

Homocysteine Inhibits Hydrogen Peroxide Breakdown by Catalase

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Abstract: Catalase, an antioxidant enzyme responsible for degradation of hydrogen peroxide, is protective in many diseases. The amino-acid homocysteine has been suggested to be a pro-oxidant with elevated levels linked to the oxidative stress seen in Alzheimer's and other diseases. This study shows that homocysteine inhibits the breakdown of hydrogen peroxide by catalase. Physiological concentrations of homocysteine inhibited catalase breakdown of hydrogen peroxide. The inhibition of catalase was by generation of the inactive catalase compound II and prevented by ethanol or NADPH but unaffected by iron. Physiological concentrations of homocysteine also inhibited cellular catalase but were not cytotoxic. Homocysteine enhanced the toxicity of the amyloid- β peptide in an *in vitro* model of Alzheimer's neurodegeneration. The antioxidant vitamin E blocked the toxicity of amyloid- β plus homocysteine but not the catalase inhibition. Catalase inhibition by homocysteine may increase the levels of hydrogen peroxide and play a role in the pathology of disease.

Keywords: Homocysteine, catalase, compound II, amyloid- β , Alzheimer's, ethanol, NADPH, iron, vitamin E.

INTRODUCTION

Hydrogen peroxide (H_2O_2) is one of the more stable reactive oxygen species and plays a role in the pathologies of many diseases including Alzheimer's disease [1]. Catalase is an antioxidant enzyme found in many cell types and considered to play a major role in the removal of H_2O_2 [2-4]. There are over 300 forms of catalase and the enzyme is found in most organisms [5]. Human catalase is a heme protein [6, 7], which also contains an NADPH binding site [8-10]. The structure of human catalase has been determined and the key residues responsible for activity identified [11, 12]. The active form of human catalase is a tetramer [2, 3, 11].

Loss of catalase activity is associated with increased susceptibility to oxidative stress [13, 14] while over expression studies suggest a role for the enzyme in longevity [15]. A number of compounds have been reported to inhibit the activity of the enzyme including the amino acid cysteine [16-19]. The inhibitory actions of cysteine were originally studied with incubations of mM concentrations of the amino acid with catalase [2, 3, 19]. These conditions are not representative of the physiological levels of this amino acid, which are normally in the μM range, and the observed inhibition is unlikely to occur naturally [20].

Catalase breakdown of H_2O_2 causes the formation of compound I [10, 16] which can then breakdown either H_2O_2 or ethanol. compound I can be converted to an inactive form of catalase, compound II [10, 16], and this inactivation can be prevented by excess ethanol or reversed by NADPH. An alternative model suggests that NADPH prevents formation of compound II [11]; both models are illustrated in Fig. (1). Chemical inhibition of catalase by 3-amino-1,2,4-triazole (3AT) or 3,3'-diaminobenzidine can be prevented by addition of ethanol and NADPH [10, 16]. Cysteine has been reported

to inhibit catalase activity by the formation of compound II [2, 3].

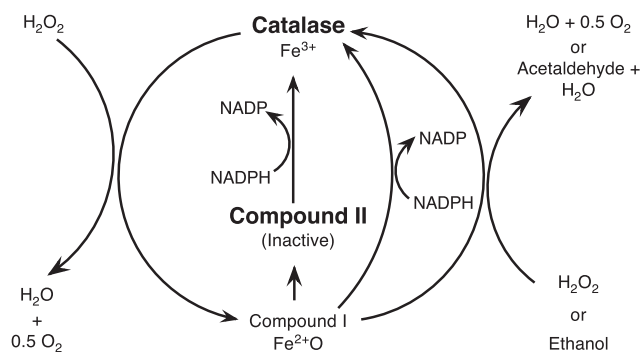


Fig. (1). Schematic representation of the breakdown of H_2O_2 and formation of the inactive compound II form of Human catalase.

Homocysteine is a non-essential sulfur-containing amino acid that is derived from methionine metabolism and closely related to cysteine [21, 22]. Homocysteine has been suggested to directly generate H_2O_2 [23-25]. Normal blood levels of homocysteine are in the range 5 – 15 μM , with around 70% of this bound to plasma proteins [26]. Within tissues homocysteine is also found in both free and protein bound forms, with similar ratios of bound to free found in most tissues, except the cerebellum which was shown to have high levels of free homocysteine [27]. Cells are able to export homocysteine into the extracellular medium in culture [27]. Elevated homocysteine levels are found in many diseases including Alzheimer's [28], atherosclerosis [29] and type 2 Diabetes [30]. Reduced catalase activity is also a feature of Alzheimer's [31], atherosclerosis [32], and Diabetes [33] raising the possibility of a direct link between elevated homocysteine and reduced catalase activity.

In models of Alzheimer's disease homocysteine enhances the toxicity of the amyloid- β peptide [34-37]. The strong links between homocysteine [38] and neurodegenerative

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disease suggests that these models would be useful to study if homocysteine has any action on catalase. Inhibition of catalase in these cell models increases amyloid- β toxicity [18] in a manner similar to that reported for homocysteine enhancement of amyloid- β toxicity. The ability of antioxidants, such as vitamin E, to prevent the enhanced toxicity of amyloid- β in the presence of homocysteine suggests a role for reactive oxygen species in the process. Vitamin E also blocks the enhanced toxicity of amyloid- β in the presence of the catalase inhibitor 3-amino-1,2,4-triazole [18].

In this report the effects of homocysteine on cellular catalase in a neuronal (human SH-SY-5Y) cell line are investigated. The direct effects of homocysteine on Human catalase activity are also investigated. Finally the effects of homocysteine on amyloid- β induced toxicity with and without vitamin E protection are investigated in an *in vitro* model of Alzheimer's disease.

METHODS

Cell Culture

The human neuroblastoma SH-SY-5Y cell line was routinely grown in a 1:1 mixture of HAM's F12 and Eagle's minimal essential medium containing 1% non-essential amino acids and supplemented with 10% foetal calf serum and antibiotics (penicillin and streptomycin; 1% each) in a 5% CO₂ humidified incubator at 37°C. The cells were passaged when confluent and medium was replaced every 2-3 days. Cells were used 4-10 days after plating. Cultures were differentiated for 7 days with 10 mM retinoic acid.

For combined cell viability and intracellular catalase activity experiments 5×10^4 cells were incubated in 24 well dishes in 1 ml medium with test substances for 24 hours. The cells were harvested, an aliquot taken for viability measurement and the remainder precipitated for catalase measurement.

For cytotoxicity experiments 5×10^3 cells were incubated in 96 well dishes in 100 μ l culture medium and test substances for 24 hours. Cell viability was determined by trypan blue dye exclusion with at least 100 cells counted per well or by MTT reduction [17]. After incubation with test substances MTT (10 μ l: 12mM stock) was added and cells incubated for a further 4 hours. Cell lysis buffer [100 μ l/well; 20% (v/v) SDS, 50% (v/v) N,N-dimethylformamide, pH 4.7] was added and after repeated pipetting to lyse cells the MTT formazan product formation was determined by measurement of absorbance change at 570 nm. Control levels in the absence of test substances were taken as 100% and the absorbance in the presence of cells lysed with Triton X-100 at the start of the incubation period with test substances taken as 0%.

Catalase Activity

Catalase EC 1.11.1.6 from human erythrocytes (Sigma, Dorset, UK) was used for all incubation experiments. Catalase activity of the standard preparation was determined by spectrophotometric measurement of H₂O₂ breakdown [39]. For all studies aliquots of this material were used as the standard catalase of known activity.

Activity of catalase (25 kU l⁻¹) incubated with test substances was determined [17, 40] after incubation in 60 mM

sodium-potassium phosphate buffer at 37°C in a total volume of 100 μ l. After incubation catalase activity was determined by mixing 50 μ l sample with 50 μ l substrate (6.5 μ mol H₂O₂ in phosphate buffer) for 60 secs, adding 100 μ l of 32.4 mM ammonium molybdate and measurement of absorbance change at 405 nm. Catalase activity was calculated from a standard curve (0-100 kU l⁻¹).

For catalase activity measurements in cell extracts the cell precipitates were washed with PBS, sonicated and protein concentration plus catalase activity of cell lysates determined [17, 18].

Sulphur containing amino acids are known to interact with molybdate [41] and tests were carried out to check whether the test substances (cysteine, homocysteine and methionine) had any effect on the absorbance change observed at 405 nm due to the interaction of ammonium molybdate with H₂O₂. Results showed no changes in the absorbance in the presence of these amino acids at concentrations up to 500 μ M. Dilution curves with catalase were also prepared and the effects of adding these amino acids after the addition of the ammonium molybdate to the reaction were also tested, again without any effect at concentrations up to 500 μ M.

Catalase Immunoreactivity

Catalase standards and cell lysates were diluted in 50 mM bicarbonate buffer and added to ELISA plates. After overnight incubation and blocking unbound sites with 5% w/v Marvel the coated plates were incubated with anti-catalase antibody (Calbiochem). Bound antibody was de-

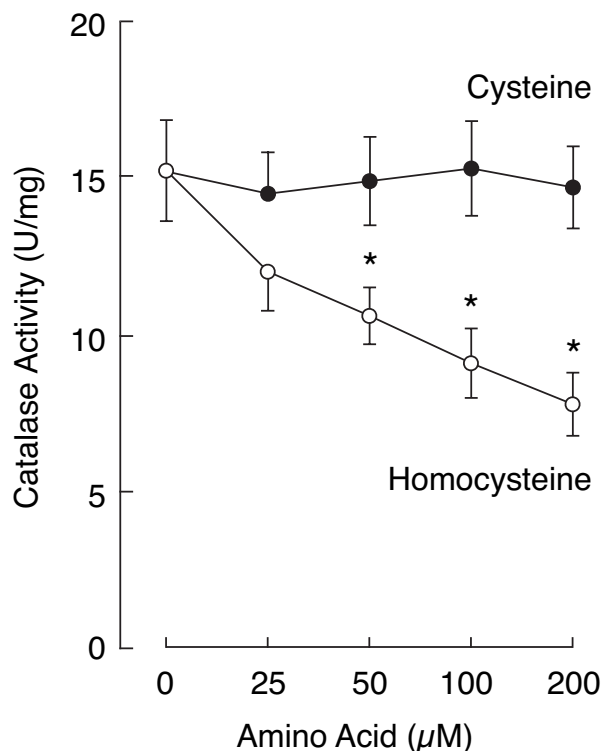


Fig. (2). Effect of homocysteine or Cysteine on the catalase activity. SH-SY-5Y neurons were incubated for 24 hours in the presence of homocysteine (open circles) or cysteine (closed circles). catalase activity was measured in cell lysates and results are expressed as mean \pm sem (n = 8). * = P < 0.05 vs Control (0 μ M amino acid); one-way ANOVA.

tected using an alkaline phosphatase second antibody plus p-nitrophenylphosphate substrate and cell lysate levels determined by interpolation from the standard curve [18].

Data Analysis

Data are expressed as means \pm sem catalase levels are expressed as % inhibition of control (untreated) catalase. For cytotoxicity experiments data are expressed as % dead (trypan blue stained) cells. The significance of differences between data was evaluated by one-way analysis of variance (ANOVA). A P value of <0.05 was considered statistically significant [17, 18].

RESULTS

Effect of Homocysteine on Cellular Catalase Activity

Homocysteine at concentrations between 25 – 200 μ M significantly decreased the cellular catalase activity of SH-SY-5Y neurons in culture when added for a 24 h period (Fig. 2). The related amino acids cysteine and methionine had no effect at similar concentrations (25 – 200 μ M). The levels of immunoreactive catalase were unchanged by 200 μ M homocysteine, 200 μ M cysteine or 200 μ M methionine when compared to control cells (Fig. 3).

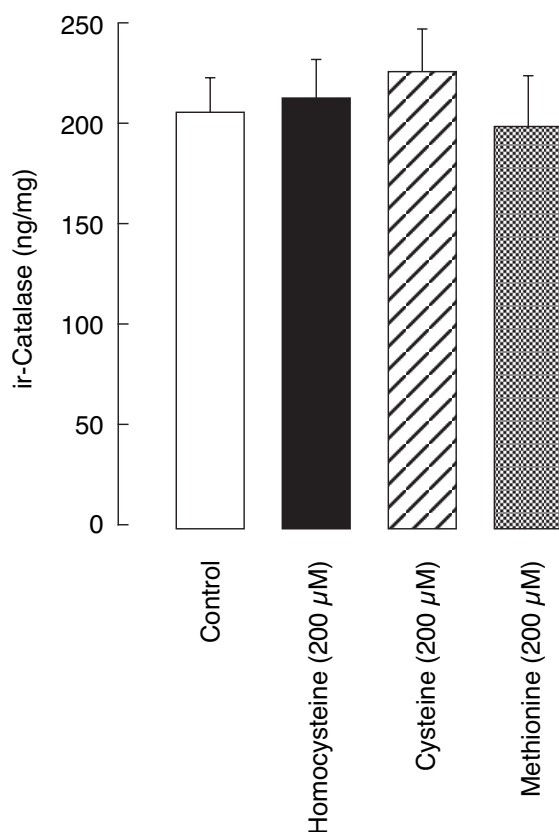


Fig. (3). Effect of homocysteine, Cysteine and Methionine on the catalase levels. SH-SY-5Y neurons were incubated for 24 hours in the presence of homocysteine, cysteine or methionine. catalase immunoreactivity and protein concentrations were measured in cell lysates and results are expressed as mean \pm sem (n = 8).

Effect of Homocysteine on Catalase Breakdown of H₂O₂

The effects of homocysteine plus the related amino acids methionine and cysteine on catalase breakdown of H₂O₂

were examined. Incubation of human catalase with homocysteine caused a significant dose dependent inhibition of H₂O₂ breakdown (Fig. 4).

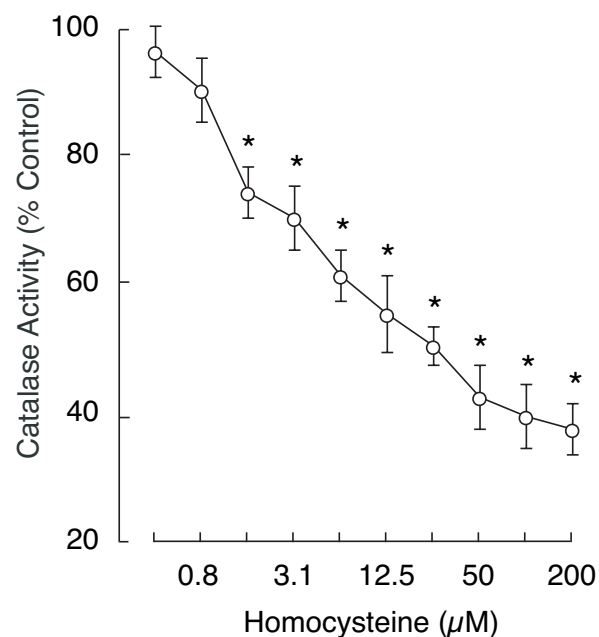


Fig. (4). Effect of homocysteine on the catalase activity. Human catalase (25 U/ml) was incubated for 45 min with homocysteine (0 – 200 μ M). Catalase activity was then measured and results interpolated from the standard curve. All results are expressed as a % Control (25 U/ml catalase alone) and are expressed as mean \pm sem (n = 8). * = P < 0.05 vs Control (0 μ M homocysteine); one-way ANOVA.

The inhibition of catalase activity was time dependent with significant levels of inhibition detected after pre-incubation for 15 min with homocysteine. Pre-incubation for 30 min or pre-incubation for 45 min with homocysteine showed increased levels of catalase inhibition. Pre-incubation for 1 h or longer showed similar levels of activity inhibition to that seen for a 45 min pre-incubation.

In the presence of sub-maximal doses of amyloid- β 25-35 or 3-amino-1,2,4-triazole the effects of homocysteine were additive. The related amino acids methionine and cysteine had no effect on catalase activity and neither compound had any effect on catalase activity in the presence of the known catalase inhibitors amyloid- β 25-35 or 3-amino-1,2,4-triazole.

Effects of Ethanol and NADPH on Homocysteine Inhibition of Catalase

To determine if homocysteine inhibition of catalase was by formation of the inactive compound II, catalase was incubated in the presence of ethanol or NADPH.

Results showed that the inhibition of catalase by homocysteine was significantly reduced in the presence of ethanol (20 nM) plus H₂O₂ (1 mM); control incubations with H₂O₂ (1 mM) alone had no effect (Fig. 5). Inhibition of catalase by homocysteine was also significantly reduced in the presence of NADPH (20 μ M), whilst control incubations with NADP (20 μ M) had no effect (Fig. 6).

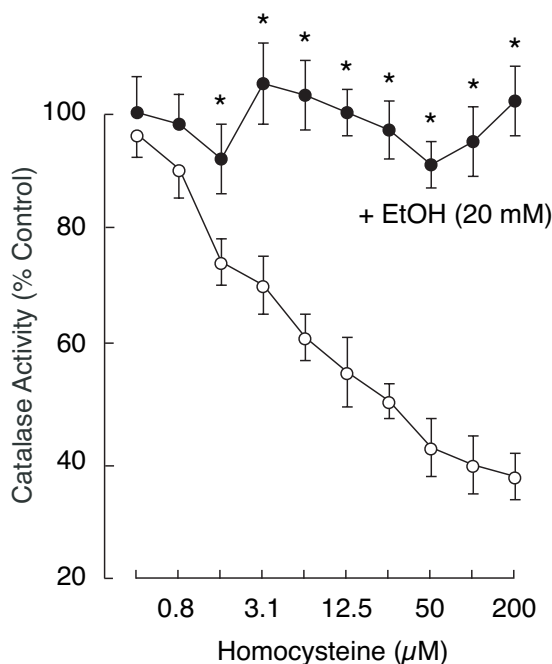


Fig. (5). Effect of ethanol on homocysteine inhibition of catalase activity. Human catalase (25 U/ml) was incubated for 45 min with homocysteine (0-200 μM) in the presence (closed circles) or absence (open circles) of 20 mM ethanol plus 1 mM H_2O_2 . Catalase activity was then measured and results interpolated from the standard curve. All results are expressed as a % Control (25 U/ml catalase alone) and are expressed as mean \pm sem (n = 8). * = $P < 0.05$ vs Control (homocysteine alone); one-way ANOVA.

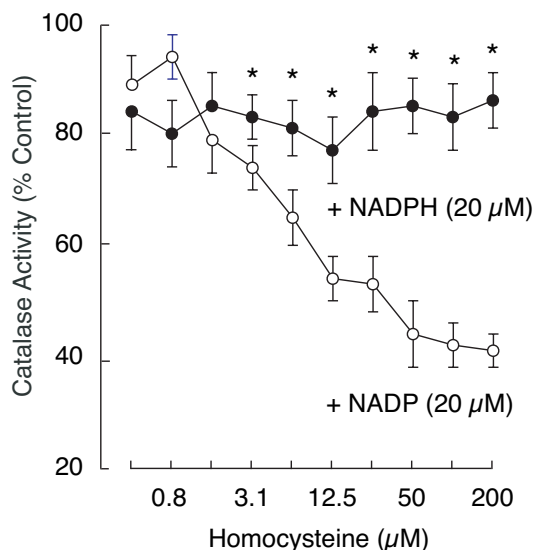


Fig. (6). Effect of NADPH on homocysteine inhibition of catalase activity. Human catalase (25 U/ml) was incubated for 45 min with homocysteine (0-200 μM) with 20 μM NADPH (closed circles) or 20 μM NADP (open circles). Catalase activity was then measured and results interpolated from the standard curve. All results are expressed as a % Control (25 U/ml catalase alone) and are expressed as mean \pm sem (n = 8). * = $P < 0.05$ vs homocysteine plus NADP; one-way ANOVA.

These results suggest that homocysteine inhibition of catalase activity is mediated by the formation of the inactive compound II form of catalase.

Effects of Iron on Homocysteine Inhibition of Catalase

Human catalase activity requires iron [11] bound to an active site at residue 358 and it is possible that an interaction between homocysteine and iron could influence enzyme activity.

Incubation of catalase with homocysteine in buffer supplemented with 25 μM FeCl_2 had no significant effect on the inhibition of catalase activity by homocysteine (Fig. 7). Incubation of catalase with homocysteine in buffer supplemented with 25 μM MgCl_2 also failed to alter the catalase inhibition induced by homocysteine. These results suggest that homocysteine interactions with the iron in the heme group of catalase is not the main cause of homocysteine induced catalase inhibition.

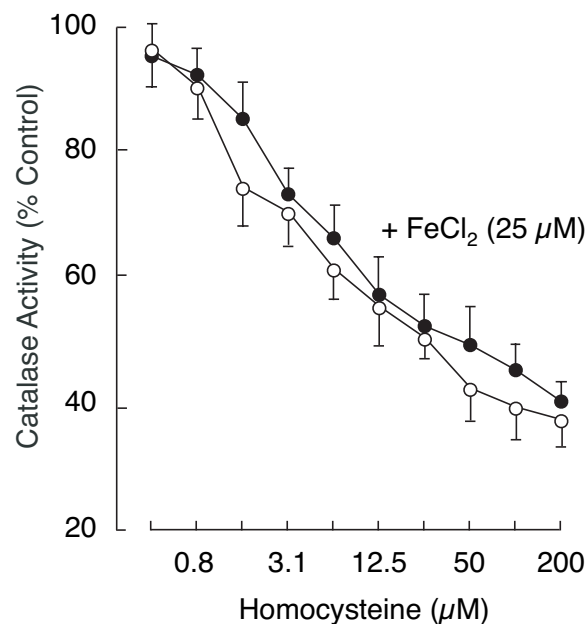


Fig. (7). Effect of FeCl_2 on homocysteine inhibition of catalase activity. Human catalase (25 U/ml) was incubated for 45 min with homocysteine (0-200 μM) in the presence (closed circles) or absence (open circles) of 25 μM FeCl_2 . Catalase activity was then measured and results interpolated from the standard curve. All results are expressed as a % Control (25 U/ml catalase alone) and are expressed as mean \pm sem (n = 8).

Effect of Homocysteine on an *In Vitro* Model of Alzheimer's Neurodegeneration

Amyloid- β toxicity is enhanced by inhibition of catalase activity [18]. Since homocysteine inhibits cellular catalase activity (Fig. 2) it is possible that this amino acid would increase amyloid- β toxicity. Cytotoxicity experiments were performed using differentiated human SH-SY-5Y neurons. Cells were incubated with the amyloid- β 1-42 peptide (2 μM) in the presence or absence of homocysteine (0-200 μM) for 24 h.

Results showed that in the presence of amyloid- β 1-42 (2 μM) addition of homocysteine caused a dose dependent decrease in MTT reduction (Fig. 8), with doses of 50 μM or greater significantly decreasing the MTT reduction in the presence of amyloid- β 1-42. Both amyloid- β 1-42 and amy-

Effects of Homocysteine Plus Amyloid- β on Cellular Catalase Activity in the Presence of Vitamin E

Since both homocysteine and amyloid- β inhibit catalase the effects of vitamin E on this process were tested. Results showed that vitamin E had no effect on the catalase activity itself and was unable to prevent the decreases seen with homocysteine or homocysteine plus amyloid- β (Fig. 11). However, there was an increase in catalase inhibition seen in the presence of homocysteine plus amyloid- β in the presence of vitamin E, which reached significance at 200 μ M homocysteine plus 2 μ M amyloid- β .

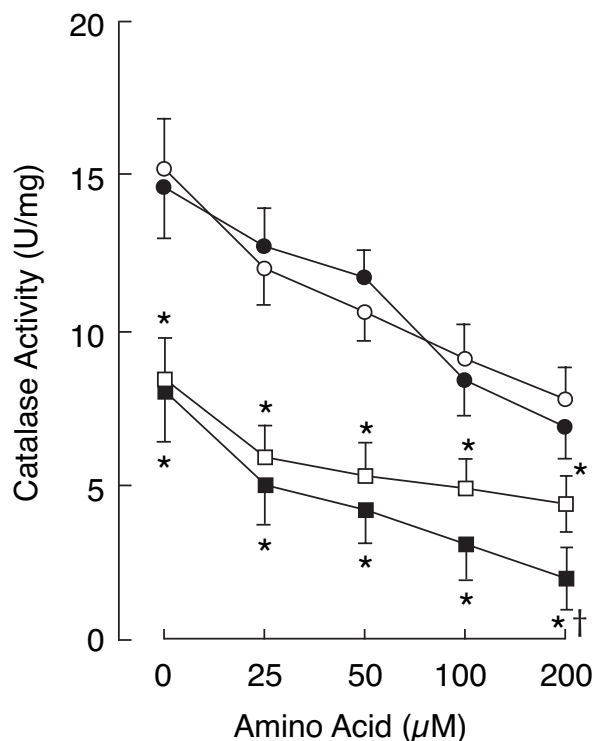


Fig. (11). Effect of vitamin E on homocysteine and amyloid- β inhibition of catalase activity. SH-SY-5Y neurons were incubated for 24 hours in the presence of homocysteine alone (open circles), homocysteine plus vitamin E (closed circles), homocysteine plus 2 μ M amyloid- β (open squares) or homocysteine plus 2 μ M amyloid- β plus vitamin E (closed squares). Catalase activity was measured in cell lysates and results are expressed as mean \pm sem ($n = 8$). * = $P < 0.05$ vs Control (homocysteine alone); † = $P < 0.05$ vs homocysteine plus amyloid- β (open squares); one-way ANOVA.

DISCUSSION

The results from this study show that homocysteine can directly act on catalase and inhibit the breakdown of H_2O_2 . The association of homocysteine with disease states in which oxidative stress is a key component of the pathology is undisputed [22, 23, 28-30]. Further there have been considerable studies in which homocysteine has been suggested to directly contribute to the oxidative stress [25, 28, 29, 34-38]. The suggestion that homocysteine can itself generate H_2O_2 , with the assistance of Copper, has provided a proposed mechanism for this contribution [44-46]. However, at physiological concentrations in the μ M range direct generation of H_2O_2 by homocysteine is unlikely to be relevant [47].

The mechanism of homocysteine inhibition of catalase is shared with a number of inhibitors including 3-amino-1:2:4-triazole [11, 16] and amyloid- β [17]. In the case of 3-amino-1:2:4-triazole the compound has been suggested to interact with a Histidine residue [11] and modify the substrate channel. Both these inhibitors of catalase, 3-amino-1:2:4-triazole and amyloid- β , bind to the enzyme [11, 48]. Homocysteine has the ability to bind proteins and to form disulphide bridges with cysteine residues within proteins [49]. Early studies identified 16 cysteines in Human catalase [50], however since catalase is normally a tetramer [2, 3, 11] this corresponds to 4 cysteines/molecule in agreement with the amino acid sequence of Human catalase [11]. It has been shown that oxidation of the cysteine residues has no effect on the activity of the enzyme [50]. However, thiols such as β -mercaptoethanol and dithiothreitol have been shown to inhibit catalase activity, raising the possibility that modification of the cysteine residues may alter the enzyme activity. Some of these thiol compounds also modify the heme group of catalase and this may be the cause of the enzyme inactivation [51-53]. In the case of homocysteine the results from the present study show no reversal of the enzyme inhibition in excess FeCl, suggesting that a direct interaction with the Heme Fe is unlikely. This cannot exclude modification of the heme group by other interactions induced by homocysteine.

It is unknown if homocysteine would interact directly with the cysteines present in catalase and which of them would be the potential target if it did. The four cysteines are located in the β_6 region, two in the wrapping loop between and one in the C-terminal alpha-helical region. None are implicated in the Heme, H_2O_2 or NADPH binding in catalase [11]. Further structural studies would be required to determine whether this is the mode of action of homocysteine.

The additive effects of 3-amino-1:2:4-triazole and amyloid- β in terms of induction of cell death have been reported [18]. In this study additive effects of homocysteine and amyloid- β were observed. The ability of both to inhibit cellular catalase was observed and the effect was additive. The actions of vitamin E as an antioxidant and neuroprotective agent are well documented [1, 18, 42, 43] and in this study they prevented the toxicity of amyloid- β alone and with homocysteine. The inhibition of catalase by amyloid- β [17] was thought to be contributory to the neurotoxicity of the peptide [18] but not causal. The ability of vitamin E to prevent the neurotoxicity of amyloid- β but not the effects on catalase indicate that vitamin E is acting downstream of the inhibitory actions of amyloid- β on catalase. The observation that in the presence of vitamin E there is an additive effect on catalase inhibition and the significant increase in catalase inhibition compared to cells not treated with vitamin E is an interesting observation. In a previous study [18] amyloid- β inhibited total cellular catalase activity but significantly increased the catalase activity of the surviving cells. In the vitamin E plus amyloid- β treated group there was little cell death and therefore the cell population will have included those cells with lower catalase levels which were more susceptible to the toxic effects of amyloid- β . It is also likely that the inhibition would be more pronounced in cells with lower catalase activity and this may be responsible for the observed effects.

Why is Homocysteine Inhibition of Catalase Important to Disease Pathology?

Homocysteine is an amino acid and can be readily taken up by cells [54]. Catalase is primarily an intracellular component and its protective actions in many tissues such as neurons are probably mediated by the catalase associated with mitochondria [15]. These two features make it highly likely that under physiological conditions homocysteine and catalase will come into contact and the inhibition of H₂O₂ breakdown is likely to occur. Homocysteine has also been shown to effect Glutathione Peroxidase gene expression [55] and to directly inhibit the enzyme at physiological concentrations [56]. It is likely that loss of one of these antioxidant enzymes such as occurs in Acatalasemia, the inherited deficiency of catalase [13], the other H₂O₂ enzymes can compensate in part for the loss of their antioxidant partner. Since the combination of catalase and Glutathione Peroxidase activities in many disease states linked to oxidative stress are key to the removal of H₂O₂ any inhibition of both enzymes will prove more deleterious. Inherited deficiencies in catalase are associated with Hyperhomocysteinemia [57] and the ability of homocysteine to inhibit Glutathione Peroxidase [56] may further reduce the ability to remove H₂O₂ in these individuals. This may explain the observed increased susceptibility of Acatalasemic individuals to diseases associated with oxidative stress such as Diabetes and Atherosclerosis [57]. Hyperhomocysteinemia has the potential to dramatically reduce the H₂O₂ removal facilities and aggravate oxidative stress.

The strong links between oxidative stress and Neurodegenerative disorders such as Alzheimer's [1] plus the protective role of catalase in these disorders [18, 42, 58-60] suggests that any endogenous inhibitor of catalase has potential to contribute detrimentally to the disease pathology. Combined with the inhibition of Glutathione Peroxidase activity homocysteine is likely to be a major contributor to Neurodegenerative disorder pathology, which has been linked to oxidative stress in most cases [61].

From the results of this study and the study of Durmaz and Dikmen [56] a new proposed mechanism can be put forward – that homocysteine inhibits the breakdown of H₂O₂ and this allows any stimulus for H₂O₂ generation to drive oxidative stress.

CONCLUSIONS

Homocysteine directly inhibits catalase breakdown of H₂O₂ by conversion of the enzyme into the inactive compound II form. This action occurs at physiological concentrations and can be observed in cells. The inhibition of catalase may be the mechanism for homocysteine enhancement of amyloid- β toxicity in an Alzheimer's disease model. This action of homocysteine, in combination with its ability to inhibit Glutathione Peroxidase, may play a role in oxidative stress and contribute to the pathology of diseases associated with Hyperhomocysteinemia.

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