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While the clinico-pathological features of sickle cell disease take the center stage, be mindful of some of the enzymatic undertones

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ABSTRACT

Many texts on sickle cell disease (SCD) usually highlight on its patho-physiological features, and the clinical correlates, turning a blind eye, as it were, to the enzymatic actions behind these features. Even when authors touch on the enzymes, they are done in a piece-meal manner. This review looks at the various studies on sickle cell disease, in which the actions of enzymes underlying the SCD condition have been considered. The search engine used is HINARI, that allows free access (particularly to researchers like some of us in the developing countries) to some top biochemical and medical journals.

KEYWORDS

Sickle;
Hemoglobin;
Hemoglobinopathy;
Oxidant stress.

INTRODUCTION

The gamut of enzymes covered

The review begins on the premise that a person with sickling hemoglobinopathy is prone to oxidant stress. There are physiologically, prooxidant and anti-oxidant processes, which should be in a delicate balance. Particularly, the various enzymatic reactions serving either as prooxidants or antioxidants are reviewed, together with other related processes.

SCD is basically a problem of the hemoglobin pigment contained in the red cell. Though the red cell is of tiny size, because it carries oxygen, the most essential molecule required by mammals to sustain life, its function is indispensable. The services of the red cells are required by all living tissues of man. In SCD persons, their red cells contain a mutant HbS instead of the wild-type HbA.

The shape of the red cell is dependent on the right compartmentalisation of the haemoglobin molecules within the red cell, vis-à-vis the other cellular contents, including the cytoskeleton and cell membrane lipid bi-layer. For its optimal functioning, the red cell metabolises glucose to provide energy and reducing equivalents. However, the composition of the red cell itself (unsaturated fatty acids in its membrane, presence of iron II, a transition metal ion, its transport of oxygen), makes it liable to prooxidant skirmishes. This is worsened in an SCD person with the mutant HbS, characterized by instability and insolubility, compared to HbA.

Superoxide anion, produced from the unstable HbS, through the action of superoxide dismutase forms hydrogen peroxide and oxygen. Then follows the action of other enzymes; catalase, glutathione peroxidase, glutathione reductase and the role of the hexose monophosphate shunt, through the action of glucose–6–phosphate dehydrogenase to make available NADPH as a

cofactor for glutathione reductase for regenerating reduced glutathione from the oxidized form. The use of two sequential enzymes, γ-glutamylcysteine synthetase and γ-glutamylcysteinylglycine synthetase or glutathione ligase for the *de novo* synthesis of the Glutathione is also featured.

By the formation of irreversibly sickled cells through oxidation and dehydration, some dense red cells are formed, with disturbed asymmetry of the red cell phospholipid membrane, externalizing phosphotidylserine on the outer monolayer. The disruption in the membrane asymmetry is linked to a disordered action of three membranal translocase enzymes; the flippase, floppase and scramblase.

The sickling of red cells leads to premature intravascular breakdown of red cells. The decompartmentalised hemoglobin or the cell-free hemoglobin, is potentially vasulotoxic, if not carried to the reticuloendothelial system, especially the spleen to be broken down. The heme is split off from the globin, giving off iron and carbon monoxide. The role of heme oxygenase in breaking down heme to biliverdin, which in turn, gets reduced by the action of biliverdin reductase to unconjugated bilirubin is mentioned. The yellow pigment, bilirubin is water–insoluble, has to form water–soluble conjugate in the liver through the action of UDP glucuronosyltransferase, which shows polymorphisms in both the coding and non–coding regions of the gene.

On one hand, Nitric oxide synthase (NOS) is looked at as it produces nitric oxide, which exerts some salutary effects on the endothelium and platelets, but on the other hand, we also look at the inimical effects due to the inactivation of NO by cell–free hemoglobin from hemolysis as it causes endothelial dysfunction and stimulating the expression of adhesive molecules. How the free radical gaseous NO exerts its action via the soluble guanylate cyclase to reduce the intracellular calcium ion concentration, as a means of modulating vasomotor tone, is briefly considered. Next, how phosphodiesterases reduce the levels of cyclic nucleotides is featured. Another off–shoot of hemolysis is the release of arginase from red cells to deplete the levels of L–Arginine, the same substrate for NOS. The possibility of NO, through its binding to the porphyrin-like corrin of methionine synthase to cause the accumulation of asymmetric dimethyl arginine (ADMA), a competitive inhibitor of arginine to NOS is brought on board. Other enzymes producing ROS are xanthine oxidase, cyclooxygenase, lipooxygenase and NADPH oxidase. Also not left out are the phospholipases; phospholipase A_2 and secretory phospholipase. The role of the respiratory burst NADPH oxidase, augmented by myeloperoxidase of polymorphonuclear leucocytes is also covered. Thus it appears the clinical condition of a SCD patient could be a symphony or cacophony of enzymatic actions in the steady state or crises.

Clinical correlates of the sickling pathophysiology

Sickle cell disease is said to be the first molecular disease to have been discovered due to the efforts of Pauling et al.[1] who used the technique of moving boundary electrophoresis to unravel the mutation in hemoglobin, the conjugated protein involved in the transport of oxygen in vertebrates.

The α–globin is made up of 141 amino acid residues, while the β–globin, 146 residues. In a normal hemoglobin designated as HbA ($\alpha_2\beta_2$), the sixth position of the β–globin chain is occupied by glutamic acid, a dicarboxylic amino acid. The mutation giving rise to SCD occurs in the β-chain, giving rise to β^s (found in HbS), in which the sixth amino acid is valine, a hydrophobic amino acid.

It is this single and simple difference in the genotype of SCD patients that are responsible for the deficiency in the function of HbS, as compared to HbA. The change from a charged to a neutral, hydrophobic amino acid creates a sticky patch on the molecular surface that causes aggregation upon deoxygenation,[2] seen as polymerization of the deoxy HbS into a gel.[3]

The main pathophysiological feature of SCD is that the polymerization of the deoxygenated HbS leads to erythrocyte rigidity, distortion, membrane damage and hemolysis.[4,5] It is the cycles of erythrocytes sickling which cause the cells to become fragile for lysis to occur reducing the RBC life span from the normal 120 days to about 10 days producing chronic hemolytic anemia.[6]

The polymerized deoxyHbS renders the red cells non–deformable to traverse the small capillaries. As a consequence, sickle cell patients suffer repeated vaso–occlusive events characterized by ischemia–reperfusion

injury and inflammation.[7,8] These chronic vascular insults lead to numerous end–organ complications such as vascular necrosis of bones, retinal infarctions, stroke, acute chest syndrome, pulmonary hypertension, splenic infarction, kidney failure and skin ulceration.[6] Diggs and Ching,[9] in (1934), were the first to propose the link between polymerization vaso–occlusion and the clinical manifestations of SCD. Their hypothesis emphasized the role of irreversibly sickled cells in initiating stasis, hypoxemia and vaso–occlusion.

The pathophysiology of sickling has been linked to the insolubility and instability of HbS, leading to the dehydration and adhesivity of red cells containing this hemoglobin variant. However, it can be inferred that the insolubility of HbS, culminating in the dehydration, sickling and adhesivity of red cells are the consequences of the instability of HbS. The vaso–occlusive events are now seen to be attributable to the oxidant stress an SCD patient faces due to the instability of HbS.[10] Erythrocytes of SCD patients are able to produce more reactive oxygen and nitrogen species than normal erythrocytes.

SCD as an oxidant stress condition: Role of superoxide dismutase, peroxidase, catalase and allied enzymes

As part of the physiological processes of the body, some oxidants are produced. However, because high levels of such oxidants could be deleterious to the body, the body has also evolved a system to neutralize the excessive levels of the oxidants that are produced. Where the capacity of the body is overwhelmed in handling the oxidants produced, then the body is said to be under oxidative or oxidant stress, or the antioxidant capacity is said to have been lowered. The antioxidant capacity of the body is enabled by the presence of endogenously elaborated enzymes and other compounds (e.g. uric acid, biliverdin, bilirubin, etc.), supplemented by some exogenously–supplied compounds.[11]

Oxidative stress can damage specific molecular targets like lipids, proteins and nucleic acids, resulting in cell dysfunction and/or death.[10,11] The accelerated production of ROS will affect the cellular redox state, and increase the risk of oxidative damage of proteins and lipids.[24] Compared with persons with normal hemoglobin, SCD patients have the greater propensity to directly generate reactive oxygen species[12,13] and/or stimulate such generation from endothelial cells.[14] SCD erythrocytes produce more reactive oxygen species (ROS), including superoxide, hydrogen peroxide and hydroxyl radicals.[12,18] However, the SCD patient has an impaired antioxidant defence.[16]

The abnormal rheological properties resulting from the polymerized HbS,[15] may also serve as a stimulus to the endothelium to produce oxidants. Unsuprisingly, sickle red blood cells at baseline, exhibit increased levels of thiobarbituric-reactive substances (TBARS).[17] In the erythrocyte, deoxygenation of hemoglobin is accompanied by the formation of superoxide, O_2^-.[19] Such a reaction occurs to a small extent in normal erythrocyte, but HbS is auto–oxidized at 1.7 times the rate of HbA, accounting for the greater generation of superoxide in SCD erythrocytes.[12]

Despite the reduced affinity of HbS for oxygen in comparison to HbA, the two are able to reversibly bind with oxygen to become oxygenated. It is particularly under hypoxic conditions that the differences between the two forms become evident. In the process of deoxygenation, the original iron II is oxidized to iron III which forms methemoglobin, and the lost electron from iron (II) picked up by oxygen molecule to form the superoxide.[20] As a free radical, superoxide is very reactive. Furthermore, in methemoglobin, the bond between iron and the heme becomes weakened to facilitate the release of iron and heme, as part of the oxidative damage of hemoglobin that will lead to its denaturation and precipitation to form hemichromes of ROS.[21] The hemichromes, which are insoluble, aggregate to form Heinz bodies.[22] The Heinz bodies, which adhere to the blood cell membrane, may themselves cause damage to the membrane.[22] Another cause of oxidative stress in SCD is the high level of free iron and iron-containing compounds such as hemin and hemoglobin in the blood of patients, resulting from hemolysis and blood transfusions.[23]

On the other hand, after the formation of the superoxide, it could dismutate enzymatically through superoxide dismutase (SOD), to produce hydrogen peroxide and oxygen. Being another ROS, H_2O_2 has to be eliminated as it can contribute to the oxidative stress. This is where catalase comes in to break down the hydrogen peroxide to water and oxygen. Another means

of handling H_2O_2 is through the use of the enzyme glutathione peroxidase (GSHPx). In the presence of the tripeptide, reduced glutathione (GSH), hydrogen peroxide is broken down to water per the following equation:

$$H_2O_2 + 2\,GSH \rightarrow H_2O + GSSG$$

where GSSG is the oxidized form of glutathione.

Similarly, lipid peroxides (intermediates formed when ROS react with unsaturated fatty acids, for example, as found in erythrocytes membrane) are also acted upon by GSHPx as part of the protective mechanism. Compared with HbA red cells, HbS red cells have been reported to generate 2 fold greater extents of H_2O_2, hydroxyl radical (OH) and lipid oxidation products (LOOH, LOO), with greatly diminished GSH levels.[25]

The Oxidation of GSH to the oxidized form,[26] leads to a decrease in the level of GSH in a cell. To restore the intracellular level of GSH, another enzyme, GSH reductase, catalyses the reduction of the glutathione disulphide (or oxidized glutathione), in a NADPH–dependent reaction. What ensures a constant supply of NADPH, a very important reducing equivalent in the erythrocyte is the efficient operation of glucose-6–phosphate dehydrogenase, the rate limiting enzyme of the pentose phosphate pathway; the enzyme that catalyses the oxidation of glucose–6–phosphate to 6–phosphogluconate in a $NADP^+$-dependent reaction.

GSH plays an important role in the protection against oxidant damage as it can be oxidized directly by ROS, and serves as an electron donor for GSPHx, which reduces lipid peroxides.[26] Thus, the major enzymes involved in the ROS defence mechanism are GSHPx, catalase and superoxide dismutase. GSHPx is linked to the balance between GSSG and GSH.[28] Through the interaction with GSHPx, GSH can neutralize hydroxyl radicals and detoxify the peroxides.

There can also be the *de novo* synthesis of GSH from its constituents amino acids in a two-step reaction. the rate limiting step is the use of the γ-carboxyl group of glutamic acid to form a γ-peptide bond with the amino group of cysteine (an unusual type of peptide bond) to form a dipeptide, γ-glutamylcysteine. In the second step, the third amino acid, glycine reacts. These two reactions, both ATP-dependent, are catalysed by cytosolic enzymes, glutamylcysteine synthetase and glutathione synthetase, respectively.[29] However, the use of this *de novo* synthesis of GSH, in energy terms is very expensive, hence the dependence on the reductive pathway.[29]

If the antioxidant defence mechanism is inadequate, the RBC is particularly susceptible to oxidant damage.[30] The three main conditions that favor free radical formation are a rich supply of oxygen, the presence of a transition metal catalyst, and a high degree of unsaturation in the lipid substrate.[31] Human erythrocytes meet all three requirements and hence are very susceptible to oxidative/peroxidative damage. Much of their metabolic activity is geared toward reductive processes that combat the threat of oxidation. If these reductive processes are deficient or overwhelmed, oxidative damage to cellular constituents occur, leading to hemolysis.[32]

Das and Nair[34] have shown decreased levels of superoxide dismutase and catalase in erythrocytes of SCD patients, implying that there is increased availability of superoxide and hydrogen peroxide in these patients.[35] It has further been demonstrated by Hammerman *et al.*[36] that exposure of endothelial cells to plasma from patients with acute chest syndrome results in the decreased activity of the antioxidant glutathione reductase system *in vitro,* favouring the formation of peroxynitrite ($ONOO^-$) from nitric oxide (NO) and superoxide.

Wetterstroem et al.[25] also showed lowered GSH levels in SCD patients. The lowered erythrocyte GSH in SCD is not due to reduced substrate availability or decreased synthesis, but rather increased consumption[33] The depletion of cellular GSH, putting the cell in double jeopardy for oxidative damage to protein and lipid components,[18] is a key factor accounting for the formation of dense RBCs that contributes to vaso–occlusive events.

Disruption of erythrocytes membrane asymmetry by imbalance in flippase, floppase and scramblase activities

In normal red cells, the phospholipids bilayer is characterized by an asymmetry in which the anionic phosphatidylserine is confined to the cytoplasmic monolayer of the erythrocyte membrane,[37,38] which has to be maintained[39] through the cooperative action of an ATP–dependent aminophospholipid translocase or

flippase (transports phosphatidylserine and phosphatidylethanolamine from the outer to inner surface[40] and a non-specific floppase (moves phospholipids from the inner to the outer membrane.[41] On the other hand, there can be the scrambling or non-specific bidirectional transport of the phospholipids across the membrane, resulting in the exposure of phosphatidylserine on the outer monolayer, a process which can be rapidly induced by a calcium–dependent scramblase.[40,42] While at physiologic cytoplasmic concentrations, the activities of flippase and floppase maintain phospholipid asymmetry,[24] at high levels of cytoplasmic concentrations of Ca^{2+} ions, scramblase is rapidly activated, accounting for the externalization of phosphotidylserine.[42,39]

Because macrophages recognize and phagocytose red cells that expose phophatidlyserine at their outer surface, the confinement of this phospholipids in the inner monolayer is essential if the cell is to survive the frequent encounters with the macrophages of the reticuloendothelial system, especially the spleen.[67] Loss of lipid asymmetry leading to exposure of phophatidylserine on the outer monolayer has been suggested to play a role in premature destruction of red cells in SCD and thalassemia.[68] The appearance of phophatidylserine on the surface is a characteristic marker of apoptotic cells and participates in the recognition and elimination by macrophages of dying, injured, senescent, or necrotic cells.[69] Furthermore, the restriction of phophatidylserine to the inner monolayer also inhibits the adhesion of normal red cells to vascular endothelial cells, thereby ensuring inimpeded transit through the microvasculature.[38]

Cycles of in vivo sickling/unsickling, with polymerization/depolymerization of HbS, and the accompanying red cell membrane changes and microvesicle formation can lead to the abnormal exposure of phosphatidylserine.[43] Other factors such as reduced flippase activity,[44] membrane oxidative damage,[24] increased levels of intracellular calcium ions[45] and dehydration[42] can disrupt the phospholipids asymmetry. The loss of membrane asymmetry produces several pathophysiologic consequences, which have been shown to occur *in vivo* in patients with hemolytic anemias, including sickle cell disease and thalassemia.[49] Potential fall-outs from phospatidylserine exposure in the hemoglobinopathies include the worsening of anemia due to enhanced phagocytic recognition and removal of cells with exposed phosphatidyllserine, apoptosis and activation of coagulation, as the exposed phosphatidylserine serves as a "docking site" for specific proteins involved in coagulation processes.[43]

The persistence of the phosphatidylserine–exposing RBC in SCD patients is believed to contribute to the morbidity of the disease, by dysregulation of hemostatic processes, increasing the patient's risk for stroke,[47] altering the adhesive properties of RBC,[48] decreasing red cell survival,[49] as well as the provision of a cellular substrate for secretory phospholipase. A2[50,51].

Conversion of ringed protoporphyrin of heme to straight chain bilirubin by heme oxygenase

Heme is an ubiquitous, hydrophobic, iron–containing compound that greatly increases cellular susceptibility to oxidant–mediated injury.[52] Heme is readily cleaved from methemoglobin[53] and rapidly intercalates into the membrane of cells. As a defence mechanism against reactive heme released in the course of hemolysis, the vasculature is induced to produce cytoprotective genes for heme oxygenase, ferritin and bilivervin reductase.[55]

Heme oxygenase (HO) is the rate-limiting enzyme in the degradative pathway of heme, as it converts heme to biliverdin, facilitating the release of iron and carbon monoxide (CO). The biliverdin is subsequently reduced to bilirubin, a yellow pigment, through the action of biliverdin reductase. There are three isoforms of heme oxygenase, one inducible isozyme, HO–1 and two constitutive forms, HO–2 and HO–3. Heme oxygenase–2 is basically expressed abundantly in the kidney.[56] The induction of HO-1 can be triggered by factors such as heme, oxidants, cytokines, ischemia, hypoxia and other stressors.[57] Some evidence supporting the induction of HO–1 in SCD include the increased production of carbon monoxide in human SCD[58] and increased expression of mRNA for HO–1 and biliverdin reductase in human SCD.[55]

Unfinished business of heme oxygenase continued by UDP–glucuronosyl transferase

The unconjugated bilirubin, produced in the reticuloendothelial system is transported in the blood to the liver, where it is conjugated and then excreted in the bile, into the gall bladder for onward secretion into the

small intestine. Bilirubin catabolism is the final step in the breakdown of heme from hemoglobin and hemoprotein turnover.[59] The primary bilirubin catabolising hepatic enzyme, UDP glucuronosyltransferase, UGT1A1, mediates the conjugation of bilirubin into a water-soluble form that is excreted in bile.[59] A nonsense mutation of the UGT1A1 gene has been identified to cause a pronounced defect in glucuronidation of bilirubin, giving rise to Crigler-Najjar syndrome.[60] There is also a mutation in the promoter region of the gene, giving rise to a less severe condition, Gilbert's syndrome, marked by varying degrees of hyperbilirubinemia, depending on the type of mutation.[61] According to Strassburg et al.,[62] there are nine UGT1A1 isoforms, expressed to varying degrees in the liver. The number of TA repeats in the TATA promoter region of UGT1A1 has been shown to be inversely associated with the transcriptional activity of UGT1A1, with five and six repeats associated with high activity and seven and eight repeats associated with low activity.[63]

The co-inheritance of Gilbert's syndrome with disorders that increase the turnover of red blood cells or their precursors, has been reported in SCD patients on hydroxyurea therapy.[64] Associations among UGT1A1 promoter variations, high indirect bilirubin levels and gallstone formation, have also been reported in SCD.[65] In a study by Maddray et al.[66] on some SCD patients, the activity of glucuronosyltransferase of jaundiced patients were two-fold greater than non-jaundiced persons. That study showed an enhanced conjugation of bilirubin in SCD patients, probably due to induction of the glucuronosyltransferase.

Beutler et al.[63] proposed that UGT1A1 promoter polymorphism may provide a balanced polymorphism that maintains bilirubin levels in a range high enough to protect against oxidative damage, but not so high as to cause a high incidence of kernicterus.

Inability of NOS to rescue the endothelium under siege from reactive species: activation of endothelium and expression of adhesive molecules

Nitric oxide (NO) is a signaling molecule, a diffusible gas, synthesized by three nitric oxide synthase (NOS) isoforms; a neuronal (nNOS), inducible (iNOS)[61,70] and endothelial nitric oxide synthase (eNOS)[71,72]. NO is synthesized from the terminal guanidine nitrogen atom of the semi-essential amino acid, L-arginine.[73] Using NADPH as cofactor, in the presence of tetrahydrobiopterin,[73] these enzymes convert arginine to citrulline and NO.

Nitric oxide is a mediator of cell-cell communication as it diffuses from production site to target cells and binds to the ferrous deoxyheme moiety of soluble guanylate cyclase (sGC), causing a conformational change in the enzyme, and hence the enzyme's activation, resulting in the increased intracellular cyclic GMP concentration.[65,74] The increase in cGMP level is the major mechanism underlying many of the cardiovascular and neural effects of NO.

Within the vasculature, the rise in cGMP induces a sequence of protein phosphorylation which ultimately causes a fall in the intracellular Ca^{2+} ion concentration, and then, smooth muscle relaxation.[66,75] Under normal circumstances, the interaction between endothelium and smooth muscle cells leads to a low vascular resistance in the pulmonary circulation.[67]

SCD patients and animal models of the disease, like the transgenic sickle mice, show a lower blood pressure than normal subjects.[70,71] The factors leading to a lower blood pressure and a lower peripheral resistance[72] in sickle cell anemia are not fully defined, but postulated mechanisms include, altered levels of vasoactive substances and an altered vascular reactivity to vasoactive stimuli.[71] Sickle cell anemia patients show a higher NO production.[66,75] The blunted vasodilatory effect of acetylcholine in transgenic sickle mice is consistent with an increased NO/NOS activity that would result in a depressor effect on the vascular tone. Furthermore, the diminished arteriolar response to sodium nitroprusside is also accounted for by an increased generation of endothelial NO, resulting in continual stimulation of guanylate cyclase-cGMP activity in the vascular smooth muscle.[66] It has been further shown[66] that the arteriolar responses to acetylcholine and sodium nitroprusside are not altered after blockade with indomethacin, suggesting that these responses are independent of prostacyclin activity. Indomethacin is a non-steroidal anti-inflammatory agent that inhibits the action of cyclooxygenase but not NOS. Nitric oxide inhibits the upregulation of cell adhesion molecules and monocyte adhesion to endothelial cells.[74] Nitric oxide also acts to regulate platelet function, preventing platelet aggre-

gation,[68] and adhesion to endothelial cells.[69]

Nitric oxide, besides the mediation of vasodilation through cGMP-dependent pathways, also inhibits endothelin-1-induced vasoconstriction.[79]

Rother et al.[85] observed that the release of hemoglobin by lysed erythrocytes into the blood plasma, unveils its vasculotoxic potential by directly impairing endothelial function and generating inflammatory and oxidative stress. Moestrup and Moller[86] also showed how the decompartmentalised/free hemoglobin form a high-affinity complex with haptoglobin (a plasma protein), to display a neoepitope, which is then recognized by CD163, the hemoglobin-scavenger protein expressed on the reticuloendothelial cells. Receptor-ligand endocytosis of the complex rapidly clears it from the blood plasma, and further stimulates the expression of an antioxidant pathway involving heme oxygenase-1 and biliverdin reductase.[85]

Decreased nitric oxide bioavailability, resulting in endothelial dysfunction contributes to chronic vasoconstriction, together with hemostatic activation and vascular smooth muscle proliferation, features also associated with pulmonary hypertension, priapism, and cutaneous leg ulceration in SCD and other disorders in which intravascular hemolysis is so severe as to overwhelm the hemoglobin-scavenging mechanism.[8,87] Despite the beneficial actions of nitric oxide, in the presence of oxygen and oxygen-related compounds, it preferentially and rapidly forms the powerful oxidants, nitrite (NO_2^-), nitrate (NO_3^-) and peroxynitrite ($ONOO^-$).[88,89] The formation of these reactive nitrogen species nullify the beneficial effects of NO, as there is preferential shunting towards the toxic metabolites of nitrogen.[90]

Phosphodiesterases in strategic control of concentrations of cyclic nucleotides

The events in the hemodynamics of smooth muscle contraction are regulated by extracellular stimulation through alteration of intracellular cyclic nucleotide levels, which are determined by a balance between their production and degradation.[90] The 3'5'cyclic phophodiesterases (PDEs) are phosphohydrolases that regulate the cellular levels of the second messengers, cAMP and cGMP, by controlling their rates of degradation, and therefore, signal transduction.[91-93] There are eleven different isoforms of the PDEs in mammalian tissues, and many of these PDEs are tightly connected to different physiological functions in the body, and by inference, different pathological conditions.[91,93] Therefore, the PDEs have become therapeutic targets, as there is the possibility of designing isoform-selective inhibitors that can target specific functions and pathological conditions, without causing non-specific side effects.[91]

PDE5A is one isoform, with high expression in the lung[94] and penis,[95] where it serves as a regulator of vascular smooth muscle contraction through the control of cGMP concentration. In the lung, inhibition of PDE5A opposes smooth muscle vasoconstriction, and so PDE5 inhibitors are being used in clinical trial for the treatment of pulmonary hypertension.[94] Using a transgenic model of sickle cell mouse, De Francesschi et al.[96] found that under prolonged hypoxia, there was upregulated PDE4 gene expression, and that rolipram, a PDE4 inhibitor prevented the hypoxia-induced PDE4 gene upregulation, and so prevented the development of pulmonary hypertension, most likely through modulation of vascular tone and inflammation. Neutrophils from sickle cell patients demonstrate increased adhesive properties *in vitro*.[92] But treatment of SCD neutrophils with nitric oxide donors or a stimulator of sGC, decreases the adhesive properties of these cells.[93] There is the PDE9A isoform which is highly expressed in neutrophils, with even higher expression in SCD patients, and that *in vitro* inhibition of this enzyme in leucocytes decreased their adhesive properties.[97] The use of sildenafil (Viagra) for the management of erectile dysfunction is linked to the inhibition of PDE5A in the penile carvenosal smooth muscle, enhancing the relaxation of the smooth muscle by NO and cGMP and thereby stimulating penile erection.[95]

NO modulation of homocysteine concentration, a stimulus to ADMA to compete with L-Arginine

Nitric oxide also modulates homocysteine concentration by inhibiting methionine synthase, the enzyme involved in the synthesis of methionine from homocysteine and 5-methyltetrahydrofolate,[98] resulting in hyperhomocysteinemia. The hyperhomocysteinemia that occurs in SCD, apparently enhances the production of asymmetric dimethylarginine (ADMA), which competes with L-arginine as the substrate of NOS.[98,99] In the absence or deficiency of L-arginine, NOS becomes

"uncoupled" as the normal flow of oxygen within the NOS is diverted so that the enzyme preferentially produces superoxide rather than NO.[99] Hyperhomocysteinemia may produce vascular dysfunction and promote oxidative stress by increasing the levels of ADMA.[100] The increased plasma homocysteine levels induces endothelial dysfunction by way of increased generation of reactive oxygen species and decreased NO bioavailability.[101]

Methionine synthase contains cobalamin (vitamin B12) as a cofactor.[102] Just as NO is able to exert its effect on sGC by binding to the heme which contains iron, it is also able to bind to the corrin group of methionine synthase, as cobalamin is similar in structure to heme.[102] Iron and cobalt are transition metals, and the porphyrin ring of heme and the corrin ring of cobalamin are substituted tetrapyrrole rings.[102] ADMA, an endogenous competitive inhibitor of NO, is a modified amino acid derived from proteins that have been posttranslationally methylated and subsequently hydrolysed.[103] ADMA is generated from the hydrolysis of ubiquitous proteins containing methylated arginine residues. The methyl groups can be distributed symmetrically or asymmetrically on guanidium nitrogen of arginine, giving several isomers, with the ADMA being the most predominant.[104] This metabolite is thought to be liberated during the turnover of red cells during hemolysis.[105] Crossby,[106] earlier in 1955, had suggested ADMA might be produced from the breakdown of proteins contained in sickle red cells which turn over at a rate twenty times normal. The levels of ADMA are slightly increased in SCD but become significantly elevated in patients with elevated pulmonary pressure, indicating that NOS inhibition may contribute to pulmonary hypertension.[107] The elevation of ADMA in SCD persons has also been found to be associated with production of soluble vascular cell adhesive molecules-1 (svCAM-1), a marker of endothelial activation, normally suppressed by nitric oxide.[108] Furthermore, ADMA is associated with lower hemoglobin levels, suggesting its correlation with severe intravascular hemolysis.[107] ADMA and similar methylated arginines can be partially excreted in the urine.[109] Furthermore, an enzyme, dimethylarginine dimethylaminohydrolase (DDAH) can hydrolyse ADMA to L-citrulline and dimethylamine.[110] Reduced clearance of ADMA in renal failure is associated with endothelial dysfunction, reversible by the administration of L-arginine.[109] In conditions like hypertension, hyperhomocysteinemia and hyperglycemia,[110,111] where there is oxidative stress, ADMA is elevated to a level that can inhibit NOS activity. In each of these conditions, the elevation of ADMA is due to the oxidative stress that impairs the ability of DDAH to hydrolyse ADMA.[111]

It has been proposed that mild hyperhomocysteinemia of sickle cell patients may contribute to the vascular complications of the disorder.[112,113] In a study to examine the relationship between stroke and plasma homocysteine concentration, SCD patients with a history of stroke had significantly higher plasma levels than those without stroke.[114]

Antagonistic action of arginase to NOS

Arginase is present predominantly in the liver and kidney, and is also present in red blood cells.[116] It hydrolyses L-Arginine to urea and ornithine, being the central enzyme of the hepatic urea cycle and the principal route for the disposal of excess nitrogen from amino acid and nucleotide metabolism.[117] Increased arginase activity is correlated with increased intravascular hemolysis, and to a lesser extent, with markers of inflammation and soluble adhesion molecules.[118] Elevated arginase activity will cause a decrease in the arginine/ orthinine ratio, a situation that can cause elevated pulmonary artery pressure,[115,119] pulmonary thromboembolism[120] and progressive thrombosis, all of which contribute to pulmonary hypertension.

In SCD, bioavailability of arginine and nitric oxide is decreased by several mechanisms linked to hemolysis. L-Arginine, the substrate for nitric oxide synthesis is deficient in SCD[121]. Destruction of erythrocytes also results in the release of arginase, which catalyses the conversion of L-Arginine to ornithine,[122] shifting arginine metabolism toward ornithine production, and decreasing the amount available for nitric oxide production. The bioavailability of arginine is further decreased because ornithine and arginine compete for the same transporter system for cellular uptake.[123] Furthermore, endogenous synthesis of arginine from citrulline may be compromised by renal dysfunction, a common problem in SCD.[122] This state of resistance to nitric oxide is accompanied by a compensatory up-regulation of

NOS and non-nitric oxide-dependent vasodilators.[124] Under conditions of low arginine concentration, NOS is uncoupled, producing reactive oxygen species instead of nitric oxide,[120,125] potentially further reducing nitric oxide bioavailability in SCD and enhancing oxidant stress.[115] Arginase is also able to inhibit iNOS activity via the generation of urea and by sensitization of NOS to its endogenous inhibitor, ADMA.[126]

Phagocytic action of neutrophils due to activity of NADPH oxidase

Phagocytic leucocytes are an important part of the innate immune system that has evolved to respond rapidly to the presence of invading bacteria, fungi and parasites. Neutrophils are one type of phagocytes in humans, which are able to ingest bacteria into intracellular compartments, phagosomes, from where they produce cytotoxic agents against the microbes.[127] The NADPH oxidase, also called the respiratory burst oxidase, is a phagosomal and plasma membrane-associated enzyme complex that is dormant in quiescent neutrophils, but are rapidly assembled when cells are activated by a variety of inflammatory stimuli.[127,128]

When faced with pathogens, neutrophils are stimulated to induce a respiratory burst, characterized by intense uptake of oxygen, to form superoxide, the first product of NADPH oxidase.[129] Superoxide has minimal antibacterial action,[130] and dismutates to produce hydrogen peroxide. The toxicity of the peroxide is greatly potentiated by the heme enzyme, myloperoxidase (MPO), which uses the peroxide to convert chloride to HOCl.[131]

$$Cl^- + H_2O_2 \rightarrow HOCl + H_2O$$

Therefore, oxidants are believed to be the main components of the neutrophil defence system.[127]

When polymorphonuclear neutrophils (PMNs) are under chronic oxidative stress as in β-thalassemia, or following *in vitro* exposure to oxidants, their ability to generate respiratory bursts is compromised, and their antibacterial functions may be less effective.[132] Stimulation of leucocytes from SCD patients release more superoxide anion than similarly treated cells from normal subjects.[133] It has also been demonstrated by Amer et al.[134] that the oxidative stress in SCD renders the PMN bactericidal action defective, partly accounting for the susceptibility to recurrent infections in SCD patients.

Xanthine oxidase (XO) and reperfusion injury

One major source of superoxide in reperfused tissue of heart, kidney, intestines and liver is xanthine oxidase (XO), and is released into the circulation following a variety of pathogenic events.[17] Mammalian xanthine oxidoreductases (XOR) catalyse the hydroxylation of hypoxanthine and xanthine, the last two steps in the formation of uric acid. XOR can exist in two forms; xanthine dehydrogenase (XDH) which uses NAD+ as electron acceptor, and xanthine oxidase (XO), which utilises molecular oxygen as the electron acceptor, leading to the formation of superoxide anion and hydrogen peroxide.[135] In a cell, XDH is the dominant form but can be readily converted to XO, either by oxidation of SH residues or by proteolysis.[136] The conversion of XDH to XO is of major interest as it has been implicated in diseases characterized by oxygen radical-induced tissue damage, such as post-ischemic reperfusion injury as in SCD.[17]

The mechanism of XO-mediated reperfusion injury was suggested by Granger et al.[137] They hypothesized two steps that could predispose tissues to reperfusion injury. The first is the breakdown of ATP to AMP, and to hypoxanthine, to provide the substrate for XO. The second step is the intracellular conversion of XDH to XO, which would compel the enzyme to use dioxygen instead of NAD+ as the electron acceptor or oxidant. During pathological conditions such as hepatocellular damage, ischemia-reperfusion,[138] and in SCD,[17] organs rich in xanthine oxidoreductase (XOR) can release this pro-inflammatory enzyme into the circulation, and plasma XO levels increase.

During ischemia, XOR can be converted to XO by either proteolytic cleavage of the amino terminus or more rapidly, by intramolecular and mixed disulfide formation.[136] Repeated interruption of blood flow and the resultant transient ischemia associated with SCD can provide the basis for ischemic liver injury and elevated plasma XO levels.[17] In support of this, XO has been shown to be increased in vessel walls and the plasma, both clinically and in a transgenic, knockout murine model of SCD that expresses exclusively human HbS.[17] Mice expressing human β^S-hemoglobin display indices of increased lipid oxidation and aromatic hydroxylation reactions and, upon exposure to hypoxia, had about 10% increase in the conversion of liver and kidney xanthine

oxidoreductase (XOR) to the O_2^- and H_2O_2-producing oxidase form.[139]

Arachidonic acid; hydrolytic product of phospholipases action, in a tug of war between cyclooxygenase and lipooxygenase

The anti-inflammatory and anti-microbial actions of leucocytes through the respiratory burst are complemented by other effects produced by the various metabolites from arachidonic acid, released from the leucocyte membrane. The view that vasoactive processes may contribute to the initiation and/or maintenance of vaso-occlusive disease is supported by the fact that circulating levels of vasoactive species, such as endothelin and prostanoids, are increased in SCD patients, especially during crisis.[140] There is evidence for an elevated renal prostaglandin synthesis in nephropathy associated with sickle cell anemia.[141]

Lungs perfused with sickle RBCs, or sickle RBCs plus autologous platelets revealed high perfusate levels of the arachidonate metabolites, thromboxane A_2, and prostaglandin E_2. In a study by Baggiolini et al.,[142] it was proposed that polymorphonuclear neutrophil (PMN) activation, as may occur in response to infections or other injurious agents, increases sickle RBC retention/adherence in the pulmonary circulation of isolated perfused rat lung via a mechanism or mechanisms, which involve the release of platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) from the activated PMN. Such PMN activation, as seen in inflammation and infection,[143] initiates vaso-occlusion through the release of phospholipid products, which increase sickle RBC retention/adherence in the pulmonary circulation.

Apart from the biomechanical changes of sludging by vaso-occlusion, seen with PMN activation, a series of enzymes; phospholipases C, D and A2 [(PLC), (PLD) and (PLA_2)] are involved in the generation of phospholipid products by the PMN, while NADPH oxidase is activated.[143] Of particular interest are oxidants and the products of membrane phospholipids and PLA_2, lyso-PAF-acether, and free arachidonic acid.[42,142] The lyso-PAF-acether is inactive, but when acetylated by an acetyltransferase, its metabolically active product, PAF is formed.[144] PAF and LTB_4, on being formed, could stimulate increased sickle RBC retention in the pulmonary circulation. The major product of arachidonic acid, which is secreted by human PMNs, is the 5-lipoxygenase product LTB_4.[145] Both PAF and LTB_4 can cause the release of other inflammatory mediators, and influence vascular permeability, cell infiltration, and PMN adhesion to vascular endothelium.[144,146]

The enzyme that catalyses a key reaction leading to the formation of prostaglandins from arachidonic acid is the cyclooxygenase, while the lipooxygenase catalyses the formation of the leukotrienes. High concentrations of superoxide have been found to re-orient the arachidonic acid pathway in cells towards the production of thromboxane A_2,[147] causing an imbalance between prostacyclin and thromboxane A_2.

Thromboxane, a prostaglandin, is a vasoconstrictor, while prostacyclin is a vasodilator. Cyclooxygenase (COX) catalyses the rate-limiting step in the conversion of arachidonic acid to prostaglandins,[148] which are important mediators of acute and chronic inflammation, development and immune functions.[149] There are two isozymes; COX_1 and COX_2, the constitutive and inducible forms respectively. The constitutive isoform appears to regulate many normal physiologic functions in several cell types, whereas the inducible form, is usually expressed at low levels in most tissues and cells, but is significantly induced by a wide range of inflammatory stimuli such as lipopolysaccharide, cytokines and chemicals.[152]

Transgenic knockout Berkeley (BERK) mice, which express exclusively human α- and β-S-globins with very low levels of γ-globin (<1%), are associated with increased oxidative stress, sickling, hemolytic anemia and reduced availability of NO. The compensatory response in these mice include induction of COX_2 and eNOS, both catalyse the generation of vasodilators. Kaul et al.[151] suggested the induction of COX_2 and the associated vasodilation is a compensatory response to accomplish adequate oxygen delivery under anemic conditions.

Secretory phospholipase A2 type IIa (sPLA2) and inflammation

Secretory phospholipase A2 has a strong affinity for phospholipids that are negatively charged (e.g. phosphatidylserine and phosphatidylethanolamine) at physiological pH.[152] Normal mammalian cells do not

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appear to act as targets for sPLA2, but when PS becomes externalized on membranes, the phospholipids become vulnerable to hydrolysis by sPLA2.[50,153] Secretory phospholipase A2 is a low molecular weight, ubiquitous enzyme that is elevated in inflammation. Its action on lipids on red cell membrane with externalized PS will give lysophopholipids, and arachidonic acid, precursor of thromboxane and leukotrienes. On PS exposure, $sPLA_2$ can also generate lysophosphatidic acid, which affect vascular integrity.[51] It has further been shown[51] that the elevation of sPLA2 after injury predicts hypoxemia, and is related to multiorgan failure. In addition, elevation of sPLA2 is predictor of acute chest syndrome in SCD.[47]

Isoprostanes, oxidant-generated prostanoids

Unlike prostaglandins that are formed through the action of cyclooxygenase enzyme, isoprostanes are formed as a result of free radical-mediated peroxidation of arachidonic acid, independent of COX. It is those isoprostanes that contain F-type prostane rings, isomeric to PGF2α that are referred to as F_2-Isoprostanes.[132] These F_2-Isoprostanes are the most studied class of such compounds, and because of their stability, they afford the most accurate measure of oxidant stress.

Isoprostanes are vasoconstrictive oxidant-generated prostanoids, increased in states of oxidative stress attended by altered vascular reactivity.[155] F_2 isoprostanes are formed *in situ* on the plasma membrane, and then released into the bloodstream via the action of yet unknown phospholipases.[132] They are speculated to be formed where free radicals like .OH and NO_2^- attack unsaturated lipids like arachidonic acid.[132] An important structural distinction between isoprostanes and cycloxygenase-derived prostaglandins, which affords marked differences in biological activities is that the former contain chains that are predominantly cis to the prostane ring, while the latter have exclusively trans side chain.[134] A second important difference is that the isoprostanes are formed *in situ*, while esterified to phospholipids, and are subsequently released by phospholipase(s),[132] prostaglandins are generated only from free arachidonic acid. The phospholipase (s) responsible for the hydrolysis of isoprostanes from phospholipids is unknown. Previous work showed various secretory phospolipases from lower animals were able to release the isoprostanes, but it is unknown whether analogous mammalian enzymes possess this activity.[132]

CONCLUSION

This review on SCD has focused on how over a score of enzymatic actions could account for the pathophysiological features of the commonest hemoglobinopathy. It is noteworthy that these enzymes are not those involved in mainstream metabolic processes for generation of energy or elaboration of tissue components. The pathophysiological features of the disease is fundamentally linked to the aberrant HbS structure, which causes the deoxygenated HbS to polymerise, distorting the erythrocyte membrane, making the HbS-containing red cell rigid and non-deformable as it sickles. The imbalances between generation and utilization of oxidants, vasodilators and vasoconstrictors, procoagulant and anticoagulant, etc. giving rise to heterogenous clinical features have been considered. But whatever the clinical features; anemia, acute chest syndrome, stroke, priapism, cholelithiasis, renal damage, etc., there are some underlying enzymatic defects, and these have been the focus of this review. Could the varied defects in the plethora of enzymatic activities be the reason for the clinical heterogeneity of SCD?

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