Effect of Dialysis on Heme Oxygenase-1 (HO-1) Expression in Peripheral Blood Leukocytes of End Staged Renal Diseased Patients

Sathyapriya Kandavelu and Parameswari Chenthchery Somasundaram
Department of Biochemistry, Bharathi Women’s College, North Chennai-600 108, Tamilnadu, India

Corresponding Author: Parameswari Chenthchery Somasundaram, Department of Biochemistry, Bharathi Women’s College, North Chennai-600 108, Tamilnadu, India Tel: +91-9941140770

ABSTRACT

Many End staged Renal Diseased and hemodialysis patients are in a state of chronic inflammation induced by the dialysis process which further enhances oxidative stress. The study aim was to explore the critical role of Heme oxygenase-1, a potent antioxidant, in patients subjected to various dialysis conditions. The study population contained randomly selected end-staged renal failure patients (n = 64) who have been treated by hemodialysis for at least 6 months, between the age group of 20-50 with creatinine levels greater than 5 mg dL\(^{-1}\). About 20 samples were collected from predialysis patients, 22 samples were collected from patients undergoing dialysis and 22 samples were collected from post dialysis patients. The samples were subjected to antioxidant enzyme analysis followed by flow cytometric and immunofluorescence studies HO-1 expression was found to be elevated in all the dialysis conditions but higher elevation was observed in post dialysis patients. The results suggest that HO-1 might have some link with the disease progression and though upregulated like other stress proteins, it’s antioxidant capacity has been ineffective to cease the disease invasion.

Key words: Heme oxygenase, dialysis, leukocytes, flow cytometry, renal failure, reactive oxygen species

INTRODUCTION

End-Staged Renal Disease (ESRD) is a state of microinflammation, with increased activation of cytokines and augmented stress (Raj et al., 2005). The host defenses in patients with chronic renal failure, while broad in scope can be correlated to the abnormal function of two cell types, the phagocytic cells and lymphocytes (Pesanti, 2005). Polymorphonuclear leukocytes (PMNLs) are a possible source for the formation of superoxide radicals and inflammatory mediators which promote ROS and inflammation (Abu El-Asrar et al., 1995).

Hemodialysis is as an alternative way of treatment in the chronic kidney failure patients (Shafipour et al., 2010). Haemodialysis (HD) may induce repetitive bouts of oxidative stress, primarily through membrane bio-incompatibility and endotoxin challenge (Abraham et al., 2003). While alterations in pro- and anti-oxidant capacity start during the early stages of renal failure, they are most pronounced in patients on dialysis. However, there is some conflicting evidence as to whether the onset of regular dialysis improves or worsens oxidative stress. Also, excessive treatment using iv iron to treat anemia in CKD patients may lead to iron overload state, with resultant production of free radicals and cellular damage (Reddy et al., 2011).
Activation of PMNs is a well-recognized feature in dialysis patients which may represent an important pathway for tissue damage and LDL oxidation in vivo.

ROS is considered to be a major contributing factor to endothelial dysfunction, including endothelial cell apoptosis, abnormalities in cell cycling and delayed replication which can be reversed by antioxidant agents or by increased expression of antioxidant enzymes (Zou et al., 2002; Balla et al., 1992; Baynes, 1991). Tolerance to oxidative stress is provided by stress proteins such as the inducible heme oxygenase (HO-1), a 32 kda enzyme, whose expression of HO-1 is up regulated during exposure to oxidants, UV-A irradiation and a series of agents including cytokines, hormones, heme and heavy metals (Stocker et al., 1990). Three isoforms of HO have been identified; inducible HO-1 and two constitutively expressed HO-2 and HO-3 (Perrella and Yet, 2003). A growing body of evidence suggests that overexpression of HO-1 may protect organ/tissues from immune-mediated injury either through prevention of oxidative damage or via a local immunomodulatory influence on infiltrating inflammatory cells. The antioxidant effects of HO-1 arise from its capacity to increase reduced glutathione levels and to degrade heme, as well as from the elaboration of biliverdin and bilirubin which have potent antioxidant properties (Descamps-Latscha et al., 1991).

Poole et al. (2005) studied that in endotoxemic condition, induction of HO-1 would lead to increased production of the vasodilator CO, lower blood pressure and decrease renal function. Interestingly, another study shows that the pre-induction of HO-1 by hemin prevented dialysis membrane-induced monocyte apoptosis, whereas inhibition of HO-1 activity enhanced dialysis membrane-induced monocyte apoptosis (Bhaskaran et al., 2005). Ferenbach et al. (2010) determined that administration of macrophages modified to overexpress HO-1 would protect from the kidneys renal ischemic reperfusion injury in mice.

The levels of HO-1 in leukocytes of chronic renal failure patients have been studied to a lesser extent. Moreover, to date, the precise effect of HO-1 expression during dialysis has not been clearly explained. The aim of the present study was to analyze the levels of HO-1 in the peripheral blood leukocytes of the patients, during the various stages of dialysis, for this will facilitate further studies to understand the relationship between the antioxidants and HO-1 in CRF patients and the effect of dialysis on HO-1 expression.

MATERIALS AND METHODS

Chemicals: Mouse anti-human Heme Oxygenase-1, goat anti-Mouse HRP conjugated antibody and goat anti-Mouse phycoerythrin conjugated antibodies were procured from (StressGen, Victoria, Canada). All other chemicals and reagents used were of analytical grade.

Study subjects: The study was conducted between 2009-2010 and the population contained randomly selected end-stage renal failure patients (n = 64) who have been treated by hemodialysis for at least 6 months, between the age group of 20-50, with creatinine levels greater than 5 mg dL⁻¹. About 20 samples were collected from predialysis patients, 22 samples were collected from patients undergoing dialysis and 22 samples were collected from post dialysis patients. Blood samples were collected in heparinized vials two hours before dialysis and two hours after dialysis from the same patients. As controls we studied adult individuals without renal diseases (n = 30). Exclusion criteria for participation in this study were acute infections, malignant diseases, cardiovascular events (myocardial infarction, stroke) and diabetic nephropathy. The study was performed according to the rules of the Declaration of Helsinki and approved by Institutional Ethical Committee (CSP01/2007).
Isolation of leukocytes from peripheral blood: Peripheral blood leukocytes were isolated from blood as per the method of Saxena et al. (1980). The protein content in the Peripheral Blood Leukocytes (PBL) was determined by the method of Lowry et al. (1951). The lipid peroxides in the PBL was estimated using the method described by Ohkawa et al. (1979). The level of reduced Glutathione (GSH) in the cells was estimated as described by Moron et al. (1979).

Estimation of antioxidant enzymes: The activity of Superoxide Dismutase (SOD) in the cells was estimated using the method described by Misra and Fridovich (1972). The activity of catalase in the cells was assayed described by Beers and Sizer (1952). The activity of Glutathione peroxidase (Gpx) was estimated as described by Rotruck et al. (1973). The activity of Glutathione-S-Transferase (GST) in the cells was estimated using the method obtained from Habig et al. (1974).

Flow cytometry analysis of heme oxygenase-1: Flow cytometric analysis on the PBL was performed as per the method described by Gray and Darzynkiewicz (1987).

Quantification of heme oxygenase-1 by ELISA: Quantification of Heme oxygenase-1 was assessed by ELISA as per the method of Deshpande (1996).

Statistical analysis: Results were expressed as Mean±SD. Differences between groups were analyzed, p values<0.05 was considered significant.

RESULTS
Antioxidant status lowered in the CRF group: To study the antioxidant status in the PBL of CRF patients, biochemical assays were performed. Figure 1 shows the comparison of various

![Graph showing antioxidant levels](image)

Fig. 1: Enzymatic and non enzymatic antioxidants in peripheral blood leukocytes. Results are expressed as Mean±SE (n = 64). ***p<0.001 in Group 1, control; group 2, CRF Patients. Enzymatic and non enzymatic antioxidants in PBL. Results are expressed as Mean±SE (n = 42). ***p<0.001 in Group 1, control; group 2, CRF Patients. Enzymatic and non enzymatic antioxidants in PBL. Results are expressed as Mean±SE (n = 42). ***p<0.001 in Group 1, control; group 2, CRF Patients
Fig. 2(a-d): Continue
Fig. 2(a-d): HO-1 production *ex vivo*. Peripheral blood leukocytes separated and stained with human anti-HO-1 antibody. The levels of HO-1 expression were compared between the dialysis groups and the control group. (a) represent the predialysis group and (b) shows the dialysis group. (c) and (d) denote the post dialysis and control groups, respectively non-enzymatic and enzymatic antioxidants levels between the control and CRF group. It was found that the antioxidants showed significant reduced activity/levels when compared to the control group.

**Increased expression of HO-1 expression in Post dialytic condition:** HO-1 is a proven antioxidant; hence studies were performed to assess the levels in the PBL of CRF patients. Figure 2(a-c) depicts the flow cytometry analysis in the leukocytes of chronic renal failure patients under pre, post and during dialysis conditions, respectively. Figure 2d represents the control group. There was concomitant increase in HO-1 expression from pre to post dialysis condition, with the latter presenting maximum elevation.

**ELISA studies also indicate rise in HO-1 activity:** ELISA studies on HO-1 expression were executed to support the FACS results. The leukocytes of the post dialysis patients exhibit nearly more than ten times the normal value (p<0.05) of expression of HO-1 as compared to the control group.
Fig. 3 (a-b): HO-1 expression in various groups. (a) Comparison of heme oxygenase-1 activity and (b) HO-1 expression compared between groups. Results are expressed as Mean±SD (n = 64) ***p<0.05 HO-1 expressed in group 1, control; group 2, Patients before dialysis; group 3, Patients during dialysis; group 4, post dialysis patients.

group and the pre and during dialysis group (Fig. 3a-b) which is in accordance with the results obtained earlier.

**DISCUSSION**

Hemodialysis represents a chronic stress status for its recipients. Evidence has accumulated that oxidative stress is present in Hemodialysis (HD) patients (Nath et al., 2000). The susceptibility to oxidative stress in HD patients is mediated by abnormal oxidant and defective antioxidant production. Changes in free radical concentration levels may be indirectly caused by activity changes of antioxidant enzymes (Uma Devi and Chinnaswamy, 2008).

Polymorphonuclear leukocytes (PMN) are the first line of defense against foreign invaders and constitute the major cell type involved in certain types of acute and chronic inflammatory diseases. There is increasing evidence that leukocytes, particularly neutrophils, mediate tissue injury and play key roles in the development of renal failure (Witko-Sarsat et al., 2000). The inflammatory response consists of a complex cascade of orchestrated signals resulting in increased permeability of blood vessels, changes in blood flow and migration of leukocytes from blood to affected tissues (Robbin et al., 1999).

Free radicals are eliminated from the body by their interaction with non-enzymic and enzymic antioxidants such as uric acid, albumin, bilirubin, Vitamins E, C, A, glutathione, glutathione peroxidase, superoxide dismutase and catalase (Marjani et al., 2007). With regard to antioxidant deficiency, it is well established that: (i) the uraemic milieu per se induces a deficiency in enzymatic system constituents and cofactors (Zn²⁺, Se⁶⁻, Mg²⁺) and (ii) this is further aggravated by the
dialysis procedure which provokes a loss of major non-enzymatic antioxidant molecules such as Vitamin C. The presence of increased plasma levels of oxidatively damaged lipid products (malondialdehyde, thiobarbituric reactive substances, isoprostanes), DNA and proteins [Advanced Oxidation Protein Products (AOPP), chlorodityrosine] reflects the oxidative stress resulting from dialysis-induced oxidant burden and insufficient availability of antioxidants. Ideally, the circulating levels of these markers can serve to assess the quality of the hemodialysis procedure and allow the evaluation to follow the efficacy of antioxidant therapeutic strategies. Only a few studies have concentrated on the assessment of antioxidant status in the PBL of renal failure patients. The decrease in the activities of SOD, catalase, GST and GpX and GSH with increase in LPO levels in the leukocytes, clearly indicates the degree of oxidative stress encountered by these cells in circulation which might be further aggravated due to dialysis in line with the previous reports.

Heme oxygenase-1 (HO-1) is an ubiquitous enzyme, profoundly present in the microsomes of spleen. Macrophages express high levels of collagenase, proinflammatory cytokines, colony-stimulating factor, inhibitory protein 1 alpha, chemotactant protein 1 which are mediators of inflammation (EL-Batouty et al., 2003). The production of inflammatory cytokines by tubular cells and interstitium of the kidneys results in a concentration gradient that recruits inflammatory cells from the microvasculature to the matrix and then through the injured tubular basement membrane, allowing interactions with tubular epithelial cells (Zhang et al., 2003). Hence, present study evaluated the alterations in HO-1 expression in leukocytes, since these cells reflect the degree of adversity in the affected kidney.

Several studies have suggested that HO-1 provides protection against cellular oxidative damage (Zhang et al., 2003) but the mechanisms of the protective effects are not completely known. Also a number of studies have reported that HO-1 is induced under infectious diseases and inflammatory diseases in macrophages and monocytes however, the detailed induction mechanism of HO-1 and its function in macrophages have yet to be understood (Rabb and Star, 2001).

Numerous reports support the protective role of HO-1 as an antioxidant, in various pathological conditions but there are no reports which concentrated the role of HO-1 in ESRD patients under dialysis. In the present investigation, HO-1 levels were highly elevated in PBL (with maximum augmentation in post dialysis condition), as compared to other antioxidant parameters studied. However, this rise did not contribute to any reduction in the extent of free radical production, implicating its inefficiency in combating oxidative stress and is in par with the results of Suttner and Denny (1999).

A previous report demonstrates that HO-1 preconditioning and an increase in HO-derived CO and bilirubin regulate the levels of antioxidant genes, such as EC-SOD and catalase, subsequently decreasing endothelial impairment in experimental diabetes (Abraham et al., 1995). However, in our case there is no possibility of pre induction of HO-1 in our patient population, since it was already upregulated due to the severity of the disease and was further ameliorated due to strenuous conditions like dialysis.

HO activity, however, may not be protective in all instances. Suttner and Denny (1999), using a tetracycline regulatable system, demonstrated that low levels (<5-fold) of HO-1 expression are protective, whereas high levels (>15-fold) of overexpression actually worsen cell injury caused by hyperoxia in hamster fibroblasts. In many conditions, the overexpression of HO-1 has associated with the resolution of inflammation. In vitro, HO-1 has been shown to be cytoprotective against oxidative stress but this protective effect has not been observed uniformly in vivo (Platt and Nath, 1998). The present study indicates the possible detrimental role of HO-1 in CRF
patients, as witnessed by rise in inflammatory cytokines levels (unpublished data). Thus we interpret that hemodialysis through up-regulating HO-1 levels in leukocytes of CRF patients, aggravates disease progression.

CONCLUSION

Present investigation has demonstrated significant increase in the expression of HO-1 in the peripheral blood leukocytes of the CRF patients, with the post dialysis patients showing marked elevation in HO-1 expression. The decrease in the levels of the antioxidant parameters studied might be due to the rise in the free radicals. However, the subsequent elevation in the HO-1 levels (being a potent antioxidant) is a fact yet to be resolved. Dialysis has been proved to enhance production of free radical, thereby leading to additional oxidative stress in renal failure patients. Though previous studies claim that prior induction of HO-1 during dialysis can prevent the free radical damage, HO-1 elevation in present study population could have probably protected the patient from the worsening effects of dialysis. Hence, further investigations are necessary for better understanding of the role played by HO-1 in renal failure, as this will ensure the prevention of disease progression.

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REFERENCES


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