Hypothesis

Vampires, Pasteur and reactive oxygen species
Is the switch from aerobic to anaerobic metabolism a preventive antioxidant defence in blood-feeding parasites?

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Abstract Several species of parasites show a reduction of their respiratory activity along their developmental cycles after they start to feed on vertebrate blood, relying on anaerobic degradation of carbohydrates to achieve their energy requirements. Usually, these parasites choose not to breathe despite of living in an environment of high oxygen availability such as vertebrate blood. Absence of the 'Pasteur effect' in most of these parasites has been well documented. Interestingly, together with the switch from aerobic to anaerobic metabolism in these parasites, there is clear evidence pointing to an increase in their antioxidant defences. As the respiratory chain in mitochondria is a major site of production of reactive oxygen species (ROS), we propose here that the arrest of respiration constitutes an adaptation to avoid the toxic effects of ROS. This situation would be especially critical for blood-feeding parasites because ROS produced in mitochondria would interact with pro-oxidant products of blood digestion, such as haem and/or iron, and increase the oxidative damage to the parasite's cells.

Key words: Hemoglobin; Energy metabolism; Free radical; Parasite; Antioxidant

1. The paradox: not to breathe in an oxygen-rich environment

For most organisms the use of molecular oxygen is the key to achieve complete oxidation of foodstuff and maximise energy yield from nutrients. Facultative aerobes clearly choose to breathe when they are allowed to – yeast being the most classic example. When oxygen tension is not high enough to oxidise reduced substrates to CO_2 and water through respiration in mitochondria, most organisms rely on anaerobic glycolysis to obtain energy. The well-known fact that glucose utilisation is higher under anaerobic than under aerobic conditions – the so-called Pasteur effect – is not much more than a consequence of almost pure thermodynamics: all organisms must optimise their energy budget. However, several intravascular parasites, such as *Schistosoma*, *Angiostrongylus*, *Dirofilaria* and *Plasmodium*, seem, at first glance, not to conform to this rule [1–7]. In the particular case of helminths, free-living parasite stages are dependent on endogenous substrate stores, which are aerobically degraded through the Krebs cycle. After getting into their host's blood vessels, they undergo a marked down-regulation of oxidative metabolism and, generically, adult parasite stages are almost completely dependent upon the host's carbohydrate supplies for their energy requirements, which are mainly utilised by means of fermentation [3]. So, these parasites constitute a paradox because, while they live in oxygen-rich environments, such as vertebrate blood, they turn their metabolism towards fermentation. Why do these blood-feeding parasites choose not to use oxygen?

2. Energy metabolism

In *Schistosoma mansoni*, it was demonstrated that the sporocysts transform into cercariae, which degrade their glycogen stores to CO_2 by aerobic metabolism [8,9]. Cercariae then penetrate the vertebrate skin and reach the portal circulation in a few days. Together with all dramatic morphological changes that occur during their transformation into schistosomula, *S. mansoni* switch from aerobic to anaerobic-based glucose degradation, which results in a several-fold increase in lactate production [2,3,8,9]. Moreover, there is a clear decrease in expression of key mitochondrial enzymes such as malate dehydrogenase and cytochrome oxidase [10]. After transforming into schistosomula, *S. mansoni* is able to survive for hours or even days in the presence of cyanide, a respiratory poison [8,11]. Concomitant with all these metabolic changes, developing schistosomula begin to feed on red blood cells a few days after infection [12].

Regarding *Angiostrongylus cantonensis*, a helminth that inhabits pulmonary and heart blood vessels of rodents and humans, several studies showed that the adult worms degrade carbohydrates essentially through glycolysis, releasing lactate, acetate and alanine as end-products, even under aerobic conditions [4,13]. Similarly, in the dog heartworm microfilaria *Dirofilaria immitis*, no Pasteur effect is observed and adult individuals remain alive after 24 h in a completely anaerobic environment, with most glucose being degraded into lactate with no acetate formation [5,6].

The energy metabolism of intraerythrocytic stages of malaria parasites was previously thought to consist primarily of ATP production through the glycolytic pathway, and the

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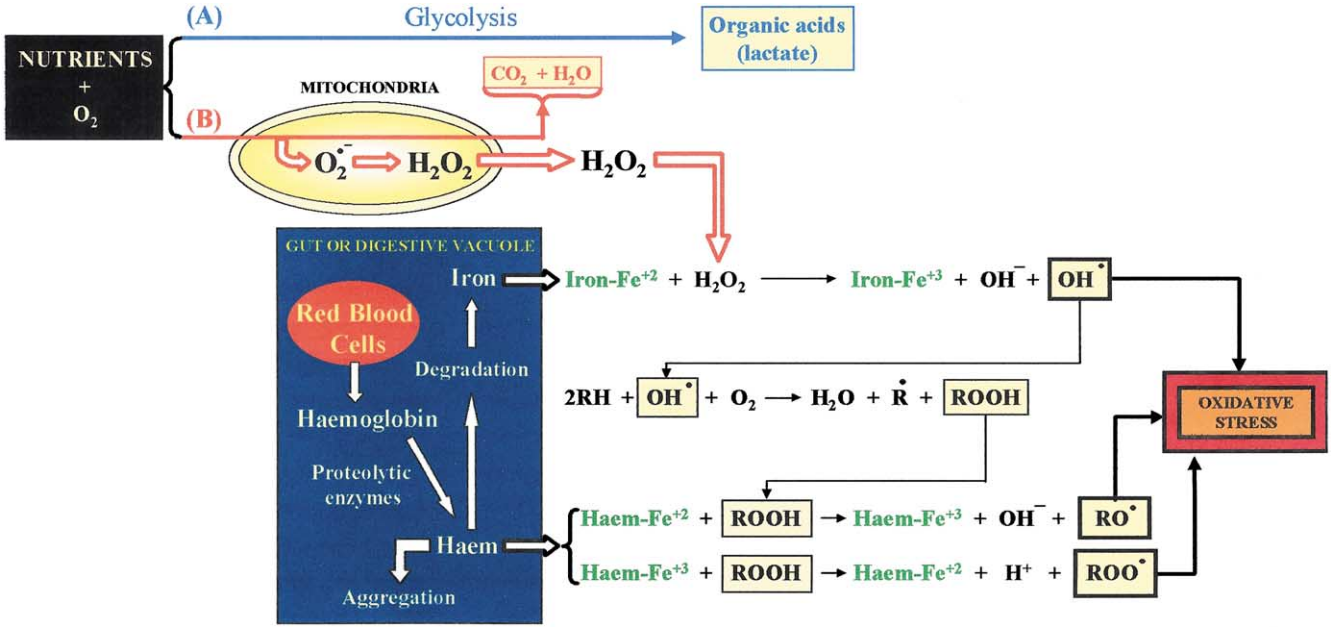

Fig. 1. Oxygen consumption, haemoglobin degradation and oxidative stress in blood-feeding parasites. Mitochondrial respiration and haemoglobin digestion are flux-generating reactions for production of ROS and are potentially synergetic. As a result, respiratory arrest should attenuate oxidative stress. Reactions of haem and iron leading to the formation of radical species are discussed in the text and were based on [28,32,39,40]. $O_2^{\bullet}$ = superoxide anion radical; $OH^{\bullet}$ = hydroxyl radical; ROOH = organic hydroperoxide; $ROO^{\bullet}$ = lipid peroxyl radical; $RO^{\bullet}$ = lipid alkoxyl radical; $R^{\bullet}$ = alkyl radical; RH = unsaturated fatty acid.

physiological relevance of mitochondria was unclear. It has been shown that most ATP produced in *Plasmodium* is formed by glycolysis, with glucose being degraded into lactate and with no evident Pasteur effect [14]. However, despite having functional mitochondria, it has been demonstrated that *Plasmodium* parasites are adapted to low oxygen tension since cultures grow optimally at 3% oxygen tension [15–18]. On the other hand, in completely anaerobic conditions *P. falciparum* cultures did not grow. Recent studies have shown the presence of a respiratory chain in *Plasmodium* mitochondria, although their levels of electron transport activity appear to be much lower than in mammalian cells [19]. It has been shown that mitochondria of asexual stages of *P. falciparum* contain few cristae, lack enzymes required for the Krebs cycle and appear to lack respiratory complex I of the electron transport chain [19–21]. Mitochondrial activities appear to be primarily anabolic rather than serving as a significant source of energy production. In particular, the respiratory chain is essential for de novo pyrimidine biosynthesis, because it provides a sink for electrons transferred from dihydroorotate dehydrogenase [22]. Moreover, Murphy and colleagues showed that about 25% of the parasite's oxygen consumption is resistant to cyanide, which was attributed to an alternative respiratory pathway [23]. These findings, together with the absence of several enzymes of the Krebs cycle, suggested that the primary function of oxygen in *Plasmodium* might not be to act as the final electron acceptor in mitochondria but rather to act as a substrate of metalloprotein oxygenases.

3. Oxidative stress and antioxidant defences

The only reason for restricting respiration in these parasites should be if the adaptive cost to be paid for respiration became too high. In this way, it is very well established that oxidative stress is an unavoidable consequence of aerobic metabolism. Estimations indicate that, at physiological oxygen levels, about 1–3% of the oxygen in mitochondria is reduced by electrons leaking from the electron transport chain as superoxide anion, which can be converted into other reactive oxygen species (ROS) such as hydrogen peroxide and the highly toxic hydroxyl radical [24–26]. To avoid the toxic consequences of ROS generation, also known as 'the dark side' of respiration, all aerobic organisms have developed an entire array of antioxidant defences [27,28]. ROS production by mitochondria would be especially critical for blood-feeding organisms since free inorganic iron and haem – the most common biological iron chelator – are also known to be potent generators of ROS [28,29]. So, in haematophagous parasites, additional ROS generation and oxidative damage to biomolecules would be expected if they kept their aerobic-based metabolism. The reactions involved in the formation of ROS in mitochondria, haem and iron release in the parasite's gut and their interactions, which ultimately lead to oxidative stress, are summarised in Fig. 1.

Interestingly, *Schistosoma*, *Angiostrongylus*, *Dirofilaria* and *Plasmodium* reside, temporarily or definitely, in vertebrate blood where they digest haemoglobin, resulting in the release of large amounts of haem prosthetic group [2–7,30,31]. Part of this haem is eventually decomposed, either enzymatically by haem oxygenase to biliverdin, or non-enzymatically through reduced glutathione resulting in free iron [32,33].

Iron is a well-known generator of free radicals, acting mainly through the classic Fenton reaction (Eq. 1), which decompose hydrogen peroxide (H_2O_2) into hydroxyl radical ($OH^{\bullet}$), the most reactive form of oxygen free radicals:

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + Fe^{3+} \quad (1)$$

Haem is an essential molecule to living aerobic organisms and plays an essential role in various biological reactions such as oxygen transport, respiration, drug detoxification and signal transduction [34]. However, like iron, haem in a free state is very toxic since it promotes oxidation of many biomolecules such as lipids, proteins, DNA and even of cellular structures like membranes [35–37]. Some reports have shown evidence of participation of haem (free or bound to haemproteins) in Fenton-type reactions [38–40]. However, formation of $OH^{\bullet}$ radical induced by haem has been a difficult task and in fact has not yet been demonstrated [32]. Notwithstanding, there is a consensus in the literature regarding production of highly reactive alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals upon interaction of haem with organic hydroperoxides, in Fenton-type reactions as shown in Eqs. 2 and 3 [32,39,40]:

$$\text{Haem} - Fe^{3+} + ROOH \rightarrow \text{Haem} - Fe^{2+} + ROO^{\bullet} + H^{+} \quad (2)$$

$$\text{Haem} - Fe^{2+} + ROOH \rightarrow \text{Haem} - Fe^{3+} + RO^{\bullet} + OH^{-} \quad (3)$$

Hence, avoiding deleterious effects of free haem is mandatory for all living cells [32]. A special situation is found in blood-feeding organisms, which can ingest several times their own weight in blood and digest it to peptides, amino acids and free haem. Therefore, blood-feeding organisms face an intense oxidative stress condition upon degradation of host haemoglobin. Thus, to overcome this oxidant aggression, several mechanisms have evolved in order to protect these organisms against iron and haem deleterious effects [32,41–43]. In malaria parasites it has been demonstrated that free haem is sequestered inside the digestive vacuole as a dark brown crystalline aggregate called malaria pigment or haemozoin (Hz) [41]. This pigment has recently been found in other blood-feeding organisms such as *Rhodnius prolixus*, an insect [44,45], in the helminth *Schistosoma mansoni* [46] and in the parasitic protozoan *Haemoproteus columbae* [47]. However, in these organisms, haem aggregation is not completely efficient, since a large portion of the haem is available to promote oxidative stress (unpublished results, [46,48]). In trophozoite stages of *Plasmodium*, only about 30% of the haem is converted into Hz suggesting that non-aggregated haem exists in the food vacuole [48]. Besides, in *Schistosoma* a significant amount of free haem is found in adult females [46]. Therefore, the protection achieved from haem aggregation is complemented by other mechanisms such as antioxidant haem-binding proteins like the *Rhodnius* haem-binding protein from the haemolymph of a blood-sucking insect, that is capable of interacting with haem and form complexes that do not promote free radical generation [32,33,43]. Indirect evidence of the existence of oxidative stress in the worm is that, in spite of the marked reduction in respiration – which should be accompanied by a proportional decrease in the production of free radicals – the transition from the cercariae to the red cell-eating adult female is paralleled by a dramatic increase in the levels of antioxidants such as superoxide dismutase, glutathione reductase, glutathione peroxidase, cytochrome *c* peroxidase, glutathione *S*-transferase (GST) and glutathione [49–51]. Moreover, it has recently been demonstrated that a novel class of antioxidant enzyme, thioredoxin peroxidase, plays a significant role in *Schistosoma*–host interactions by neutralisation of hydrogen peroxide [52].

In support of this view, a recent report has indicated that several antioxidant enzymes are regulated at gene expression level during *S. mansoni* development [53]. Quantitative mRNA determination of *S. mansoni* antioxidant enzymes such as cytosolic Cu–Zn superoxide dismutase (CT-SOD), signal-peptide-containing SOD (SP-SOD), glutathione peroxidase (GPX), and GST showed that all these enzymes are developmentally regulated [53]. The adult worms have the highest level of specific mRNA when compared with larval stages. Interestingly, immunolocalisation of antioxidant enzymes in schistosomula and adult worms indicated that GPX, SP-SOD, and CT-SOD are associated with the adult tegument and gut epithelium, whereas schistosomula showed little immunofluorescence. The presence of these enzymes in regions exposed to oxidative stress should allow adult worms to evade radical attack derived from both host immune response and haemoglobin digestion products.

4. Anaerobic metabolism: a preventive antioxidant defence?

Choosing anaerobiosis, reducing oxygen consumption – and lowering ROS production by the parasite metabolism – may be a strategy to avoid oxidative stress produced by haemoglobin degradation in blood-feeding parasites. 'Life without air' was the charming way in which Louis Pasteur described the anaerobic metabolism of yeast in his classic work proposing it as a solution for an organism that has been deprived of oxygen. However, for these parasite-vampires, living without air – but in the presence of air – may be the only way not to get rusty.

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