Chapter 3

Homocysteine in Red Blood Cells Metabolism – Pharmacological Approaches

Filip Cristiana, Zamosteanu Nina and Albu Elena

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/47795

1. Introduction

Red blood cells are responsible for oxygen transport from lung to tissues. Oxygen transport depends on reduced state of iron (Fe^{2+}) in hemoglobin. To accomplish their delivery function erythrocytes must accommodate to the environment conditions as they change along the vascular branches. Both element oxygen (O_2) and iron are able to quickly shift their oxidative state in response to different external/internal emerging stimuli. Moreover in erythrocyte nitric oxide (NO●) a vasodilalatory messenger is present. All these elements act in normal condition in well established mechanisms but they may generate alone or together high reactive species, named free radicals, that damage red blood cells as well as vascular endothelium. Free radicals may be generated from both oxygen and nitrogen and are known as reactive oxygen species (ROS) respectively nitrogen reactive species (RNS). However erythrocytes have intracellular enzyme/non enzyme defense system. When reactive species are quickly and intensely generated under external/internal stimuli the activity of antioxidant defense system is overwhelmed. Free radicals generation is triggered by normal, adaptive or pathological stimuli such as: superoxide detoxification, decreasing oxygen saturation in vascular branches, shear stress or atherosclerosis, ischemic attack and bacterial infections.

One of the most potent oxidant agents in living cells is homocysteine (Hcy) a metabolic compound from methionine metabolism. The already mention metabolism requires vitamin B_{12}, B_6 and folic acid involvement. Every deficiency in vitamins supplies or enzymes activity triggers the onset of different diseases and erythrocyte is first affected in megaloblastic or Biermer anemia. A secondary consequence of vitamins deficiency is hyperhomocysteinemia. HyperHcy represents a high risk in cardiovascular diseases and not only. Nowadays is generally accepted that Hcy disturbs the normal endothelial function, promoting thrombosis and inhibiting fibrinolysis through many mechanisms which can possibly integrate and are

not mutually exclusive; oxidative processes, decreasing NO bioavailability and specific protein targeting.

The already specified free radicals are not all "bad". NO● can be regarded as a "good radical" but it is inactivated by many Hcy-dependent Mechanisms that finally impair its vasodilatatory function. Thus damaging Hcy effects expands to the environment where erythrocytes move and act. As a consequence directly and indirectly Hcy has a big impact on erythrocytes whose deformability in shear stress is crucial for circulatory function. Taken into account all these factors pharmacological approaches envisage lowering homocysteine levels by different ways such as: vitamins B supplementation, antioxidant drugs, hypotensive agent, antithrombotic drugs etc. Some of these patterns such is vitamins supplementation proved to have limited clinical benefits while others as nitrite/nitrate are still in debates. Because most pathological processes mentioned above involve oxidative pathway mechanism, pharmacological presentation will focus on drugs with antioxidant properties

As a conclusion the effect of elevated homocysteine appears multifactorial affecting both the vascular wall structure, function as well as erythrocytes metabolism.

2. Homocysteine and red blood cells

2.1. Red blood cells oxidative-reducing balance

Red blood cells are responsible for oxygen transport from lung to tissues. Their function depends on reduced state of iron (Fe^{2+}) in hemoglobin (Hb). Both element oxygen and iron are able to quickly shift their oxidative state in response to different emerging stimuli. Literature shows in [1] that hemoglobin may undergo oxidative reaction in the process of releasing oxygen. In this process iron is oxidized to Fe^{3+} and reactive oxygen species are generated. The bigger the reactive species release is the higher normal deformability and flexibility of erythrocytes is disturbed.

2.1.1. Reactive species generation

In erythrocyte are found together oxygen (O_2), nitrogen oxide (NO●) and iron (Fe^{2+}). All these elements act in normal condition in well established mechanisms but they may generate alone or together reactive species, named radicals, that damage red blood cells as well as vascular endothelium.

A radical is a chemical species that possesses a single unpaired electron in outer orbitals, and is able to independently exist, known also as free radical. Radicals are highly reactive in extracting an electron from any neighbor molecule in order to complete theirs own orbitals. There are two main groups of free radicals: ROS or reactive species of oxygen, RNS or reactive nitrogen species. ROS and RNS can act together damaging cells and causing nitrosactive stress. Therefore, these two species are often collectively referred to as ROS/RNS.

Transitional metals (Fe belongs to this group) particularly behave. They have a single unpaired electrons in theirs outer orbitals, but they don't behave as free radicals because within cells they are attached to proteins in most cases. However they are able to catalyze electron transfer in many processes and sometimes generate radicals.

2.1.2. Reactive oxygen species

Reactive species of oxygen refers to a group of highly reactive O_2 metabolites, including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical (OH•), that can be formed within cells. Reactive oxygen species are constantly formed as byproducts in normal enzymatic reaction in all human cells through normal aerobic processes as mitochondrial oxidative phosphorylation or as necessary products in neutrophils in order to kill invading pathogens. The above mentioned phenomena are consuming oxygen processes.

Erythrocytes must save oxygen for delivering it to the cells; as a consequence red blood cells lack mitochondrion the main oxygen consumer within cell. In this particular condition the source of ROS in erythrocyte may be the carried oxygen itself.

In order to understand oxygen behavior an inside in its structure is needed. The ground state of oxygen is triplet oxygen meaning that the molecule has two unpaired electrons occupying two different molecular orbitals.

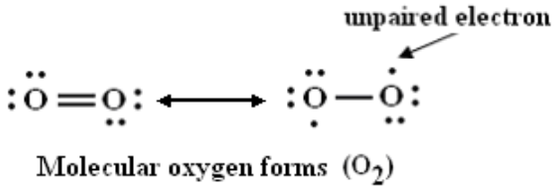

Figure 1. The structure of oxygen molecule

These electrons can't travel both in the same orbital because they have parallel spin (they spin in the same direction). As a consequence molecular oxygen is paramagnetic and from this feature it was concluded that the structure in the right may be assigned to O_2 (figure1). Although O_2 is very reactive from thermodynamic standpoint its single electrons cannot react rapidly with already paired electrons in the covalent bond of organic molecules (abundant in living cells). As a consequence it is harmless to these molecules. Instead molecular oxygen can rapidly react with single unpaired electrons from transitory metals (e.g. Fe, Cu, Mn). One mole of properly chelated cooper could catalyze consumption of all of the oxygen in an average room within one second in [2].

In fact oxygen O_2 is both kinetically stable thus not reactive and very reactive promoting fast reactions, depending the surrounding conditions.

Within cells where transitional metals are bounded to proteins (in metal containing proteins or enzymes) oxidative attack of O_2 tend to be slow, meaning that a first single electron is relatively difficult to add. As a consequence superoxid radical ($O_2^{\bullet -}$) will form very slow.

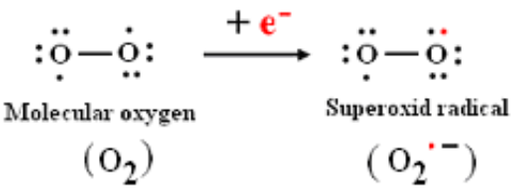

Figure 2. Superoxide radical generation

Once an electron acquired additional electrons are easier added, further reactions quickly occur in [3] and reactive species of oxygen are generated.

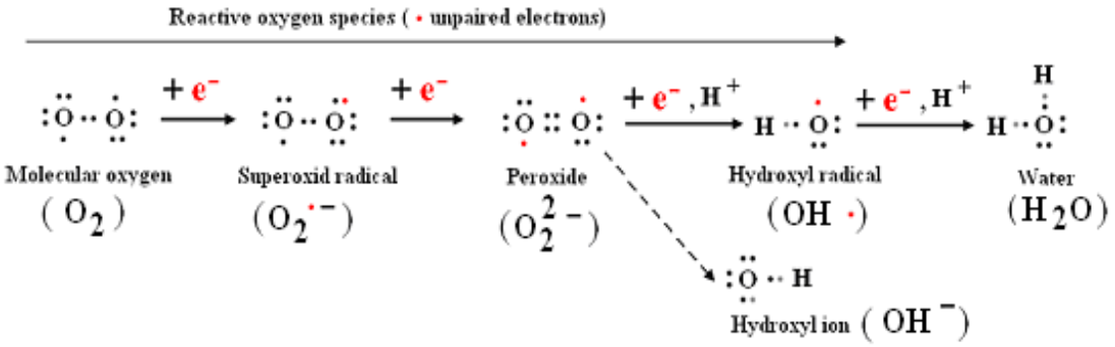

Figure 3. Reactive oxygen species generation

Superoxide ($O_2^{\bullet -}$) is formed when molecular oxygen (O_2) gains an additional electron, producing a molecule with only one unpaired electron (figure 2), generating a very reactive free radical. When accepts an electron, superoxide is reduced to hydrogen peroxide, which is not a radical. In the next one-electron reduction step hydrogen peroxide generates water and the hydroxyl radical (OH •) which is probably the most reactive free radical. A final electron acceptance (figure 3) reduces hydroxyl radical to water in [4].

Superoxide radical ($O_2^{\bullet -}$) is a reactive radical, however it cannot diffuse to far having limited lipid solubility. Instead it might react in the presence of ferric iron with de hydrogen peroxide generating the most potent hydroxyl radical through a non-enzymatic reaction known as Haber-Weiss reaction.

The reaction takes place in two steps that involved ferric iron and superoxide as follows:

$$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\bullet$$

The general non-enzymatic reaction occurring in living cells is:

$$O_2^{\bullet -} + H_2O_2 \rightarrow OH\bullet + OH^- + O_2$$

It appears that superoxide radical is also a source of hydroxyl radical in ferric iron presence.

Hydrogen peroxide (H_2O_2) is not a free radical but still classified as a ROS because it generates the most powerful hydroxyl radical (OH •) in the presence of transitional metals (Fe^{2+}, Cu^{+}), the reaction above. Hydrogen peroxide (H_2O_2) is lipid soluble and as a

consequence it can diffuse through lipid membranes. No matter where it meets proteins (including hemoglobin) that contain transition metals Fe^{2+}, Cu^{+}, H_2O_2 generates OH• at the specific site where these metals are located thus damaging protein structure.

Another reactive species (but not a radical) derived from molecular oxygen is singlet oxygen, designated as $^1O_2$. Singlet oxygen ($^1O_2$), a highly excited state created when molecular oxygen absorbs sufficient energy to shift an unpaired electron to a higher orbital, can be formed from superoxide radical in [5]:

$$2\ O_2^{\bullet -} + 2H^{+} \rightarrow H_2O_2 + {}^1O_2$$

Singlet oxygen is even more reactive than the hydroxyl radical, although it is not a radical.

As a conclusion the most reactive radical is hydroxyl (OH •) which indiscriminately extracts electrons from any other molecules around it whereas superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are more selective in their reactions with biological molecules [6].

All the above reactions and processes take place in all human cells including erythrocytes. As for the other cells the main source for free radicals is mitochondrion, in the particular case of red blood cells the main source for radicals is the carried oxygen. Molecular oxygen is carried in order to be delivered to tissues and as a consequence it is found, for a short period of time, free, thus unbound. In this state it might be prone to generate the above described radicals.

Oxygen binds to hemoglobin at the ferrous iron. The ferrous state (Fe^{2+}) of iron is a condition for hemoglobin normal function. However a small percent of Fe^{2+} is slowly converted by O_2 to ferric form (Fe^{3+}) in resulting methemoglobin. An enzymatic system, methemoglobin reductase quickly restores Fe^{3+} to Fe^{2+} and reduces methemoglobin back to hemoglobin. Binding of oxygen to the iron in the hem is considered not to change the oxidation state of the metal. However oxygenated hem has some of the electronic characteristics of a Fe^{3+}–OO^{-} peroxide anion [3]. Misra and Fridovich demonstrate that the $Fe^{3+}O^{2-}$ complex is able to generate superoxide radical in [7] during the normal molecular oxygen transport to tissues through the hemoglobin auto-oxidation. Thus hemoglobin auto-oxidation causes superoxide formation within erytrocyte.

Other researchers show that hemoglobin may undergo oxidative reaction in the oxygen releasing process. Balagopalakrishna and coworkers demonstrate in [8] that at intermediate oxygen pressure, where hemoglobin partially releases molecular oxygen, the superoxide radical production increases. They show that superoxide radical is released in the hydrophobic hem pocket. The process in slow enough thus the formation of superoxide was followed for more than 15 min, and thus detected by low temperature electron paramagnetic resonance technique.

Being a radical superoxide reacts fast with other radicals or alternatively it is efficiently scavenged through the specific superoxide dismutase (SOD) activity.

When collides with other radicals $O_2^{\bullet -}$ gives birth to new reactive species as follows:

- $O_2^{\bullet -}$ reacts with itself generating molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) a source for hydroxyl radical.

- $O_2^{\bullet-}$ reacts fast with NO• radical generating toxic peroxinitrite ($ONOO^-$), a reactive nitrogen specie (RNS). At physiological pH, $ONOO^-$ rapidly protonates to peroxynitrous acid, ONOOH. This powerful oxidizing and nitrating agent can directly damage proteins and lipids.

Thus, any system producing $O_2^{\bullet-}$ and NO• can cause biological damage, and erythrocytes make no exception in [6].

Hydrogen peroxide is not a radical because it doesn't have any unpaired electron. The limited reactivity of H_2O_2 allows it to cross membranes and to become widely dispersed. Even hydrogen peroxide is not a radical it can generates the short-lived but very active hydroxyl radicals via Harber-Weiss non-enzyme reaction in [9]. The hydroxyl radical (OH•), which is highly reactive, diffuses only a short distance before it reacts with whatever biomolecules it collides with. Recent study consider that the high and indiscriminate reactivity of the hydroxyl radical minimizes its ability to diffuse and makes it more damaging within cell or in the environment where it is generated in [10]. This consideration becomes more important when the oxidative events prevail within a specific cellular compartment. Hydroxyl radical are especially dangerous because it can initiate an autocatalytic radical chain reaction. Being so harmful cells carefully control hydroxyl radical by limiting the availability of both Fe^{2+} and H_2O_2 in [6].

The non-enzymatic decomposition of hydrogen peroxide described by Haber-Wiess and especially the mechanism through which hydroxyl radical (OH•) acts was highly debated.

Some researchers consider that hydroxyl radical is responsible for damaging cellular component on behalf of a radical mechanism. Others consider that ferryl ion ($Fe(IV)O^{2+}$), an oxidizing species where iron is in high oxidation state (Fe IV) in [11,12] is an active intermediate responsible for chain reaction propagation. Another group consider that conditions inside cell dictates whether metallo-oxo species or hydroxyl radical (OH•) is the main oxidant [in 13]. Some other like Prousek concluded in [14] that both oxidising species can be formed in living cells.

As a general conclusion in erythrocytes ROS are produced both accidentally and physiologically in different enzyme-catalyzed reactions (figure4).

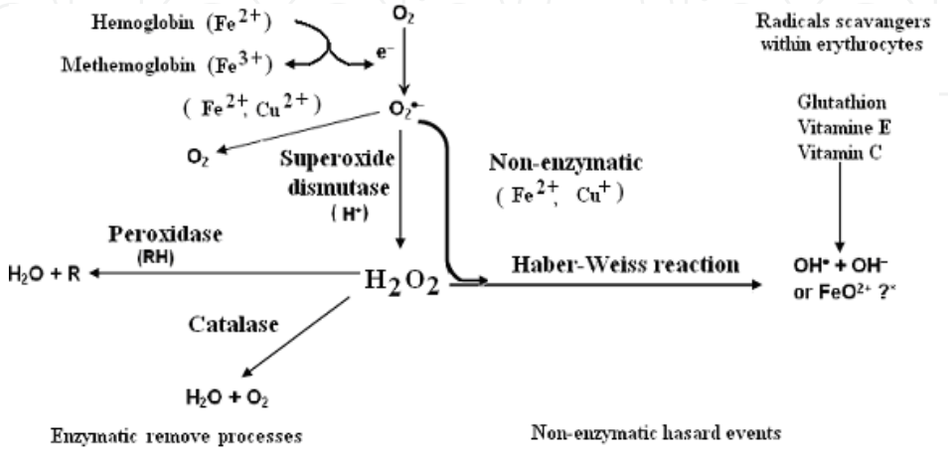

Figure 4. ROS generation in erythrocyte.

2.1.3. Damaging effects of ROS

ROS are considered "bad" radicals because can they indiscriminately interact with any biological molecule they meet causing DNA, lipids and protein damage. As a missing nucleus cell erythrocyte can undergoes the last two lesional processes but endothelium can undergoes all three mentioned injuries. Erythrocytes are particularly affected by oxidation of polyunsaturated fatty acids in membrane phospholipids which causes their peroxidation, degradation and fragmentation [15]. During lipid peroxidation other reactive species as peroxyl radicals are generated in a succession of chain reactions (fig.5). This reactive intermediates amplify the injury at the place were they are formed.

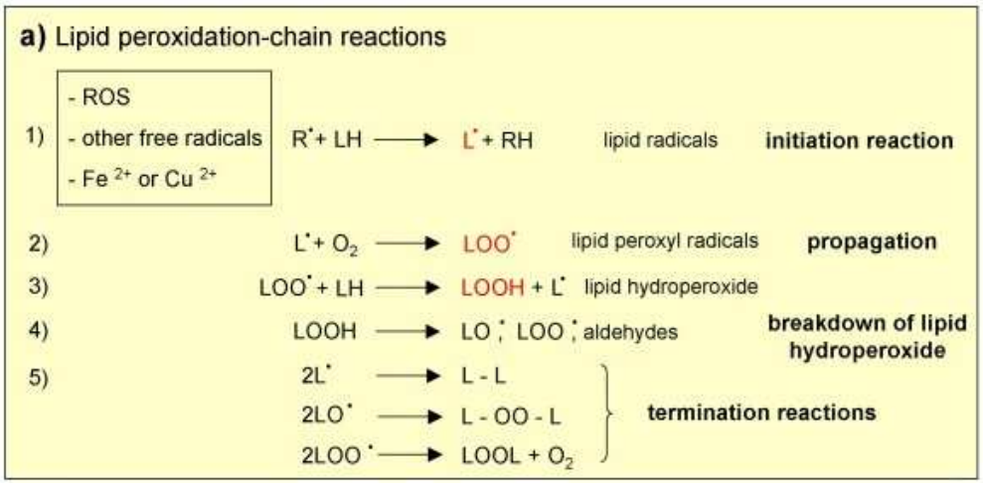

Figure 5. Lipid peroxidation and radical and non-radical intermediates formation [taken from 15]

Erythrocytes also undergo the amino acids and/or whole proteins oxidation. Protein oxidation leads to inactivation (if targeted proteins are enzymes), fragmentation, aggregation of fragments and/or increased susceptibility to proteolysis [**15**]. In addition if injured proteins belong to erythrocytes skeleton the deformability of erythrocytes is impaired [16]. ROS attack doesn't limit to erythrocytes it also affect endothelium cells, which in turn influence erythrocyte metabolism. In nucleus containing endothelium cell beside lipids and proteins oxidation ROS cause DNA injuries. Free radicals can interact with DNA leading to strand breaks or structural changes such as adduct formation (figure 6) [15].

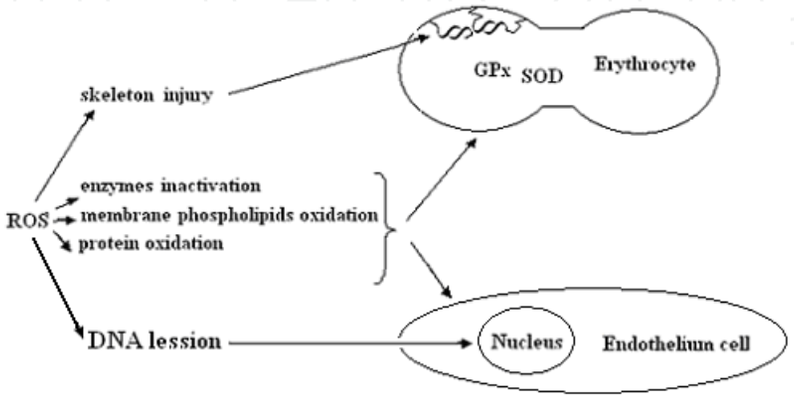

Figure 6. Over simplifying scheme of ROS damaging activity in erythrocytes and in endothelium cells

2.1.4. ROS signaling

Until recently reactive oxygen species were considered only oxidizing damaging factors. But it was demonstrated by in [**15, 17**] that ROS can be also "good" as they act as signaling molecules. In fact both authors show that ROS are neither "good" nor "bad", temporal length and intensity of free radicals generation make the difference between physiological, adaptive or pathological effect. Thus oxidizing molecules is not the end "of the road" for reactive species alternatively they trigger cellular responses which depending on the intensity of ROS attack, prepare the cell to survive or on contrary trigger cell death (figure 7).

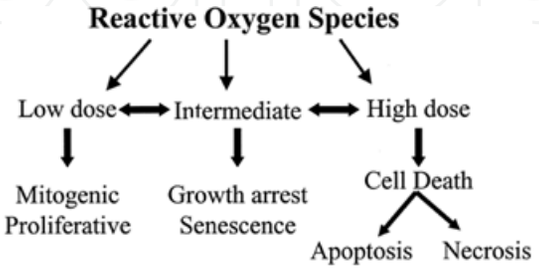

Figure 7. Cells response under ROS attack (taken from 17)

Being highly reactive ROS can intercept cell signaling pathways within successive steps in cascade events modulating the functions of many enzymes and transcription factors. Oxidative stress triggers cellular response by activating many signaling pathways. ROS can directly or indirectly modulate a) the function of different types of enzymes, b) the transcription factors activity and c) the activity of ion-channels.

a. Enzymes modulated by ROS include both kinases and phosphatases. The big class of kinase includes both tyrosine kinase as Src, Ras, JAK2, Pyk2, PI3K, and the mitogen-activated protein kinase (MAPK). The three best-characterized MAPK subfamilies are c-Jun N-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK) [18]. All these MAPK pathways are structurally similar, but functionally distinct. Importantly, ERK, JNK and p38 MAPK have all been shown to be activated by oxidative stress [19]. ERK and JNK are important in recruiting c-Fos and c-Jun to the nucleus where they activate the transcription factor AP—1 (activator protein -1), whereas activation of p38 and inhibitory kappa kinases (IKK) is important in the transcriptional activation of NF-κB. Both of these factors are important in regulating the diverse genes, which play key roles in the pathogenesis of inflammation, and in regulation of cell cycle, proliferation, and apoptosis. ROS may inhibit tyrosine phosphatase activity further contributing to tyrosine kinase activation.
b. ROS also influence gene and protein expression by activating transcription factors, such as the already mention NFkB and activator protein-1 (AP-1) and hypoxia-inducible factor-1 (HIF-1).
c. ROS stimulate ion channels, such as plasma membrane Ca2+ and K+ channels, leading to changes in cation concentration. The cytosolic Ca^{2+} level can be increased by ROS in various cell types, including epithelium cell, through the mobilization of intracellular Ca^{2+}stores and/or through the influx of extracellular Ca^{2+} [15]. The ROS-mediated increase

in Ca^{2+}concentration contributes to the oxidative stress-mediated activation of PKC and to the transcriptional induction of the AP-1 proteins c-Fos and c-Jun [20] (figure 8).

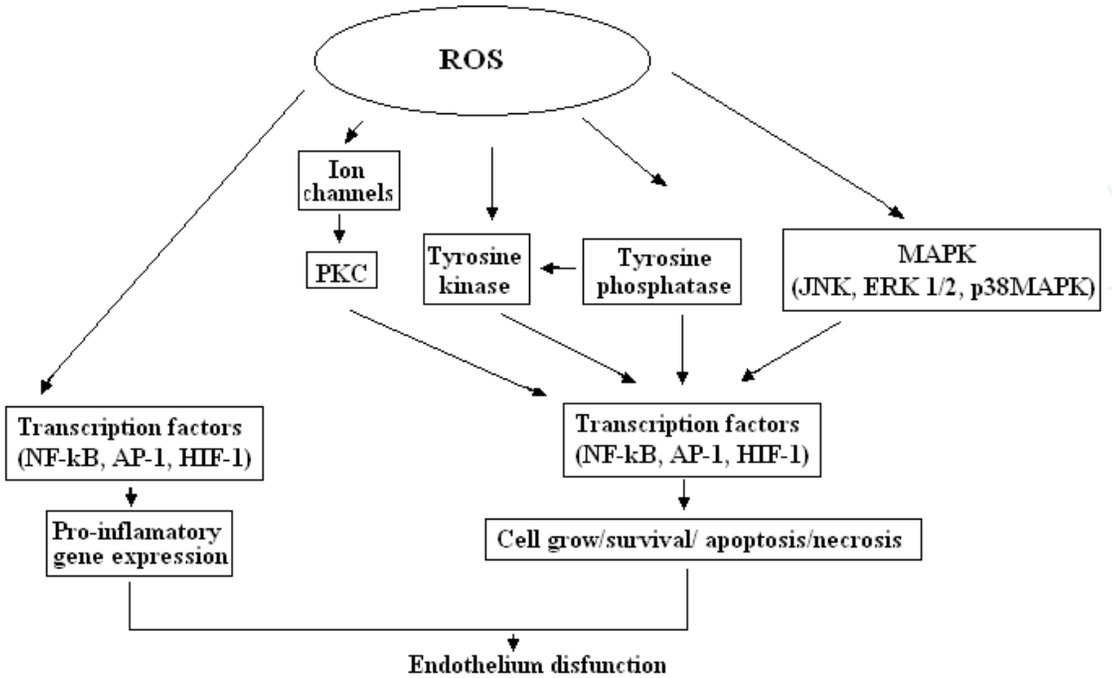

Figure 8. Major pathways activated by ROS generation (modified from **21)**.MAPK=mitogen-activated protein, JNK=c-Jun N-terminal kinases, ERK=extracellular signal-regulated kinases, NFκB=nuclear factor κB, AP-1=activator protein-1, HIF-1= hypoxia-inducible factor-1, PKC=protein kinase C

More details about the cellular response in ROS and other radical and non-radicals species attack in oxidative events can be found in [15,17,19]

2.1.5. Scavenging ROS

Erythrocytes have an impressing antioxidant enzyme and non-enzyme system that deals with an important amount of free radicals. Superoxide dismutase and glutathione peroxidase are the most efficient antioxidant enzyme in red blood cells.

Erythrocytes superoxide dismutase remove $O_2^{\bullet -}$ by catalyzing its dismutation, one $O_2^{\bullet -}$ being reduced to H_2O_2 and another oxidized to O_2 (figure 9).

$$2\,O_2^{\bullet -} + 2\,H^+ \xrightarrow{\text{Superoxide dismutase}} O_2 + H_2O_2$$

Figure 9. Superoxide dismutase activity

The dismutation of superoxide $O_2^{\bullet -}$ by SOD is very efficient having the largest k_{cat}/K_M (an approximation of catalytic efficiency) of any known enzyme (~7 x 10^9 $M^{-1}s^{-1}$) [22]. SOD catalyst activity is limited only by the frequency of collision with superoxide. That means the reaction rate is limited only by the diffusion of superoxid radical. Diffusion limitation becomes canceled in radicals over production thus activating the process.

As seen in upper reaction (fig.9) superoxide dismutase must work with enzymes that remove H_2O_2.

Glutathione peroxidase (GPx) removes H_2O_2 by coupling its reduction to water (figure10) with oxidation of reduced glutathione (GSH), a thiol-containing tripeptide (glu-cys-gly). The product, oxidized glutathione (GS-SG), consists of two GSH linked by a disulphide bridge, and can be converted back to GSH by glutathione reductase enzymes in [6].

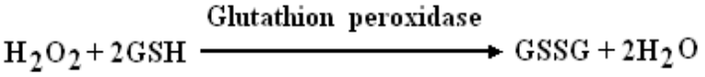

Figure 10. Glutathion peroxidase activity.

Beside enzyme antioxidant systems erythrocytes uses antioxidants agents (fig.4). These agents are preferentially oxidized by reactive species to preserve more important biomolecules and can be reversibly reduced back. For example, GSH and ascorbate can scavenge $O_2^{\bullet -}$, OH•, and also ONOOH. Tocopherols are good scavengers of peroxyl radicals and help to protect membranes against lipid peroxidation by interrupting the propagation-chain reaction (figure 5) in [6].

2.1.6. Reactive nitrogen species

Nitrogen compounds found in the body comes from exogenous sources as nitrites/nitrates or from endogen production of nitric oxide (NO•). The group of nitrogen derivatives includes:

- NO• nitric oxide a natural free radical also named nitrogen monoxide is involved in vasodilatation in mammals
- NO_2^- nitrogen dioxide or nitrite. In organism is found in its corresponding salts nitrites(from nitrous acid HNO_2)
- NO_3^- nitrate (from nitric acid HNO_3) also found in the body in corresponding salts

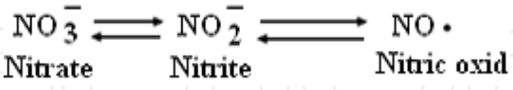

Figure 11. Nitrogen derivatives

Nitrogen derivatives convert into each other forward and backward continuously under shifting conditions within cells (figure11).

Endogenous NO• is synthesized by nitric oxide synthases (NOS) in the endothelial and other cells, where is involved in vascular physiology.

Endothelial nitric oxide synthases (eNOS) synthesizes NO• (figure 12) from L-arginine with 1,5 consumption of 1.5 NADPH equivalents and two oxygen molecules per NO• formed in [23]. The reaction requires the presence of Ca^{2+}-Calmodulin and tetrahydrobiopterin (BH_4) as cofactors in[24].

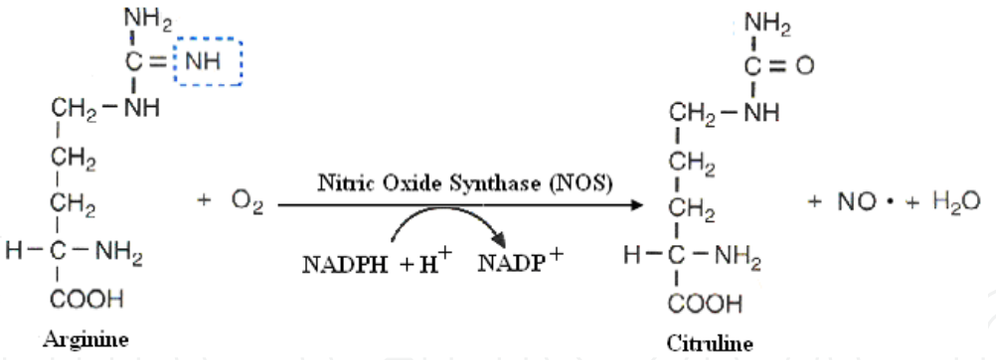

Figure 12. Nitric oxide generation

Generated NO• is a gaseous molecule with unpaired electrons and as a consequence a radical; it is lipophilic and diffuses rapidly through membranes. NO• is a messenger in many physiological processes: endothelial relaxation of the smooth muscle, inhibition of platelet aggregation, neurotransmission and cytoxicity in [25]. NO• pathology includes both low and high concentrations as follows: insufficient NO• production is involved in hypertension, the activation of platelet aggregation and atherogenesis while high NO• production generates septic shock, stroke, and carcinogenesis.

NO• released from endothelial cells diffuses through blood or to the underlining smooth muscle cells in the media where it triggers vasodilatation. In blood stream NO• will affect platelets, leucocytes and erythrocytes.

Recent studies show that:

a. e-NOS can produce NO • not only in normal oxygenation (figure 12) but also decreased oxygenation gradient across vascular branches. In addition eNOS can sometimes be a source of ROS generating $O_2^{\bullet -}$
b. endothelial cells are not the only ones able to generate NO•, blood erythrocytes are expressing functional NOS
c. Hemoglobin itself also "produces" NO• from nitrite in order to modulate vasodilatation.

a. It is only recently found that endothelial NOS may be a source of ROS generating $O_2^{\bullet -}$ depending the availability of its substrates within cell (figure 13) [23].

Endothelial nitric oxide synthetase activity is regulated by a combination of mechanisms that allow eNOS to modulate its activity under physio-pathological condition in [22]. eNOS contains 2 enzymatic domains, a flavin-containing reductase and a heme-containing oxygenase domain (Fe^{3+}) connected by a regulatory calmodulin-binding domain. Binding of the Ca^{2+}/calmodulin complex orients the other domains in such a position that NADPH-derived electrons generated on the reductase domain flow to the oxygenase domain in [26].

The oxygenase domain of eNOS contains an iron ion (Fe^{3+}) that binds oxygen on reduction Fe^{2+}, and this complex finally causes the conversion of L-arginine to NO• and L-citrulline. This sequence of events properly rules if the cofactor BH_4 "provides the connection"

between the two domains. Deficiency of arginine or BH_4 causes the reductase uncoupling from oxygenase. At the oxygenase domain intermediate Fe^{2+}-O_2 complex dissociates to form superoxide and the original Fe^{3+} group of the eNOS)[27]. Thus eNOS releases $O_2^{\bullet -}$ instead of NO• (figure 9).

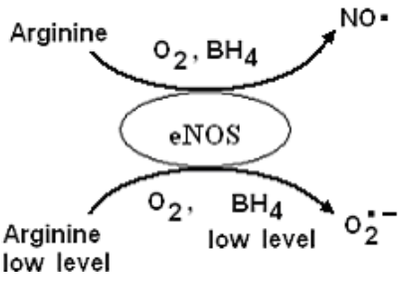

Figure 13. Endothelial NOS differently behaves generating either NO• or $O_2^{\bullet -}$ depending on the substrate availability.

Oxygen deficiency is known to halt the L-arginine cycle if the oxygen levels fall below a threshold level of ca [O_2] ~ 10 μM [28]. However, eNOS is not wholly inactivated in hypoxia, instead, in the presence of nitrite (figure 14), it shifts again and produces NO• in [29].

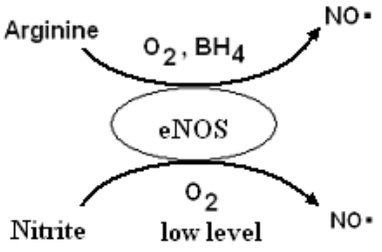

Figure 14. Endothelial NOS may generate NO• in both normal and low oxygenation

Stroes et al demonstrate in [30, 31] an intriguing activity for eNOS only, the simultaneous generation of both NO• and superoxide, even in the presence of BH_4 and L-arginine, under physiological conditions. The consequence is the production of peroxynitrite, a highly reactive molecule, by eNOS (figure 15).

Thus eNOS is a source of free radical producing "good" or "bad" radicals upon inside cell condition.

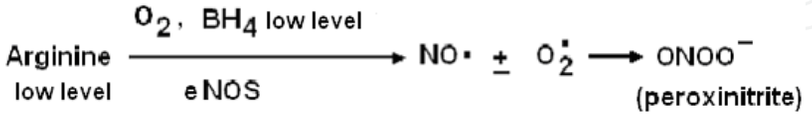

Figure 15. Peroxinitrite generation as a result of particularly eNOS activity

Peroxynitrite anion ($ONOO^-$) is a reactive species of increasingly recognized biological relevance that contributes to oxidative tissue damage. Recent research indicates in [32] that peroxynitrite is able to cross the erythrocyte membrane by two different mechanisms: in the anionic form through the anion exchange channel, and in the protonated form by passive diffusion. Entering the erythrocyte peroxynitrite causes nitration of intracellular hemoglobin, in a process that is enhanced in thiol-depleted erythrocytes.

To summarize NO• can be produced by eNOS from either L-arginine in good oxygenation physiological state or from nitrite in hypoxia. In vitamins deficiency (low BH4 levels) eNOS produces superoxide radical. Recent studies demonstrate that endothelium cells surprisingly produce ROS under hypoxia. The primary site of reactive oxygen species production was demonstrated to be complex III in electron transport in mitochondria. The paradoxically increase in ROS production under low oxygenation is still not fully understood but it is considered that reactive oxygen species released during hypoxia act as signalling agents that trigger induction of erythropoietin, endothelial growth factor and glycolytic enzymes. Systemically, these responses enhance the delivery of O_2 to cells and facilitate the production of glycolytic ATP instead of mitochondrion. Induction of these genes is mediated by "specialized" hypoxia inducible factor 1 (HIF-1) [33, 34]. As a conclusion in normal oxygenation NO is produced by eNOS. In hypoxia adaptive responses are onset; the release of NO from nitrite to sustain normal vascular function is one path. Alternatively when mitochondrion "senses" hypoxia it releases ROS as signaling molecules that activate diverse functional responses, including activation of gene expression that promote cell survival.

b. Kleinbongard demonstrates in [35] that red blood cells express functional eNOS which is located in both the internal side of the plasma membrane and the cytoplasm with a higher expression in the membrane. The enzyme has a similar activity and regulatory mechanism as the endothelial-derived NOS. Besides its vasodilatation activity NO• also regulates red blood cells deformability and inhibits platelet activation. In physiological condition where there is a normal supply of L-Arginine and subsequently a normal NO• production, nitric oxide sustains red blood cells deformability. On contrary decreased NO• levels reduces erythrocytes deformability preventing them to easily pass through microcirculation. The same effect was observed on platelet aggregation when decreased NO• levels promote thrombosis.

Ulker demonstrates in [36] that red blood cell-NOS is activated by mechanical factors and that export of NO from erythrocytes is enhanced by mechanical stress thus pointing erythrocyte contribution to the regulation of vascular tonus

c. NO• is a short life species as a consequence it quickly reacts with any encountered molecules or it is rapidly oxidized by hemoglobin in blood. In fact the general accepted theory is that Hb in the red blood cells is an extremely effective NO• scavenger in [37]. Oxigenated-Hb reacts with NO• which is rapidly converted into nitrate (figure 16). After reacting oxygenated hemoglobin is converted to methemoglobin (met-Hb). This reaction is considered to be limited only by the diffusion.

$$\underset{\text{oxyhemoglobin}}{Hb\text{-}Fe\,(II)O_2} + NO\bullet \longrightarrow \underset{\text{methemoglobin}}{Hb\,(Fe\,III)} + \underset{\text{nitrate}}{NO_3^-}$$

Figure 16. Oxyhemoglobin activity of scavenging the nitric oxide

Lundberg shows in [38] that NO• can be alternatively produced in hypoxic condition by deoxi-hemoglobin from nitrite. Inside erythrocyte Hb can interact with NO• in many ways depending on its oxygen saturation, as follows:

- in oxygenated form, oxi-Hb acts as a scavenger removing NO• as nitrate.
- in deoxygenated form deoxi-Hb acts in two different ways: first it binds NO• thus functioning as a transporter and second it reduces nitrite to generate NO• (figure17) Recently several authors suggest in [39-41] that this behavior represents a mechanism for NO• generation in regions of poor oxygenation where deoxy-Hb predominates

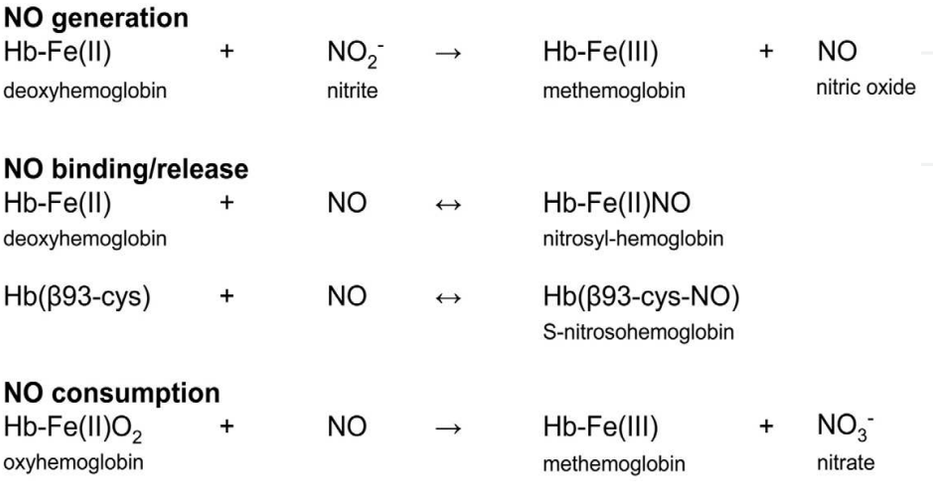

Figure 17. Hemoglobin "regulates" the NO• use/consumption, from [38]

Two recent theories try to explain the Hb involvement in nitric oxide use/consumption in vasorelaxation process in [42].

- Stamler and colleagues originally suggested in [43] a role for a thiol (SH) group in Hb as a carrier and releaser of NO•. According to this theory, the binding (formation of *S*-nitrosohemoglobin) and release of NO• from Hb are allosterically regulated so that NO• release occurs when Hb is deoxygenated.
- Cosby, Crowford et all suggest in [44] that Hb is not a transporter of NO• but rather an "enzyme" dealing with NO• depending oxygen saturation as follows: when Hb is fully oxygenated, the primary reaction is oxidation of nitrite into biologically inert and supposed "pool" nitrate. As oxygen saturation falls along the vascular tree, Hb gradually turns into a "reductase" and starts to reduce nitrite into vasodilator NO• (figure 18). The maximal nitrite reduction is observed when Hb is approximately 50% oxygenated (P_{50}) in [45,46]. Concomitantly, vasodilation is initiated at the P_{50}, ideally suited for the regulation of hypoxic vasodilation under varied physiologic and pathologic conditions (figure 19).

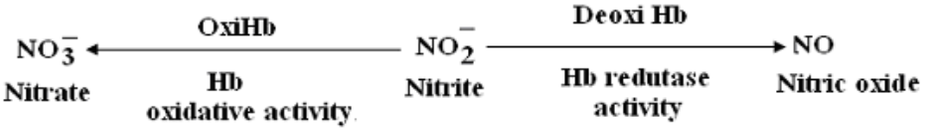

Figure 18. Hemoglobin behavior depending on oxygen saturation.

J.O.Lunderg concluded in [42] that: "in just one decade, Hb has gone from being merely a NO• scavenger to NO• carrier and now NO• generator".

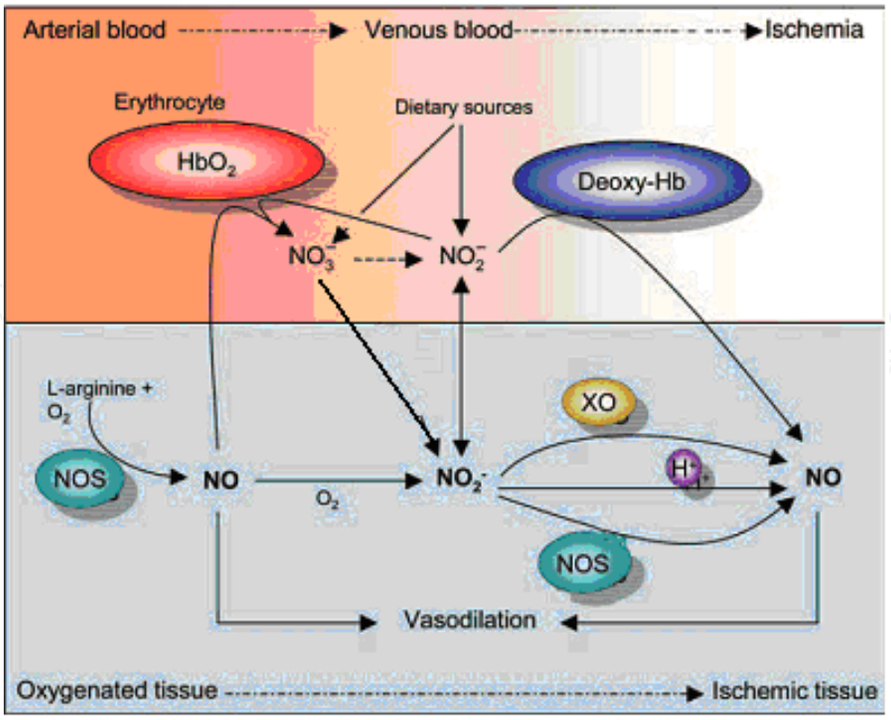

Figure 19. Hemoglobin uses NO• to generate nitrate a supposed "NO_2^-/ NO• pool" in full oxygenation and releases it with maximal activity at P_{50} oxygen saturation in hypoxia [modified from [38].

To summarize: when there is plenty of oxygen, NO• is mainly produced by endothelial NOS or by erythrocyte own NOS. Thus nitric oxide radical maintains good erythrocytes deformability and probably oxy-Hb regulates the amount of NO• by trapping its extra amount to form *S*-nitrosohemoglobin. When oxygen is scarce NO• is synthesized in low amount (as a consequence of low eNOS activity where oxygen is cofactor). In this condition NO• is saved through binding on deoxyHb which becomes a source of NO• in vascular circulation, or deoxy-Hb "catalyses" the NO• generation from nitrite. Behaving this way deoxy-Hb can maintain also red blood cell deformability in hypoxic condition. As a consequence hemoglobin may "regulates" membrane deformability along circulating branches through the way it uses/produces NO• radical. [47]

In addition two other supplementary mechanisms, endothelial xantinoxidase activity and blood pH dictate superoxid radical versus NO• generation depending on the blood oxygenation/deoxigenation status in [48,49].

2.2. Homocysteine metabolism

Homocysteine is a metabolic compound formed in methionine and betaine metabolism. The already mention metabolisms require vitamin B_{12}, B_6 and folic acid involvement and the proper activity of two main enzymes cystathionine β-synthase (CBS) and methylenetetrahydrofolate reductase (MTHFR). Every deficiency in vitamins supplies or enzymes activity triggers the onset of different disease. Megaloblastic anemia or Biermer disease affect primarily the red blood cells. Secondary to the above mentioned illnesses hyperhomocysteinemia can also install.

Hyperhomocysteinemia is considered to be involved in many diseases from cardiovascular to neurological illnesses. It is generally agreed that two general mechanisms cause hyperhomocysteinemia: one is low vitamins (B12, B6, folic acid) supplies and second the main enzymes deficiencies (cystathionine ß-synthase deficiency and methylenetetrahydrofolate reductase deficiency). Hypehomocysteinemia is today considered a severe risk factor in vascular illnesses. Many approaches envisage lowering homocysteine levels by vitamin B or oral folic acid supplementation but many recent studies show that vitamins administration fail to give a real clinical benefit and suggest that B vitamins might instead increase some cardiovascular risks in [50,51]. However not all patients with cardiovascular events or neurodegenerative diseases are enzymes deficient or poor vitamins supplied. The majority of research works report hyperhomocysteinemia associated to many diseases but the question what triggers hyperhomocysteinemia is yet to answer. An interesting hypothesis suggests that in fact hyperhomocysteinemia is more a secondary effect that amplifies in its turn the initial injury [52]. Brattström and Wilcken in [52] consider that impaired renal function due to hypertension and atherosclerosis is an important cause of the elevated plasma homocysteine found in vascular disease patients. The reasons are as follows. Atherogenesis and elevation of blood pressure commonly develop silently over many years before the emergence of clinically evident vascular events. These processes also lead to nephrosclerosis and a degree of deterioration of renal function, and this is highly relevant to the plasma clearance of homocysteine. For these reasons, the presence of vascular disease itself may contribute to an elevation in circulating homocysteine by leading to a decline in renal function. This means that because of reduced renal function, patients with either occult or clinically evident cardiovascular disease may have elevated circulating homocysteine concentrations (figure 20). This could also explain the relation between plasma homocysteine and the severity of atherosclerosis.

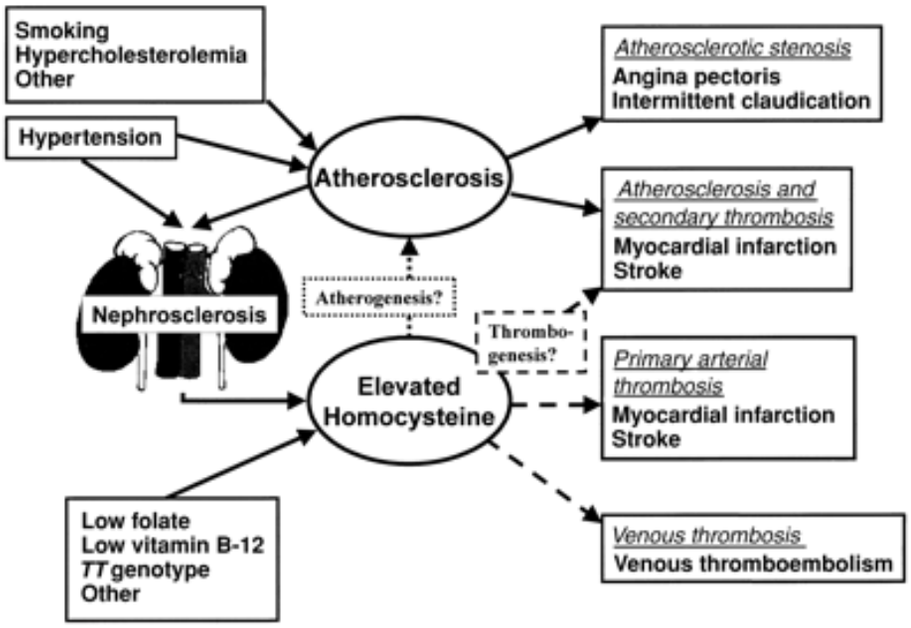

Figure 20. Proposed mechanisms for the causes of hyperhomocysteinemia (taken from [52])

2.3. Hyperhomocysteinemia a disturbing factor of the endothelial function

Homocysteine refers to all species that contain and can release homocystein including homocystine (the dimer of homocysteine) and mixed cysteine-homocysteine disulfide or homocysteine bound on proteins. In fact the major form of homocysteine in circulation, around 70% is protein bounded.

In early data normal levels of homocysteine were admitted to be around 15μM/L. It was found that homocysteine slightly increases with age in [53]. Levels of 15-30 μM/L corresponds to mild, 30-100 μM/L to moderate and more than 100 μM/L to severe hyperhomocysteinemia in [54].

Nowadays it is considered that concentrations below 9 micro mol/L are an appropriate target level for therapy in [55].

Nowadays it is generally accepted that homocysteine promotes thrombosis with simultaneously vasodilatation inhibition. It is considered that homocysteine triggers its effects by three distinct mechanisms which can possibly integrate and are not mutually exclusive; oxidative processes, decreasing NO• bioavailability and specific protein targeting (figure 21).

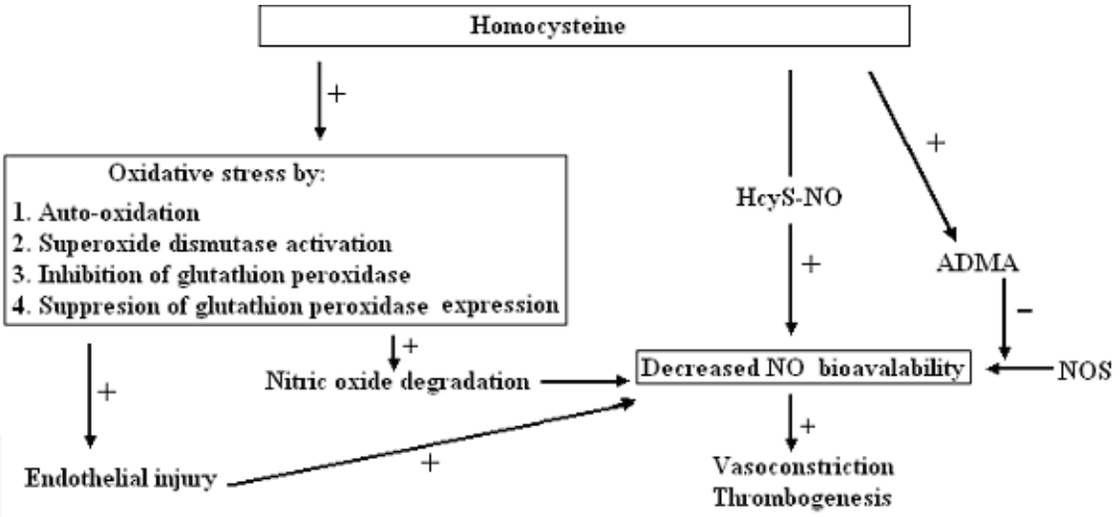

Figure 21. Homocysteine adverse effects. (+/— represent activation/inhibition processes)

Even the precise path was not yet established, it seems that Hcys inhibits some "good" factors and activates same "bad" factors that finally influence the processes of thrombosis-fibrinolysis and constriction–vasorelaxation and which are summarized in (figure 21) [56].

Amongst "good" factor can be included: NO•, GPx, eNOS, protein C, tissue Plasminogen Activator (tPA), annexin II. Amongst "bad" factor can be included: ADMA, $O_2^{\bullet-}$, H_2O_2

Homocysteine directly and indirectly influences erythrocytes metabolism. It directly affects the erythrocyte antioxidant enzyme systems promoting free radical generation. Indirectly Hcy decreases NO• bioavailability and modifies the environment where erythrocytes move and act.

2.3.1. Homocysteine pro-oxidative activity

Homocysteine is involved in reducing–oxidative processes by reacting either with itself or with different compounds. In other words homocysteine can submit auto-oxidative as well as oxidative processes.

Thiols (RSH) can auto-oxidize in the presence of transition metal catalysts and molecular oxygen, leading to the formation of reactive oxygen species (ROS). Hcy like all containing thiol group undergoes oxidation to disulfide (RS-SR) in O_2 presence at normal pH. It was found that cooper catalyses Hcy (noted with general formula RSH) auto-oxidation, even in low homocysteine concentration, yielding hydrogen peroxide and thus promoting ROS generation in both extra and intracellular compartments through reaction proposed by Starkebaum in [57]:

$$2\ \text{Homocysteine (RSH)} + O_2 \rightarrow \text{Homocystine (RS-SR)}$$

$$\text{Homocystine (RS-SR)} + \text{superoxid } (O_2^{\bullet -}) \rightarrow H_2O_2$$

Hydrogen peroxide generated by the copper catalysed auto-oxidation of homocysteine was involved in the mechanism of toxicity by the demonstration of the reduction in endothelial damage with the addition of catalase in [58].

Homocysteine was proved to generate superoxide radicals which promote vasoconstriction. Lang et al. demonstrates in [59] that the inhibitory effect of homocysteine on endothelium-dependent relaxation is caused by an increase of the intracellular levels of $O_2^{\bullet -}$ in the endothelial cell and provide a possible mechanism for the endothelial dysfunction associated with hyperhomocysteinemia.

Cysteine is also a thiol circulating aminoacid related to homocysteine and its concentration is 20 to 30 times higher than Hcys one. In fact Cys is the main circulatory thiol but there was found no correlation of Cys with free radicals generation. Instead a strong association of hyperhomocysteinemia with F_2-isoprostane was found. F_2-isoprostane is an indicator of in vivo lipid-peroxidation and its association with Hcy lead to the conclusion that this amino acid is involved in free radicals generation in [60] thus pointing Hcy as pro-oxidative agent.

Hcy involvement in ROS generation was also indirectly proved in connection with antioxidant enzyme system modulation SOD and GPx.

The activity of superoxide dismutase, an important antioxidant enzyme in vascular tissue, was measured along with homocysteine in homocystinuric patients and found to be positively associated with homocysteine levels. This strong relationship can be regarded as a protective antioxidant response to homocysteine-induced oxidative action and as indirect evidence that Hcy represents a source of free radicals in [61]. In our study we found an increased superoxide dismutase activity in red blood cells lysate in experimental induced hyperhocysteinemia in rats. We consider this increased response in enzyme activity as evidence for free radicals' production in [62].

Homocysteine may affect glutathione peroxidase activity, thus altering the microenvironment in the propagation of ROS in [63]. Our study on GPx activity, in installed hyperhomocysteinemia, was consistent with these reported data. GPx activity in red blood cells lysate significantly decreases as a consequence of experimental induced hypehocysteinemia in rats. We considered that increased amount of free radicals consume the GSH enzyme cofactor which subsequently trigger the enzyme activity decay in [62]. As a consequence GPx activity is lowered in hyperhomocysteinemia thus disturbing the detoxification process of H_2O_2 within cell.

Upchurch in[63] demonstrates that homocysteine reduces mRNA levels of glutathione peroxidase, indicating that the expression of this enzyme is inhibited and/or down-regulated.

Even it was attributed to different causes such as: a decrease in enzyme activity, a down regulation from high homocysteine levels or an inappropriate gene expression of GPx, the decrease in GPx activity in Hcys presence is generally reported.

Homocysteine-induced oxidative stress was proved to be generated within vascular cells in [64]. Our data show that in installed hyperhomocysteinemia the intracellular space is more affected than the extracellular, circulatory one. We found significant changes in antioxidant enzyme systems within erythrocyte (we worked on erythrocyte lysate) as compared with total antioxidant capacity (TAC) in plasma in [62].

As a conclusion hyperhomocysteinemia by promoting free radical generation affects both erythrocytes and endothelial cells as well in [65,66].

2.3.2. Homocysteine decreases NO bioavailability

The second hypothesis considers that Hcy acts to prevent NO• bioavailability. This process is considered to have, at least partially, the same oxidative basis. In living organisms, including in human, endothelial-derived nitric oxide performs the following function: regulates vessel tone by promoting vasodilatation, inhibits platelet activation, adhesion and aggregation, limits smooth muscle proliferation and modulates endothelial–leukocyte interactions in [56]. Homoysteine was proved to limits NO bioavailability thus promoting the contrary processes: vasoconstriction, thrombosis and fibrinolysis inhibition.

There are proposed many patterns for homocysteine impairing NO• bioavailability (figure 22).

A first process that limits NO• bioavailability seems to be more a protecting mechanism than a harmful one. Homocysteine reacts with nitric oxide to form S-nitroso-homocysteine, which has some of the properties of nitric oxide. It markedly inhibits platelet aggregation, is a potent vasodilator and does not support hydrogen peroxide generation. This represents much more a protective mechanism against the adverse effects of homocysteine than a limiting process in NO• bioavailability in [56].

However prolonged exposure to high homocysteine concentrations impairs nitric oxide production. Thus in hyperhomocysteinemia the limited bioavailability of nitric oxide could be due to S-nitrosothiol formation in [67].

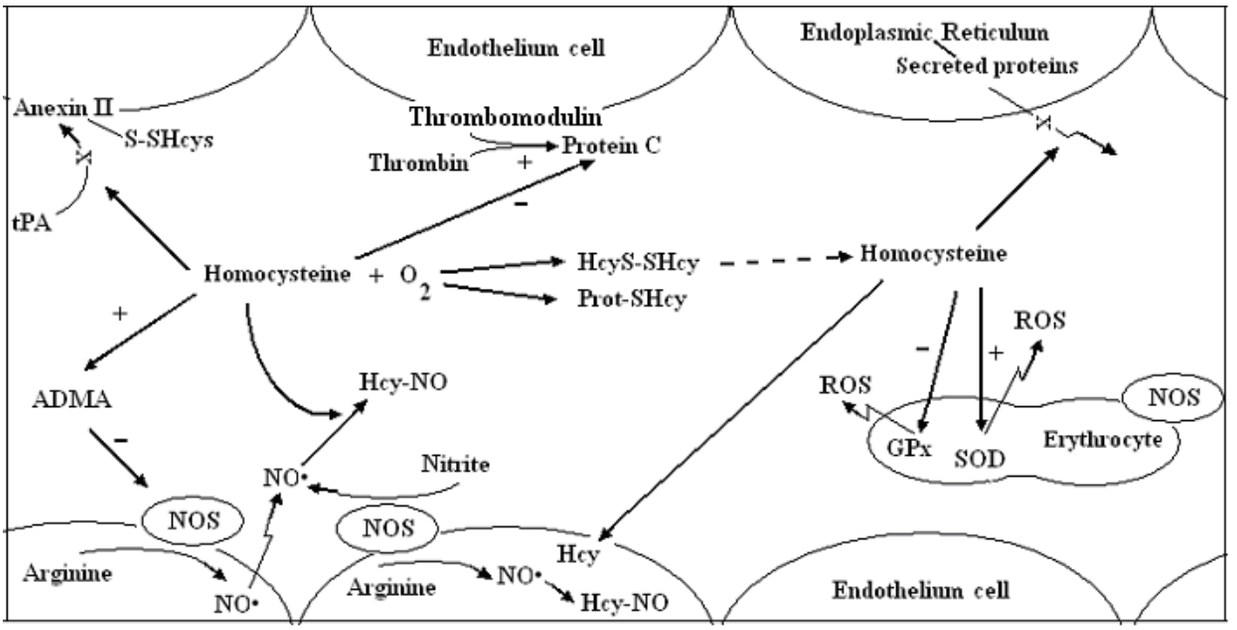

Figure 22. Proposed mechanism through which Hcy inhibits some "good" factors and activates same "bad" factors thus influencing thrombosis-fibrinolysis respectively constriction–vasorelaxation processes. tPA, ADMA represents tissue plasminogen activator/respectively asymmetrical dimethyl arginine.

A second process that limits NO• bioavailability is nitric oxide trapping/degradation by other radical species. NO• is trapped by superoxide to form peroxinitrite thus being inactivated. This mechanism was confirmed by many experimental data in [63, 68].

Nitric oxide can be alternatively degraded by hydrogen peroxide as a consequence of GPx activity inhibition through Hcy-dependent mechanism. Homocysteine seems to be the only amino acid amongst all circulating others capable to inhibit glutathione peroxidase activity in vitro. Cysteine is also capable of generating free radicals and is present in serum at concentrations four times higher than homocysteine but cysteine doesn't prove inhibiting properties on GPx activity. Experimental data show that Hcy inhibits GPx activity and also suppresses the cellular GPx expression thus promoting the increase of hydrogen peroxide concentration in [64]. Hydrogen peroxide promotes in its turn free radicals generation and peroxinitrite production thus decreasing NO• availability.

A third mechanism that limits NO• bioavailability is the decrease in NO• synthesis through Hcy-dependent asymmetrical dimethylarginine (ADMA) generation. ADMA is produced by methylation of specific arginine residues of certain cellular proteins. Most of these proteins are found in the nucleus. When the proteins are degraded, ADMA and other isomers are released to the extracellular space where especially ADMA acts as potent endogenous inhibitors of NOS enzyme in [69].

Methionine loading was proved to induce hyperhomocysteinemia. In methionine loading there is S-adenosylmethionine accumulation and as a consequence a high proteins methylation. Asymmetrical dimethylarginine (ADMA) the product of degradation from methylated proteins competitively inhibits NO-synthetase activity. Elevated ADMA levels found in hyperhomocysteinemia are supposed to inhibit NO• synthesis thus decreasing the NO availability in [70].

2.3.3. Homocysteine own action

A third way that homocysteine acts is targeting specific proteins which are located within cell, on cell membrane or in the extracellular space.

Two important intracellular proteins, already mentioned, targeted by Hcy are the antioxidant enzyme GPx which activity decreases and SOD which activity increases in homocysteine presence (fig22). Hcy alters other intracellular proteins disturbing the redox potential of endoplasmic reticulum and Golgi apparatus thus inhibiting the surface expression and secretion of proteins in [71,72].

Hcy targets proteins located on both membrane surface and within cell. Jacobsen considers that circulatory oxidized form of Hcy enters the cell were it is converted back to reduced Hcy, in reducing environment within cell. Under reduced form Hcy impairs the binding of tissue plasminogen activator (tPA), a protein involved in the breakdown of blood clots, to annexin II in [71] by forming a disulfide bridge with Cys9 on annexin II. Thus Hcy limits the plasminogen conversion to plasmin. This results in a decreased fibrinolysis. The circulating reduced Hcy acts in the same manner with annexin II on the membrane from the vascular endothelium. Homocysteine was found to be the only circulating thiol that impairs the binding of tissue-plasminogen activator.

The atherogenic factor lipoprotein (a) [Lp (a)] competitively inhibits the binding of plasminogen to fibrin. Fibrin is a cofactor for plasminogen activation to plasmin, an important enzyme that degrades fibrin clot. Homocysteine was found to interfere in this process. Hcy and lipoprotein (a) seems to act in the same direction: homocysteine promotes lipoprotein(a) binding to fibrin and lipoprotein(a) competitively inhibits the binding of plasminogen to fibrin. The final effect is the decrease of fibrinolysis. The combination of Lp (a) plus homocysteine is a possible mechanisms for the occurrence of thrombosis in hyperhomocysteinemia in [73].

Protein C is an example of circulating proteins whose activity is inhibited by Hcy. The protein C enzyme system appears to be one of the most important anticoagulant pathways in the blood. Its activation depends on the complex thrombomodulin-trombin. Thrombomodulin is an integral membrane protein expressed on the surface of endothelial cells where it serves as a receptor for thrombin. The complex thrombomodulin-trombin activates protein C thus raising its activity. Homocysteine inhibits the function of thrombomodulin. Both thrombomodulin and protein C contain disulfide-rich domains. Reduction of these disulfide bonds by homocysteine may disrupt important structures

within these domains, resulting in impaired function in [74]. The result is the promotion of thrombotic process.

Hcy acts on both endothelial cells and smooth muscle where it generates contrasting effect. On endothelium it promotes injury and impairs DNA repair, in smooth muscle Hcy stimulates proliferation in [69]. Md S. Jamaluddin considers that Hcys promotes vascular injury through hypomethylation. When Hcys accumulates it uses adenosine, a normal constituent of all cells, to form S-adenosyl-homocysteine (SAH) a potent inhibitor of cellular methylation. By impairing methylation Hcy arrests cell growth, increases cellular SAH concentration in endothelial cells (EC) and decreased DNA synthesis thus decreasing cellular repair. This chain of events was not found in vascular smooth muscle cells in [75].

Erythrocytes are also affected by homocysteine-induced hypometilation. High intracellular SAH impairs the posttranslational methylation of membrane proteins. Reduction in membrane protein methylation was particularly observed for erythrocyte cytoskeletal component ankyrin, which is known to be involved in membrane stability and integrity. Because of hypomethylation, structural damages accumulate in erythrocyte membrane proteins, and are not adequately repaired thus affecting membrane physical properties. Erythrocyte deformability is a crucial properties for circulatory function in [76].

As a conclusion the effect of elevated homocysteine appears multifactorial affecting both the vascular wall structure and the blood coagulation system as well as erythrocytes metabolism in [77].

3. The pharmacological influences on the blood cell metabolism – Antioxidant drugs in cardiovascular risk status and roll of red blood cell antioxidant defense capacity

There are growing evidences on the role of adaptive mechanisms of erythrocyte in pathological processes: atherosclerosis, ischemic attack, bacterial infections, etc. All of this processes involve as main mechanism oxidative stress. Erythrocytes have an intracellular enzyme and non-enzyme defense system. In order to remove reactive species of oxygen, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase act together. Glutathione (GSH) participates as a co-substrate for GPx in order to detoxify H_2O_2 generated by SOD enzyme. GSH is a critical tripeptide that oxidizes to glutathione disulphide (GS-SG) inactive form after reacting with oxygen radicals. GSH proves to be essential for reactive species detoxification as a consequence it is permanently restore in its reduced active form by glutathione reductase based on nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) from Glucose-6-phosphate dehydrogenase (G6P-DH) catalysed reaction in pentose phosphate pathway. When reactive species of oxygen are quickly and intensely generated under external or internal stimulus the activity of SOD, GPx and GSH concentration are severely changed.

When erythrocytes are undergo shear stress in constricted vessels, they release ATP which causes the vessel walls to relax and dilate so as to promote normal blood flow [78].

Also, when their hemoglobin molecules are deoxygenated, erythrocytes release **S-**nitrosothiols which acts to dilate vessels, thus directing more blood to areas of the body depleted of oxygen in [35].

Using L-arginine as substrate, erythrocytes can also synthesize nitric oxide enzymatically, just like endothelial cells. The nitric oxide synthase is activated when the erythrocytes are exposure to physiological levels of shear stress, thus, nitric oxide is synthesized, exported and it may contribute to the regulation of vascular tonus [79].

Another mechanism that involves the erythrocytes in relaxing vessel walls is the production of hydrogen sulfide. It works as a signaling gas. It is believed that the cardioprotective effects of garlic are due to erythrocytes converting its sulfur compounds into hydrogen sulfide. [80]

The free radicals released by erythrocytes when they are lysed by pathogens break down the pathogen's cell wall and cell membrane, and so, they are killing them. This represents the involving of erythrocytes in the body's immune response in [81].

On the other hand, as response of injury after several stressors, including oxidative stress, energy depletion, as well as a wide variety of endogenous mediators and xenobiotics, the erythrocytes can initiate the self suicidal death (eryptosis). Eryptosis is characterized by cell shrinkage, membrane blebbing, activation of proteases, and phosphatidylserine exposure at the outer membrane leaflet. This can make the macrophages to recognized and engulf erythrocyte to be degraded. Eryptosis can be considered a mechanism of defective erythrocytes to escape hemolysis. Conversely, excessive eryptosis favors the development of anemia. Conditions with excessive eryptosis include iron deficiency, lead or mercury intoxication, sickle cell anemia, thalassemia, glucose 6- phosphate dehydrogenase deficiency, malaria, and infection with hemolysis-forming pathogens. Inhibitors of eryptosis include erythropoietin, nitric oxide, catecholamine and high concentrations of urea in [82, 83]

The red blood cell SOD activity has been found to be useful in evaluating the biochemical index of copper, zinc and manganese nutrition. The largest amount of SOD enzyme is found in liver and erythrocytes. There are two forms of SOD in human tissue. One form is present in cytosol and it is a protein containing two atoms each of copper and zinc. The other form is a much larger molecule containing four atoms of manganese and it is found in mitochondria and cytosol. Significant changes in cellular concentration of copper, manganese and zinc have the potential of altering the antioxidant activity of SOD. On the other hands, the correlation between of copper and zinc plasma level, the oxidase activity of ceruloplasmin in serum, and Cu,Zn-SOD activity in erythrocytes can be a way to investigate involvement of oxidative stress in pathological conditions, as atherosclerosis obliterans [84]

Another element involved in the function of necessary enzyme for cellular protection is selenium. Selenium functions primarily as an activator of enzymes necessary for cellular protection from oxidative damage and maintenance of normal redox potentials. A primary role of selenium in erythrocytes appears to be the activation of the enzyme glutathione peroxidase whereby glutathione (the critical tripeptide antioxidant/antitoxin for all cells) reacts with oxygen radicals. Importantly, selenium catalyzes glutathione reductase, an enzyme that maintains the glutathione in its reduced or active form [85].

Specify participation of erythrocyte enzymatic system as adaptive mechanism to different pathological processes and specify how nutritional deficiencies and oxidative drugs can interfere these systems introduces the chapter on pharmacology of erythrocyte antioxidant system.

3.1. Antioxidant drugs in cardiovascular risk status and roll of red blood cell antioxidant defense capacity

3.1.1. Probucol

Probucol has modest lipid-lowering properties. It was used for the treatment of hypercholesterolemia until more tolerable and effective cholesterol-lowering treatments, such as the HMG Co-A reductase inhibitors, or "statins," became available. Probucol lowers the level of cholesterol in the bloodstream by increasing the rate of LDL catabolism. Additionally, probucol may inhibit cholesterol synthesis and delay cholesterol absorption in [86]. Another possible mechanism of action of probucol is inhibition of ABCA1-mediated cholesterol efflux without influencing scavenger receptor class B type I–mediated efflux (ABCA1 = ATP-binding cassette transporter - member 1 of human transporter sub-family ABCA, also known as the cholesterol efflux regulatory protein is a protein which in humans is encoded by the ABCA1 gene). The inhibition of ABCA1 translocation to the plasma membrane may in part explain the reported in vivo high-density lipoprotein–lowering action of probucol in [89].

Probucol is a powerful antioxidant which inhibits the oxidation of cholesterol in LDLs; this slows the formation of foam cells, which contribute to atherosclerotic plaques.

The major mechanism by which probucol lowers LDL levels relates not to changes in the cellular mechanisms for LDL uptake or to changes in LDL production but rather to intrinsic changes in the structure and metabolism of the plasma LDL in [87]. It has been postulated that the oxidative modification of LDL might contribute to atherogenesis by facilitating lipid accumulation in macrophages (foam cells) and by inhibiting macrophage motility. LDL resists oxidative modification, however, when probucol is added to in vitro incubations or when the LDL itself is isolated from probucol-treated patients in [88]. Under the treatment with probucol xanthomatous lesions disappear which that suggest a facilitation of cholesterol transferred from tissues to the excretion or catabolic pathways. Compared with other hipolipemiants, probucol is a non hepatotoxic drug and induces a decrease of lithogenic index of bile.

In recent studies was shown that probucol protect against diabetes-associated and adriamycin-induced cardiomyopathy by enhancing the endogenous antioxidant system including glutathione peroxidase, catalase and superoxide dismutase [90].

3.1.2. The HMG Co-A reductase inhibitors, or "statins"

Specific for hypercholesterolemia status is the high production of free oxygen radicals. These can impair the endothelial function because destroying of nitric oxide (NO) and secondary affecting its beneficial and protective effects on the vessel wall. Most of the other cholesterol-lowering therapies present, also, antioxidant effects. There are two way improving antioxidant defence system in hypercolesterolemiant patients: either increasing the activities of CuZn-SOD and GSH-Px or preventing the production of the superoxide radicals.

Malone dialdehyde (MDA), more than cholesterol plasma level, is considered a marker of patients with increased risk of coronary heart disease, because MDA is a marker of lipid peroxidation. In individuals who smoke or who have diabetes are particularly prone to oxidative stress that can lead to the formation of oxidized LDL (oxLDL). Oxidatively modified LDL is considered to be highly atherogenic and can be considered a biochemical risk marker for coronary heart disease. Oxidative modification of LDL increases their ability to bind to the extracellular matrix, increasing its retention within the intima and accumulation of oxLDL in macrophages, so, it contributes to the formation of an atherosclerotic lesion.

The oxLDL accumulation within macrophages promotes the chemotaxis of monocytes into the vessel wall and initiates the various pro-inflammatory effects by different scavenger receptor pathways: CD36 class B scavenger receptors from human macrophages (activates nuclear factor kB that regulates the expression of many pro-inflammatory genes), class A scavenger receptors (modify macrophage activation), lectin-like oxidized LDL receptor - LOX-1 (the expression of endothelial cell adhesion molecule). On the other hands, the accumulation of inflammatory cells can further increase the levels of oxidative stress. Oxidative stress inactivates nitric oxide (NO) and inhibits its synthesis by endothelial nitric oxide synthase (eNOS). On this way, the vasoprotectant effect of NO (anti-inflammatory, anti-platelets, antioxidant and vasodilator) is affected [92].

Statins inhibit 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase the rate-limiting enzyme in the mevalonate pathway through which cells synthesizes cholesterol. On this way, the "statins" increase the resistance of LDL to oxidation. Statins may also exert effects beyond cholesterol lowering. These “pleiotropic” vascular effects of statins are involved in restoring or improving endothelial function: by increasing the bioavailability of nitric oxide, promoting reendothelialization, reducing oxidative stress, and inhibiting inflammatory responses.

Other effects of statins that explain their involving in preserving normal vascular function and blood flow are: inhibition of the uptake and generation of Ox-LDL, decreasing the

vascular and endothelial superoxide anion formation by inhibition of NADH oxidases via Rho-dependent mechanisms and preserving the relative levels of vitamin E, vitamin C and endogenous antioxidants (such as, ubiquinone and glutathione) in LDL particles. All these mechanisms explain a dual action of statins on oxidative stress, not only decreasing oxidants but also restoring antioxidants [92]. Statins reduce both extracellular LDL oxidation (by reducing substrate availability) and intracellular oxidative stress (by cholesterol-independent effects on NO and, indirectly, by reducing Ox-LDL) [91].

Statins themselves may be able to reduce levels of superoxide radicals, an effect that can only partially be explained by a reduction in LDL cholesterol. Rosuvastatin has been reported to reduce markers of oxidative stress in ApoE (−/−) mice [93] while fluvastatin treatment has been shown to decrease superoxide radical generation and to reduce the susceptibility of LDL to oxidation in cholesterol-fed rabbits [95, 96].

Atorvastatin has been demonstrated to inhibit angiotensin II-induced superoxide formation by NADPH oxidase in isolated rat vascular smooth muscle cells [96] and in rats in vivo [97]. In addition, statins have been shown to reduce NADPH-dependent superoxide formation by a monocyte-derived cell line in culture [98].

Another beneficial effect of statins is potentiation the synthesis of tetrahydrobiopterin, which may prevent the uncoupling of eNOS and shift the balance away from NOS-generated superoxide production to the generation of NO [99]. Statins may also be influence the endogenous antioxidants other than NO. Atorvastatin has been shown to increase paraoxonase activity and reduce the enhanced cellular uptake of oxLDL of monocytes differentiating into macrophages [100]. Long-term treatment with HMG-CoA reductase inhibitors (statins) appears to upregulate the expression and the activity of the vascular endothelial NO synthase (eNOS) pathway and increases nitric oxide availability, resulting in not only a downregulation of oxidative enzymes but also a direct scavenging of superoxide anion. As oxygen radical production is increased in various clinical settings such as hypercholesterolaemia, diabetes and hypertension, this statin-induced eNOS upregulation may play a foremost role in the vascular protective effects of these drugs. [119]. Moreover, sustained nitroglycerin (NTG) treatment is associated with an increased bioavailability of superoxide anion, likely playing a major role in the development of nitrate tolerance. The triggering events leading to this redox imbalance remain controversial as several cellular enzyme systems have been shown to be impaired by sustained in vivo exposure to NTG, including membrane bound oxidases in [121] endothelial NOS in [122] and arginine transporters [123].

Other effects than hipocholesterolemic of statins was described. Lovastatin or simvastatin has been shown to have anti-inflammatory properties. They reduce monocyte adhesion to endothelial cells, cytokine expression and MCP-1 production [101-103]. By limiting the influx of inflammatory cells statins may reduce the release of superoxide radicals and the oxidative modification of LDL. On this way statins increases the resistance of LDL to oxidation. Macrophage growth stimulated by oxLDL can also be inhibited by statins [92]

3.1.3. Fenofibrate

Very few data concerning the fibrates are available. In hypercholesterolemic patients, it has been shown that bezafibrate is more active than pravastatin in reducing the susceptibility of LDL oxidation [104]. Moreover, in diabetics, De Leeuw and Van Gaal have found that fenofibrate, but not pravastatin or simvastatin, can reduce the oxidizibility of LDL and of VLDL [105].

3.1.4. Beta-adrenergic blockers

Beta adrenergic blocking agents have also been shown to have beneficial effect on atherosclerosis. Several mechanisms of action have been suggested including an antioxidant action. All β-blockers have in vitro antioxidant activity which appears to be related to their degree of lipophilicity. In patients with CHD, Croft and coworkers showed that, while the lag time in patients with CHD is not significantly different from controls, in patients with CHD who are taking β-blockers, the lag time is higher than that observed in patients who are not taking β-blockers in [106]. When LDL are oxidized in vitro by copper or by macrophages, carvedilol, the most lipophilic β -blocker appears more potent than pindolol, labetolol, atenolol and propranolol and this is confirmed in vivo [107].

3.1.5. Angiotensin-converting enzyme (ACE) inhibitors

ACE inhibitors have been shown to have a beneficial effect in atherosclerosis. They reduce the progression of the disease in animals. These beneficial effects of ACE inhibitors have been related to an antioxidant activity against LDL oxidation that has been demonstrated. In vitro, the lag time was found to be clearly increased by the presence of captopril at concentrations close to those that can be achieved therapeutically with large doses. A similar effect is observed with *N*-acetylcysteine which contains like captopril, a sulfhydryl group. Quinapril, which lacks the sulfhydryl group, had no antioxidant activity [108]. In vivo, Aviram and coworkers have shown that the propensity of LDL to oxidation is increased in patients with hypertension and is positively correlated with the blood pressure. Giving captopril or enalapril for 3 weeks decreases the oxidizibility of LDL. That suggests that the sulfhydryl group, which is absent in enalapril, does not have any influence on the resistance of LDL oxidation [109]. Actually, the same group gave data suggesting that the antioxidant activity might be related to the decreased production of angiotensin-II (A-II) as A-II appears to increase the LDL oxidation by macrophages [110].

3.1.6. Calcium channel blocker

All calcium channel blocker are potent antioxidants in vitro and this property is probably related to their interaction with the lipid bilayer of the membranes. Lacidipine has the highest degree of interaction with the membrane Lacidipine inhibits the LDL oxidation produced by several oxidants. [111].

3.1.7. Metabolic medication - Trimetazidine

Trimetazidine (TMZ) is the first in a new class of metabolic agents, available for clinical use. In conditions of hypoxia or induced ischemia, TMZ maintains homeostasis and cellular functions by selectively inhibiting 3-ketoacyl-CoA-thiolase [112]. As a consequence, fatty acid b-oxidation is reduced and glucose oxidation is stimulated, resulting in decreased cellular acidosis and higher ATP production [113, 114]. In humans, TMZ has been shown to increase the ischaemic threshold and to relieve angina pectoris in patients with coronary artery disease. These benefits have been observed without any change in heart rate, blood pressure, and rate-pressure product at rest, during submaximal and peak exercise in [115,116]. There is also demonstration that TMZ has antioxidant properties. During acute and chronic ischemia, TMZ reduces the loss of intracellular K+ induced by oxygen free radicals and also the membrane content of peroxidated lipids [117]. In vivo, pre-treatment with TMZ (40–60 mg per day for 7 days) significantly decreases membrane malondialdehyde (MDA) content of red blood cells incubated with superoxide dismutase inhibitor diethyldithiocarbamate [118]. In humans, plasma levels of MDA were decreased after pre-treatment with TMZ during coronary artery bypass surgery [118].

4. Instead of conclusion

Mechanism of action of homocysteine is far from being elucidated. The big number of studies on this subject was gathered a lot of evidences about the role of Hcy as a major cardiovascular risk factor. All studied diseases: nephropathies, neurodegenerative illnesses, osteoporosis, atherosclerosis seems to be tributary to this homocysteine effect. It is widely accepted that involvement of homocysteine in the pathogenesis of these diseases activates prooxidative mechanisms. Therefore, the initiation of therapy of drug with antioxidant properties in such pathologies is justified. Moreover, there is clinical evidence to support this point of view. Thus, although the clinicians question the value of trimetazidine in the treatment of myocardial ischemia or degenerative deafness. [124-128] there are the clinical trials and basic research that support the benefits of this antioxidant metabolic medication. Scientific arguments exist regarding the use of atorvastatin [129, 130] or nimodipine [131] therapy for antiischemic effects and prevention of vascular events.

Author details

Filip Cristiana and Zamosteanu Nina
Dept. Biochemistry. Univ.Med. Pharm. "Gr.T.Popa", Iasi, Romania

Albu Elena
Dept. Pharmacology. Univ.Med. Pharm. "Gr.T.Popa", Iasi, Romania

5. Acknowledgement

Our research study to which we referred at the references was supported by grant PNCDII Idei 1225/2007 sustained by Ministry of Education and Research National Authority for Scientific Research UEFISCSU

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