Chapter 3

Is Homocysteine a Marker or a Risk Factor: A Question Still Waits for an Answer

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http://dx.doi.org/10.5772/intechopen.81799

Abstract

Homocysteine, a non-proteinogenic sulfur-containing amino acid, was discovered in 1932, and 30 years passed until, in 1969, for the first time, its involvement in pathology was reported. It was only in the last two decades that homocysteine has become a subject of scientific interest and has begun to be intensively studied. A large number of scientists consider homocysteine as an independent risk factor particularly for cardiovascular disease, while others indicate homocysteine as a marker of this disease. Both sides bring scientific arguments for their opinions, yet the dilemma of homocysteine characterization still persists. Although the reported studies do not lead to a unique answer, it is generally accepted that homocysteine is associated with vascular dysfunction. Numerous scientific data show that the link between homocysteine and inflammation is achieved via the reactive oxygen species (ROS) pathway. The latest data indicate hydrogen peroxide as a possible messenger in cellular signaling in physiological or pathological processes and present the consequences of disturbing the oxidation-reducing balance. In this chapter, we present the latest scientific evidences gathered from the literature for both hypotheses regarding homocysteine involvement in pathology, and we propose a possible mechanism of action for homocysteine, based on our preliminary (yet unpublished) work.

Keywords: hyperhomocysteinemia, ROS, inflammation, cell signaling, protein-tyrosine phosphatases

1. Introduction

Homocysteine (Hcy) is a non-proteinogenic amino acid that is formed in the human body in methionine metabolism. Although not forming proteins, homocysteine participates in major processes such as transmethylation, cysteine (Cys) formation, transsulfuration, etc. In the transmethylation process, homocysteine is an intermediate that allows the formation of compounds with a major metabolic role such as adrenaline, lecithin, creatine, etc. Cysteine formation, via homocysteine, is a very important process because Cys is a vital amino acid to stabilize the spatial conformations of proteins, to form the most important antioxidant agent in the body named glutathione, or to detoxify harmful compounds.

Over the past 40 years, homocysteine has come to the clinicians' attention because its high levels in blood have been associated with high risk of mortality and morbidity in many illnesses, particularly cardiovascular diseases. Patients with high levels of Hcy, also called hyperhomocysteinemia (HHcy), develop thromboembolism, premature atherosclerosis, mental retardation, bone fragility, eyes disease, and even miscarriage.

It is obvious that Hcy is related to the pathological phenomenon but the way it intervenes has not yet been elucidated. Moreover, there are researchers who believe that homocysteine indicates an already altered state [1] while others consider it a factor triggering the alteration of some functions [2]. Both opinions are based on scientific arguments, and although the debate continues, most researchers agree that there is an unquestionable link between homocysteine and vascular endothelial dysfunction [3–5]. Endothelial dysfunction may have several causes, but the major cause is inflammation. Inflammation is the vital process by which organisms respond to aggression. In the inflammatory process, a large number of pathways are activated to remove aggression and restore homeostasis [6–8]. Complex structures such as cells, proteins, but also small molecules such as reactive species, that are capable of rapidly signaling changes in homeostasis, are involved in this process. The activities of these structures need to be coordinated, and the latest data indicates that the inflammasome is responsible for this task. Recent data have found links between Hcy activity and inflammation [9]. In this chapter we present these new data that connect Hcy, inflammation, cell signaling, and reactive species.

As a conclusion, current data indicates Hcy as an amino acid that certainly plays a role in pathology, a role that needs to be elucidated.

2. Homocysteine metabolism

A short presentation of the homocysteine metabolism indicates two major pathways of transformation: the transmethylation pathway and the transsulfuration pathway (**Figure 1**).

Transmethylation pathway converts Hcy to methionine through a chain of reaction that involve the participation of methylenetetrahydrofolate reductase (MTHFR), folic acid, vitamin B12, and methionine synthase (MS).

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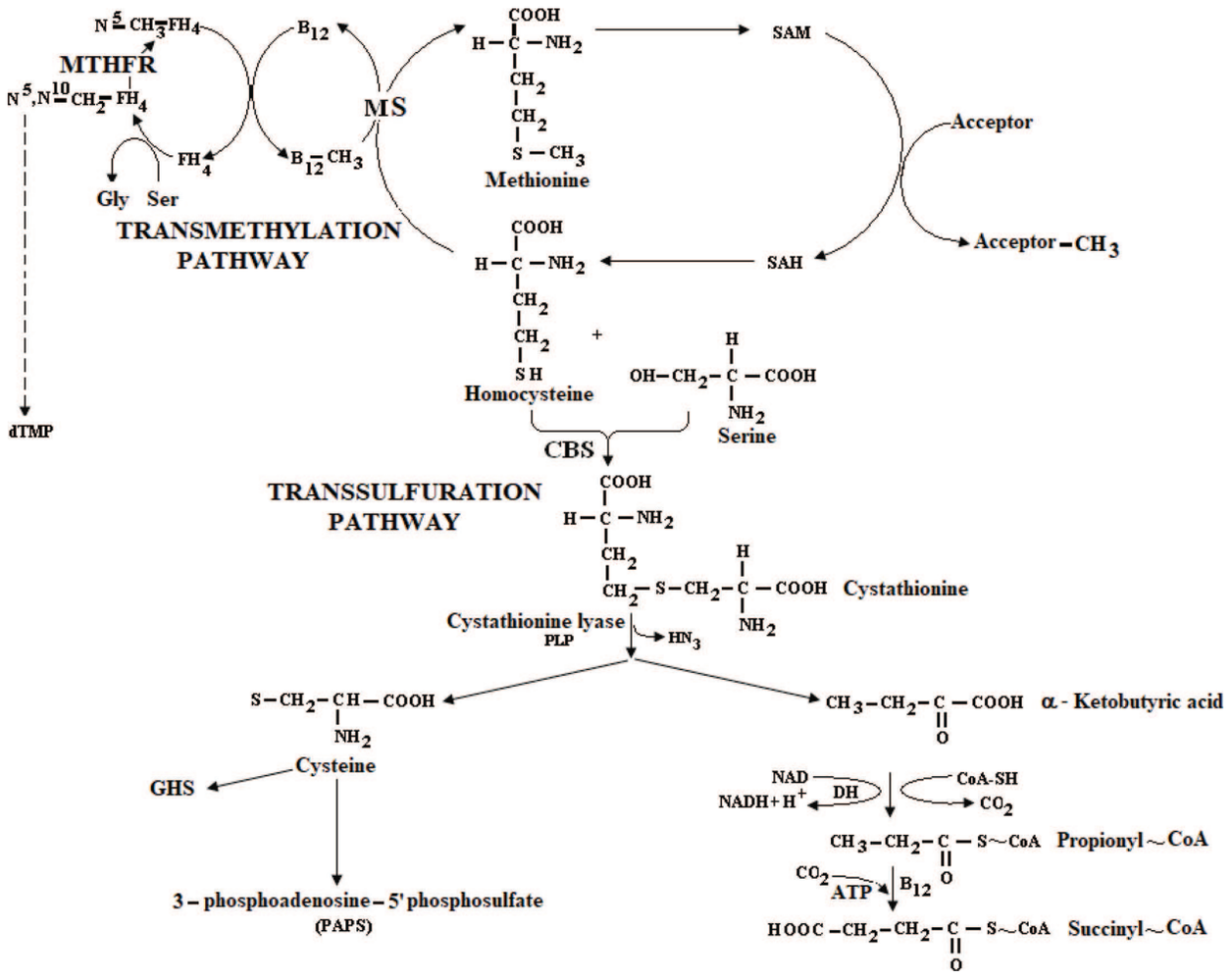

Figure 1. Main pathways of homocysteine transformation.

Transsulfuration pathway converts Hcy to cystathionine in the presence of the cystathionine beta-synthase (CBS) and vitamin B6. **Figure 1** highlights the role of tetrahydrofolate (FH4), the active form of folic acid, B12, and pyridoxal phosphate (PLP), the active form of vitamin B6 in the Hcy metabolism. A minor pathway, not shown in this figure, uses betaine to convert homocysteine to methionine.

The general methionine/homocysteine metabolism highlights the two major causes that generate HHcy: first, the enzymatic deficiencies of the enzymes acting in Hcy metabolization and, second, the nutritional deficiencies in vitamin cofactors. This last observation is the base of the therapeutic approaches that uses vitamin administration in order to decrease the homocysteine levels.

The normal concentration of homocysteine in human blood is 5–15 μM. HHcy is classified according to clinical consequences as being moderate at 16–30 μM, intermediary at 31–100 μM, and severe above 100 μM [10]. HHcy caused by the lack of vitamins is not commonly found in medical practice and it is easy to cure. The most common cause of HHcy is the enzymatic defect of different enzymes acting in this metabolism.

3. Homocysteine in pathology

3.1. Cardiovascular diseases

Currently, it is widely accepted that levels of Hcy, even at concentrations slightly higher than normal, are related to the risk of cardiovascular disease. Clinical studies indicate that a 5 μM increase in Hcy levels is equivalent to a 20 mg/dL increase in blood cholesterol [11, 12], which virtually doubles the cardiovascular risk. This suggests that between levels of Hcy and atherosclerosis there is a better correlation than between the cholesterol levels and atherosclerosis [13, 14]. However recent data [2, 15] show that a surprising 30% of cardiovascular mortality occurs in patients who do not present conventional risk factors as high LDL, hypertension, smoking, or obesity. This raises the question whether Hcy is an independent risk factor or it is a marker of a lesion process.

3.2. Diabetes

Hyperhomocysteinemia is considered a higher risk for patients with diabetes than nondiabetic patients. An exponential increase in vital risk has been demonstrated in patients presenting HHcy associated to diabetes [16–18]. The increase in Hcy levels noticed in diabetes is believed to be due to the degree of diabetes-induced nephropathy [19–21]. Thus, high levels of Hcy are found in kidney failure. This data suggest more for a marker role of homocysteine rather than a risk factor.

3.3. Neurological diseases

Seshadri [22] has shown that HHcy is associated with Alzheimer's disease and that it doubles the risk of developing the disease in patients with elevated levels of homocysteine as compared to those with normal levels. Although the mechanism that links Hcy to Alzheimer's is unknown, it is supposed that HHcy toxicity to neuronal cells is caused by possible neuronal damage following excessive stimulation caused as result of chronic central nervous system ischemia [23–25].

3.4. Bone fragility

Increased levels of homocysteine were correlated with increased risk of bone fractures in the elderly [26–31]. It seems that Hcy does not affect bone density but rather affects the structure of collagen by interfering in the transversal linkages between the collagen fibers. Thus, Hcy intervenes in tissue fortification showing more a risk factor role.

3.5. Miscarriage

Research studies notify that HHcy can be generated by the specific mutation in MTHFR. This inherited deficiency lead to a 3.3-fold increase in the risk of miscarriage in a sample group of 185 Caucasian women [32, 33]. Literature also specifies that associations between MTHFR C667T mutations to factor V Leiden and prothrombin gene mutations were identified in patients having recurrent miscarriages [34].

4. Homocysteine involvement in the endothelial function

The presented data show that in high concentration Hcy certainly plays a role in pathology. A large number of recent studies indicate that Hcy is an independent risk factor in cardiovascular disease [2, 35]. However, other studies indicate Hcy as a marker of this disease [1]. Although the reported studies do not lead to a unique answer regarding homocysteine role, it is generally agreed that homocysteine is connected to the vascular dysfunction. As a consequence, the investigation of HHcy leads to the investigation of endothelial dysfunction. Normal endothelial function consists in maintaining the vascular relaxation and the anticoagulant status. Any aggression on the endothelial homeostasis leads to changes in vascular morphology, tonicity, coagulability, etc. The intensity and time span of aggression determine the transition from a normal to a pathogenic transformation.

4.1. Endothelial dysfunction

Vascular endothelium modulates vascular tonicity by secreting a large group of vasoactive molecules such as vasodilators (e.g., NO, prostacyclin) and vasoconstrictors (e.g., endothelin, thromboxane). The ratio of these compounds showing antagonist action dictates the final vascular tonicity, and under pathological conditions, additional stimulants (mediators of inflammation) cause severe changes in vascular behavior.

Nitric oxide (NO) a natural free radical is synthesized by nitric oxide synthases (NOS) from L-arginine by many types of cells including the endothelial cells. Nitric oxide that is synthesized by endothelial nitric oxide synthase (eNOS) promotes vasodilatation; inhibits platelet activation, adhesion, and aggregation; prevents smooth muscle proliferation; and modulates endothelial-leucocytes interaction [36]. Homocysteine diminishes NO bioavailability through various processes that are, at least partially, based on oxidative mechanisms. The current literature presents three mechanisms proposed to explain the decrease in NO bioavailability in the presence of elevated levels of Hcy. The first mechanism indicates that Hcy reacts with nitric oxide to form S-nitroso-homocysteine [36, 37]. The second one considers that NO bioavailability is blocked by sequestration following reactions with other radical species. NO is trapped by superoxide to form peroxynitrite, thus being inactivated [38, 39]. The third mechanism assumes that NO synthesis is decreased by NO-synthase inhibition by asymmetric dimethylarginine (ADMA), a potent inhibitor of the enzyme produced by the degradation of methylated proteins [40]. Increased ADMA concentration was identified in an HHcy status [41]. These mechanisms are found widely presented in our previous work [42].

Eicosanoids represent a group of compounds directly involved in vascular function. They act as paracrine hormones and mediate the inflammatory response. This group includes prostacyclin (PGI2), a compound with vasodilating activity, and thromboxane TXA2, a compound with vasoconstrictive activity. Prostacyclin or prostaglandin PGI2, produced by epithelial cells, prevents platelet aggregation, decreases proliferation of smooth muscle cells, decreases pro-inflammatory cytokines (↓IL-1 and IL6), and exerts antimitogenic activity (↓VEGF and TGF-β). On contrary TXA2 promote the thrombosis and vascular constriction. In the chain of reactions that generates eicosanoids, some are of the oxidative type so they generate reactive

species. Thus, the balance between these paracrine hormones is very important for the vascular homeostasis. Research data show that HHcy is considered as a factor that prevents vasodilation, promotes vasoconstriction, and increases the risk of thrombosis, thus inducing vascular injuries [43]. In vitro studies have demonstrated that HHcy induces the release of arachidonic acid, precursor of eicosanoids, including TXA2 [44].

Endothelins are vasoconstricting peptides mainly produced by the endothelium. They constrict blood vessel promoting high blood pressure. In addition to its vasoconstrictor effects, isoform endothelin-1 (ET-1) influences cell growth, thus being involved in atherosclerosis. Epithelial cells regulate ET-1 levels in response to hypoxia, oxidized species of LDL, or pro-inflammatory cytokines. Endothelins (ET-1, ET-2, ET-3) act on two receptors that have different locations and whose activation triggers different effects: vasoconstrictive effect through ET_A receptors located in smooth muscle cells [45] and vasodilation and NO release through ET_B receptors located on endothelial cells. Recent data show that HHcy results in the upregulation of ET_A receptor expression and high blood pressure in rats [46] while decreasing ET-1 production in endothelial cells, thus impairing NO and prostacyclin production and consequently the vasodilatation [47]. Thus, HHcy disturbs the ratio between vasodilators and vasoconstrictors promoting endothelial dysfunction [48].

5. Homocysteine mechanism of action

In the endothelial dysfunction, the inflammation process is a key step, and the reactive species are present at the site of inflammation, playing multiple roles, including defense, annihilation, or cellular signaling. In this chain of events, HHcy interferes somewhere with the endothelial normal function. There are several generally accepted mechanisms for Hcy-dependent endothelial dysfunction: *reactive oxygen species* [49], *inflammatory response* [50], or *thrombotic phenomenon* [51]. These mechanisms will be presented below along with scientific evidence for each of them.

5.1. Hyperhomocysteinemia involvement in oxidative stress

Numerous researches point ROS as the potential mediators for the effects of HHcy. Generation of reactive species is considered to trigger a cascade of events leading to release of pro-inflammatory cytokines, activation of adhesion molecules, generation of intracellular messengers that activate intracellular enzymes, and cellular responses including gene activation/repression [52–54]. Many studies demonstrate that HHcy generates reactive species directly or through autoxidation [55, 56]. ROS species found in HHcy was indirectly assessed through the measurement of antioxidative enzyme activity [57–59]. In our previous work, we have found that HHcy triggers the generation of hydrogen peroxide and that high levels of homocysteine experimentally induced (by methionine loading in rat) diminish more the total antioxidant capacity inside the erythrocytes rather than in plasma [60, 61].

5.2. Hyperhomocysteinemia involvement in inflammation

Recent studies [7, 8] had advanced the idea that Hcy triggers vascular damage by promoting an inflammatory response followed by immediate effects on the vascular wall or by delayed effects on proteins and DNA structures. The inflammatory phenomenon represents the vascular tissue response to lesion agents (chemical/physical or biological) [6]. The inflammatory response consists in two actions: removal of the lesion agent and initiation of the healing process. The acute inflammation predominates the local vascular response characterized by the presence of fast-acting and low half-life components (leucocytes). In the chronic inflammation, there is a progressive change in the types of cells present at the lesion site, characterized by the dominant presence of macrophages. The crucial phase is the destruction of pathogens. This phase takes place in monocytes/macrophages and neutrophils in the respiratory burst where the reactive oxygen species are generated. ROS are as damaging to pathogens as they are to the host's tissue. Consequently, chronic inflammation is accompanied by tissue destruction. Macrophages/neutrophils are not the site for respiratory burst only, but they also secret and/or trigger the secretion of specific compounds such as cytokines. The discovery of interleukins had introduced the concept of systemic inflammation. This type of inflammation is characterized by the fact that tissue destruction is not limited to a certain tissue but involves endothelium and other organs also. In systemic inflammation, elevated levels of chemical mediators such as interleukins (IL-6, IL-8, and TNFα) are associated with atherosclerosis and diabetes [62–64]. Recently, it has been found that HHcy is associated to inflammatory markers IL-6 and TNFα [65–68].

The cells of the innate immune system continually survey the extracellular environment in order to detect the "danger" signal. To achieve this function, immune cells develop receptors that act as sensors for the "invaders." Following the foreign detection, a group of actions must be initiated and coordinated, task being undertaken by the inflammasome. Inflammasomes are key signaling platforms that act as a checkpoint that controls and regulates the inflammatory response. It consists of multi-protein complexes that assemble by pattern-recognition receptors after the detection of a "danger "signal in the cytosol of the host cell. The protein association represents the activation stage of the inflammasome that triggers the signal of inflammation which is the caspase 1 and caspase 11 activation. Activated caspases initiate the highly pro-inflammatory cytokines' interleukin-1β (IL-1β) and IL-18 production, and finally an inflammatory form of cell death termed pyroptosis is triggered. The intracellular control of the inflammasome assembly is exerted via ion fluxes, free radicals, and autophagy. Latest data indicate the inflammasome activation as a possible mechanism for homocysteine involvement in inflammation and in programmed cell death in endothelial cells [69]. Current literature also demonstrates that the activation of inflammasomes (NLRP3 complex) represent a key step in HHcy-aggravated atherosclerosis [9].

5.3. Hyperhomocysteinemia involvement in thrombogenesis

HHcy promotes thrombosis by a mechanism that integrates the already presented processes of oxidation and decreases the NO bioavailability with the modification of some specific proteins acting in the coagulation and fibrinolysis pathway. Literatures show that homocysteine initiates structural modifications of these proteins, modifications that will impair their normal

functions. Such proteins include the tissue plasminogen activator (tPA), atherogenic factor lipoprotein(a) (Lp(a)), the complex thrombomodulin-thrombin, and DNA proteins.

The tPA is a serine protease that converts plasminogen to fibrinolytic protein plasmin. Hcy forms disulfide bridge with annexin II (an important receptor for tissue plasminogen activator in endothelium), thus blocking tPA binding to this protein. As a result, tPA activity is impaired, plasmin generation is diminished, and fibrinolysis activity is decreased [70].

Activation of plasminogen depends on the binding of fibrin as a cofactor. Lipoprotein (a) is an atherogenic lipoprotein which competitively binds to fibrin, thus preventing activation of plasminogen. Hcy favors lipoprotein-a binding to fibrin, which ultimately leads to decreased fibrinolysis [71]. HHcy added to a dyslipidemia profile results in increased risk of thrombosis.

Protein C is another serine protease present in blood as zymogen. Upon activation it exerts important role in anticoagulation, inflammation, and also cell death. The complex thrombomodulin-thrombin activates protein C, thus inhibiting the thrombotic process. Hcy impairs the complex thrombomodulin-thrombin activity by forming disulfide bridges with both thrombomodulin and protein C. As a consequence, the thrombotic process is promoted [72]. These mechanisms are found widely presented in our previous work [42].

5.4. Hyperhomocysteinemia involvement in cellular signaling

The survival of the cell is by default linked to its ability to remove any type of aggression/lesion and to restore the initial healthy structure. In this process, cells develop a network of systems that is capable to communicate, to mobilize defense/healing structures, or to memorize information about the type of aggression. In this process, complex structures and small molecules are equally involved, together being able to signal any changes in homeostasis. Reactive species of oxygen and nitrogen as well as active peptides (cytokines) produced at the site of inflammation by neutrophils or monocytes/macrophages are small molecules capable of rapid signaling. They promote vascular changes and open the inter-endothelial junctions thus allowing the migration of inflammatory cells across the endothelial barrier. All the activities related to inflammatory response are coordinated by chemical signaling through reactive species signals or active peptides (cytokine) [73].

The link between reactive species and inflammation is now well documented. On the other hand, current data associate Hcy with both inflammation and reactive species. The factor that puts together all these components is not fully elucidated. Over the past two decades, many scientific evidences show that ROS serve in physiological as well as pathological processes [74, 75]. Normal levels of reactive species act as signaling molecules to regulate biological and physiological processes, while their accumulation is strongly associated with oxidative stress [76]. Current scientific data indicate that among reactive oxygen species hydrogen peroxide is the most likely secondary messenger [77]. Early data had signaled that exogenously added H_2O_2 could mimic growth factor activity and that the growth factors could stimulate the endogenous production of H_2O_2 within cells. [78–80]. A major role in cell signaling that promotes cell proliferation, nutrient uptake, and cell survival is realized by the activation of the protein-tyrosine kinases class which includes both tyrosine kinases (Src, Ras, JAK2, Pyk2, PI3K) and mitogen-activated protein kinases (MAPK) (**Figure 2**).

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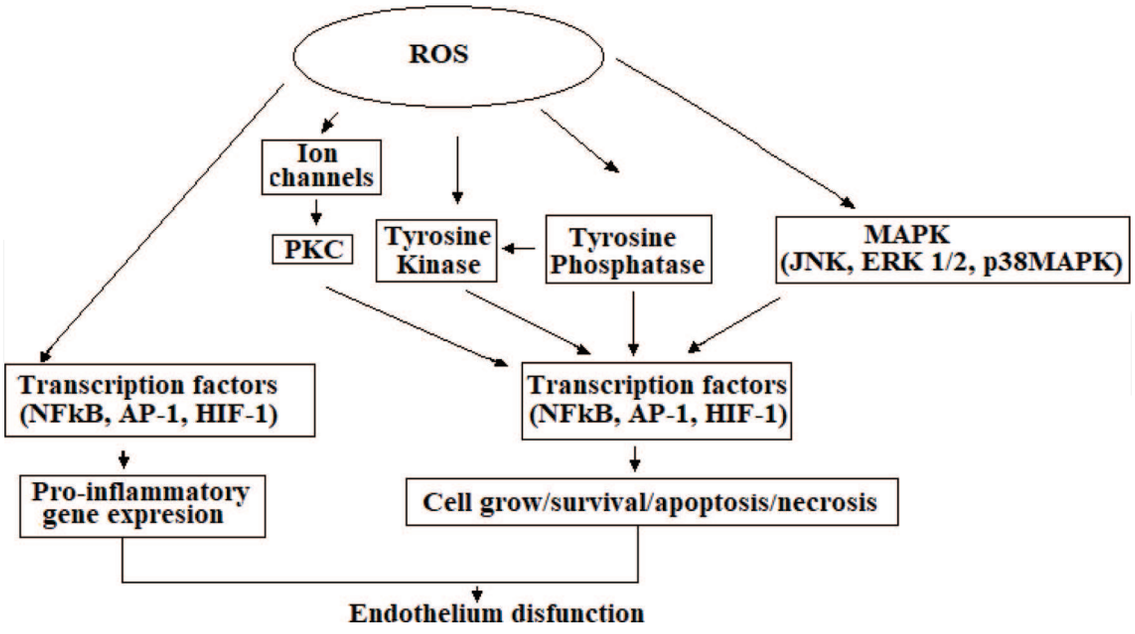

Figure 2. General signal pathway activated by ROS (modified from [81]), PKC = protein kinase, MAPK = mitogen-activated protein, JNK = c-Jun N-terminal kinases, ERK = extracellular signal-regulated kinases, NFκB = nuclear factor κB, AP-1 = activator protein-1, HIF-1 = hypoxia-inducible factor-1 C [42]. More details about the cellular response in ROS and other radical and nonradical species attack on oxidative events can be found in [82–84].

These signal transduction pathways use receptors with intrinsic tyrosine kinase activity (RTK) which leads to the phosphorylation of specific tyrosine residues located on tyrosine kinase proteins. Literatures show that hydrogen peroxide is required for optimal activation of protein-tyrosine kinases [85]. In the same time, hydrogen peroxide transiently inhibit protein-tyrosine phosphatases (PTPs) through the reversible oxidization of their catalytic cysteine [86], thus suppressing protein-tyrosine kinases dephosphorylation [87]. Thus, the activity of MAPK kinases is negatively regulated by protein-tyrosine phosphatases as depicted in **Figure 3**.

Protein-tyrosine phosphatases are specific proteins that contain cysteine residues at their active site. These enzymes remove a phosphate group attached to a tyrosine residue (such in MAP kinases), using a cysteinyl-phosphate enzyme intermediate. Latest literature data [88, 89] show that the activity of protein-tyrosine phosphatases is regulated by the reversible oxidation of cysteine residues. In the reversible oxidation, the PTPs activity results in temporarily dampening of mitogenic signaling [84, 90]. Protein-tyrosine phosphatases can suffer an irreversible oxidation to their thiol groups, in the presence of high H_2O_2 levels, [91]. As a result, their function is blocked and the mitogen signal remains continuously activated (**Figure 3**).

Cysteine is unique among the amino acids because it is the only proteinogenic amino acid containing a free SH group. The mechanism of redox signaling involves reversible H_2O_2-mediated oxidation of cysteine residues within proteins [92]. During redox signaling low/normal concentration of H_2O_2 (nM range) oxidizes the thiol group of cysteine residues to sulfenic form (Cys-SOH). As the concentration of H_2O_2 gradually increases, the sulfenic form

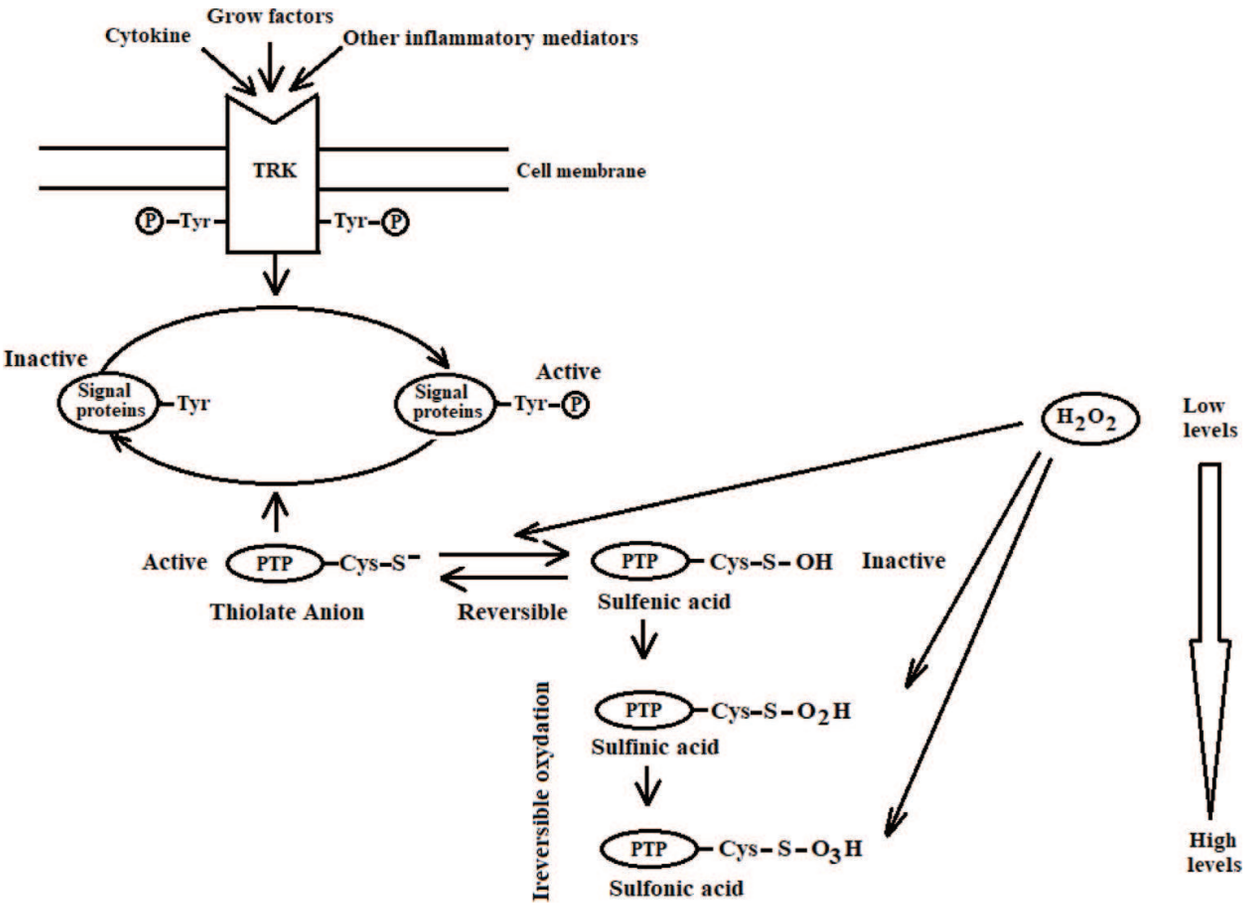

Figure 3. Hydrogen peroxide role in protein-tyrosine kinases regulation. In normal/low concentration, H_2O_2 regulates PTPs activity by promoting the reversible oxidation of the Cys residues. At high concentration of H_2O_2, PTPs becomes irreversibly inactive and, as a consequence, tyrosine kinase proteins involved in cell proliferation (MAPK) remain blocked on active form. TRK = receptors with intrinsic tyrosine kinase activity; and PTP = proteon-tyrosine phosphatases.

transforms to sulfinic (SO_2H) and sulfonic (SO_3H) forms, respectively. Unlike sulfenic modifications, sulfinic and sulfonic are irreversible transformations. As a consequence, high levels of H_2O_2 can trigger the irreversible oxidation of cysteine group.

Considering the above data, it is possible that Hcy, a H_2O_2 generator according to scientific data, may interfere in this signaling process promoting mitogenic activity.

Moreover, Hcy is very similar in structure to cysteine. Like cysteine, Hcy is an amino acid containing a free SH group. This makes possible the occurrence of disulfide bridges between the two amino acids similar to those existing between cysteine residues in some particular concentration of hydrogen peroxide. In our opinion (preliminary work, unpublished data), this may be a possible mechanism of homocysteine involvement in cell signaling that must be investigated (**Figure 4**).

All the scientific evidence presented above suggest Hcy as a risk factor for the vascular/endothelial dysfunction.

Instead some scientists investigate Hcy from the opposite point of view [93] and consider HHcy as a marker of an already altered vascular state rather than a risk factor. These authors

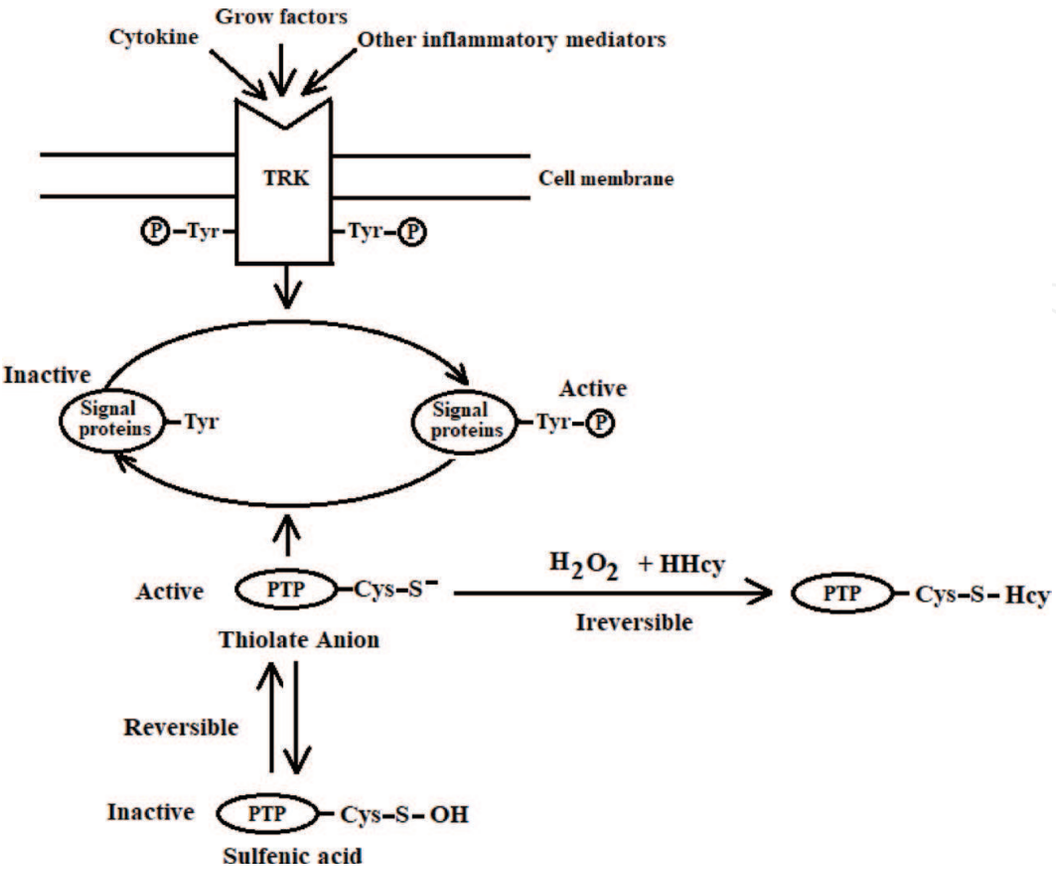

Figure 4. Possible mechanism for hyperhomocysteinemia to intercept the protein-tyrosine phosphatase regulation through disulfide bridge formation. TRK = receptors with intrinsic tyrosine kinase activity; PTP = proteon-tyrosine phosphatases; and HHcy = hyperhomocysteinemia.

consider that hypertension and atherosclerosis reach the stage where kidney function is severally impaired and Hcy removal is diminished and, consequently, its concentration rises in the blood. Atherosclerosis and hypertension are silent diseases that develop years before a vascular event occurs. The disease is accompanied by a silent decline in renal function and, as a consequence, total clearance including that of homocysteine diminishes. Thus, vascular disease contributes to the elevation of circulating Hcy as result of the progressive decline in renal function, and HHcy in fact reflects the severity of atherosclerosis. Thus, HHcy becomes a signal that the atherosclerotic disease reaches an irreversible stage.

Regardless of the classification of homocysteine as a risk factor or marker, its involvement in pathology is certain, and its role needs to be elucidated.

6. Conclusion

The study of homocysteine began when its association with cardiovascular disease was discovered. Further studies revealed its association with vascular dysfunction, and then Hcy was linked to the inflammatory phenomenon. Recently, as studies advanced, the homocysteine involvement in inflammation has been identified. The inflammatory process in turn is related

to the activity of reactive species, and recent data indicate protein-tyrosine phosphatases as key factors in regulating intracellular signaling pathways. These proteins allow regulation because they can undergo reversible oxidation phenomena due to the presence in their structure of cysteine residues bearing SH groups. The structural similarity of Cys with homocysteine draws attention to the possibility that Hcy may interfere with cysteine functions. In conclusion, the recent association of Hcy with both inflammation and the reactive species involved in cellular signaling indicates that homocysteine remains a topic of interest and attention in current research. It is obvious that HHcy is an issue of interest in contemporary medicine.

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