescence inhibition of MPO obtained by NaOCl (Fig. 6), indicating that more than the above-mentioned mechanisms may apply. In any case, it is worthwhile to notice that NaOCl depressed MPO chemiluminescence, but stimulated A9 chemiluminescence.

In a previous study [4], we found that the heterodimer calprotectin (A8/A9) enhanced MPO chemiluminescence, possibly reflecting different properties of the heterodimers and the monomers. Still, when several redox molecules participate, which especially takes place in intact PMN, it may be difficult to predict the outcome from our assay results. Adding to the complexity, it should be noted that with haemoglobin or methaemoglobin introduced to the reaction mixture, haem groups may transfer electrons, bind small molecules, act as cofactor for enzymes and even be transitory ligands [28, 29].

Cbl and derivatives of vitamin B12 markedly improved the survival of a H₂O₂/homocysteine-sensitive cell line (1-Sk-Hep) exposed to the pro-oxidants homocysteine or hydrogen peroxide [14], possibly due to an antioxidant effect of Cbl [30]. But, as discussed by the authors, the improved survival might also depend on stimulation of methionine synthase activity, a glutathione sparing effect or modification of signalling molecules such as TNF- α and EGF. Our simple assay decided between these possibilities, as the three latter alternatives could not apply to our simple assay. The possible two-phase responses obtained in some set-ups (Fig. 7) are in line with our explanatory hypothesis described above.

Chemiluminescence may be affected in different ways by erythrocyte components (Fig. 4). The high levels of O₂ and haemoglobin facilitate auto-oxidation to produce O₂⁻ and H₂O₂ and thereby to increase chemiluminescence [17], which we found. The enhanced chemiluminescence could at least partly also be due to formation of methaemoglobin, which has pseudoperoxidase activity. The methaemoglobin content in a haemoglobin solution is reported to increase from 1.3 to 2.4% during 47 days of storage in a blood bank [31]. Presumably, this would catalyse ROS production, like MPO does [29, 31–33]. A further increase (Fig. 4) of haemoglobin concentration in the assay (20 μ g/ml) reduced the chemiluminescence to background values, in line with our tentative explanation of unexpected findings (see above). S100 proteins do not contain haem, in contrast to several other oxidants and antioxidants.

The inhibitory (antioxidant) effect of Cbl (5–20 μ M) was strikingly similar on cells (PMN), RBC extract and redoxome components (Fig. 7). In relative terms, the concentration of active Cbl (5–20 μ M) is high, as compared to serum values (4–500 pM). Possibly, the Cbl is concentrated in cells, where it has its important functions. We have no explanation for the stimulation at low concentration (0.5 μ M) of Cbl. One possibility is that stimulation and inhibition have different concentration thresholds. Cytidine deaminase, added to the basic myeloperoxidase assay, inhibited chemiluminescence at all concentrations examined [4]. Therefore, because the assay can test stimulators as well as inhibitors, we cannot exclude the possibility that the main reducing activity (inhibition) from Cbl was masked by some unknown oxidative function of Cbl at low concentrations, which was lost by dilution.

By and large, the inhibitory effect of lutein on RBC extract was similar to that observed for Cbl (Fig. 7). A particular strong inhibitory effect of lutein was observed with the combination of A9 and NaOCl as target molecules (Fig. 8).

One should be aware of some caveats. MPO in the refrigerator tends to lose activity quite rapidly; the dissolved MPO should be used within a few days, or the volume fraction (concentration) of MPO in the wells should be stepwise increased with prolonged usage. It is difficult to exclude a toxic

or quenching role of haemoglobin (Fig. 4). Still another uncontrolled variable is the abundance of antioxidants in red cells (catalase, superoxide dismutase, thioredoxin reductase), the removal of which from our assay depends upon the haemoglobin separation technique used. In work with PMN, it is therefore particularly important to make sure that red cells have been efficiently removed. It should also be kept in mind that haemoglobin can be modified by about 50 haem variants [28], possibly contributing to redox variability. Still, the functional role of haem in these regulators remains elusive.

With a number of various dose–response experiments, utilizing a simple redox reaction based chemiluminescence assay and different reactants, we have obtained results that suggest that the redoxome is a so complicated system, which can react in such an unexpected way, that to foresee the results of perturbations in the intact organism is for now very difficult. Prescriptions of antioxidants to an intact organism are consequently ‘risky business’. The results further make us speculate that the proteins S100A8, S100A9 and S100B can have important redox buffering or balancing function in inflammatory conditions.

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Author contributions

AB performed the majority of the laboratory-based work and wrote part of the manuscript. RJF and IS did part of laboratory work and were involved in the writing of the manuscript. KLS designed the graphs and contributed to intellectual input and performed corrections on the manuscript. HBB was involved in creating the concept of the presented work, troubleshooting, intellectual input and writing the manuscript.

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