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Tyrosinase Inhibition Due to Interaction of Homocyst(e)ine with Copper: The Mechanism for Reversible Hypopigmentation in Homocystinuria Due to Cystathionine β-Synthase Deficiency

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Summary

Deficiency of cystathionine β-synthase (CBS) is a genetic disorder of transsulfuration resulting in elevated plasma homocyst(e)ine and methionine and decreased cysteine. Affected patients have multisystem involvement, which may include light skin and hair. Reversible hypopigmentation in treated homocystinuric patients has been infrequently reported, and the mechanism is undefined. Two CBS-deficient homocystinuric patients manifested darkening of their hypopigmented hair following treatment that decreased plasma homocyst(e)ine. We hypothesized that homocyst(e)ine inhibits tyrosinase, the major pigment enzyme. The activity of tyrosinase extracted from pigmented human melanoma cells (MNT-1) that were grown in the presence of homocysteine was reduced in comparison to that extracted from cells grown without homocysteine. Copper sulfate restored homocyst(e)ine-inhibited tyrosinase activity when added to the culture cell media at a proportion of 1.25 mol of copper sulfate per 1 mol of DL-homocysteine. Holo-tyrosinase activity was inhibited by adding DL-homocysteine to the assay reaction mixture, and the addition of copper sulfate to the reaction mixture prevented this inhibition. Other tested compounds, L-cystine and betaine did not affect tyrosinase activity. Our data suggest that reversible hypopigmentation in homocystinuria is the result of tyrosinase inhibition by homocyst(e)ine and that the probable mechanism of this inhibition is the interaction of homocyst(e)ine with copper at the active site of tyrosinase.

Introduction

Homocystinuria/hyperhomocyst(e)inemia due to cystathionine β-synthase (CBS) deficiency is an inborn error of sulfur amino acid metabolism. CBS (E.C.4.2.1.22) catalyzes the condensation of homocysteine and serine to form cystathionine. Reduced CBS activity results in elevated plasma levels of homocyst(e)ine and methionine, while cyst(e)ine is decreased. Pyridoxal-5-phosphate is a cofactor for CBS, and pyridoxine treatment will increase the conversion of homocysteine to cysteine in many patients with CBS deficiency. Folic acid and betaine are also used in the treatment of homocystinuria to stimulate the remethylation of homocysteine to methionine (Wilcken et al. 1983; Mudd et al. 1985, 1995).

Homocystinuria due to CBS deficiency is usually manifested in the 1st–2d decade of life, with involvement of the eye (lens subluxation and/or luxation, myopia, and glaucoma), the nervous system (psychomotor retardation of various degree, epilepsy, and psychiatric symptoms), the vascular system (venous thromboses and emboli), and the skeleton (Marfanoid habitus and premature osteoporosis) (Mudd et al. 1985, 1995). Light skin and brittle red to blond hair (lighter than unaffected members of the family) can also be present and are often striking in degree (Barber and Spaeth 1969; Shelley et al. 1972; Wilcken et al. 1983; Witkop 1985; Mudd et al. 1995). We recently treated two female homocystinuric patients with multisystem involvement who had light skin and blond hair and observed dramatic darkening of their hair following treatment. In this report we evaluate the effect of the different biochemical abnormalities found in homocystinuria due to CBS deficiency on tyrosinase and demonstrate that homocyst(e)ine inhibits tyrosinase, the major enzyme of the melanin synthesis pathway (Witkop 1985; King et al 1995). We propose that interaction of homocyst(e)ine with copper at the active site of tyrosinase is the mechanism for this inhibition. The resulting decreased tyrosinase activity explains the hypopigmentation found in homocystinuria.

Patients and Methods

Patients

Patient 1 is a 13-year-old female who presented with developmental delay, Marfanoid habitus, ectopia lentis, and stiffness of joints. Her blond hair was lighter than

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Table 1

Biochemical Results for the Homocystinuric Patients Before and After Treatment

	PATIENT 1		PATIENT 2		
	Before Treatment	After Treatment	Before Treatment	After Treatment	NORMAL
Total homocysteine[a]	440	240	430	60	10–20
Free homocystine	60	20	110	0	0
Cystine	20	60	10	80	30–150
Methionine	230	1400	70	30	10–60
Urine-free homocystine	1400	920	1100	NA	0 μmol/g creatinine
CBS activity[b]	0	NA	.1	NA	8–11 U

NOTE.—All data are micromolar unless otherwise labeled. NA = not assessed.

[a] To evaluate total plasma homocysteine, samples were first treated with dithiothreitol (DTT) to reduce disulfide bonds. They were then deproteinized with sulfosalicylic acid and analyzed using high pressure chromatography (Brattström et al. 1988). Plasma-free homocystine, cystine or urine-free homocystine were similarly analyzed but without the addition of DTT.

[b] CBS activity was determined in the patient's fibroblast culture by measuring the formation of cystathionine (nmol/μg protein/h), using labeled serine (Nordström and Kjellström 1992).

unaffected family members and was noted to be brittle and fragile. Her skin was pale and tanned poorly (type I skin) (Gange 1986). The family history was noncontributory. The diagnosis of homocystinuria due to CBS deficiency was confirmed by urine and plasma amino acid analysis and by the demonstration of reduced CBS activity in fibroblast cultures (table 1). Treatment consisted of a gradual increase in pyridoxine to 1,000 mg/d, betaine 6 g/d, folic acid 2 mg/d, and a methionine-restricted diet. Several weeks after initiation of therapy her plasma homocyst(e)ine levels had decreased and her cystine and methionine had increased (table 1). Darkening of her newly growing hair was noted 4 wk after initiation of therapy. A clear demarcation between the old, blond and new, dark hair was found (fig. 1). The consistency of hair also changed from a coarse to a softer texture.

Patient 2 was a 23-year-old female who had had two uncomplicated pregnancies prior to diagnosis. Ectopia lentis was found when she was examined for a gradual loss of visual acuity, and her physical examination was otherwise normal. She had blond thin hair that was different in color and texture from that of her siblings. The family history was noncontributory. Plasma and urine amino acid levels were consistent with homocystinuria, and CBS deficiency was confirmed by fibroblast enzymatic assay (table 1). Administration of pyridoxine at a dose of 250 mg/d decreased her plasma levels of homocyst(e)ine, with an increase of cystine (table 1). Her scalp and eyebrow hair color changed from light to dark brown 6 wk after initiation of therapy, along with her biochemical response to treatment.

Laboratory Studies

The terms *homocyst(e)ine* and *cyst(e)ine* are used in this study to designate the different potential states of these compounds (free, oxidized, and bound).

Cell cultures studies.—Pigmented human melanoma (MNT-1) cells were grown in Dulbecco's modified Eagle media (DMEM D5648) with 4,500 mg/l glucose, 10% FCS, and 1% penicillin/streptomycin (standard media). This media does not contain homocyst(e)ine but contains 200 μM cystine and methionine. Unless noted in the text, the experimental media used in this study contained decreased cystine and methionine concentrations (40 μM) as compared with the standard media, by making a 1:5 dilution of standard DMEM with special DMEM (D3916) containing no cystine or methionine.

Cells were plated in 24-well plates and grown in the experimental media for 7 d. The cells were grown for an additional 17 d in the presence of one of the following additions: DL-homocysteine (0–880 μM); DL-homocysteine (440 μM) mixed with copper sulfate (0–550 $\times 10^3$ nM); pyridoxine hydrochloride (0.02–0.37 μM); betaine hydrochloride (0.03–11.25 μM); or cystine (200–10 μM, the lower concentration produced by serial dilutions of the experimental media with DMEM D3916 to which 40 μM methionine was added). All compounds were obtained from Sigma Chemical. The tissue culture experiments were carried out in an incubator containing 5% CO_2, and the tissue media was changed twice a week.

Cells grown for 24 d in experimental media without the addition of the tested compounds were used as controls. After a culture period of 24 d, tyrosinase was extracted and activity was determined using tritiated tyrosine as described elsewhere (Townsend et al. 1984). Protein concentration was determined (BCA Protein Assay, Pierce Chemical). Unless specified otherwise, no external homocysteine was added to the reaction mixture in the course of a tyrosinase-activity assay.

Holo-tyrosinase studies.—MNT-1 cells were grown in the experimental media. After 24 d of proliferation, tyrosi-

Figure 1 Photograph from above of patient 1 demonstrates the increase in hair pigment after treatment. A demarcation between the old, blond, and the dark, new, hair is easily seen.

nase was extracted (holo-tyrosinase) and incubated for 2 h in a reaction mixture that contained DL-homocysteine (0–440 μM) without or with copper sulfate (0–550×10^3 nM). The mixture contained a constant ratio of 1.25 mol of copper sulfate to 1 mol of DL-homocysteine (Lerner et al. 1950).

Holo-tyrosinase incubated in a reaction mixture without the addition of any of the above compounds was used as control. After incubation, tyrosinase activity was determined.

Results

Effects on Tyrosinase Activity in Cell Culture

The activity of tyrosinase intracellularly translated in the presence of homocysteine was reduced (table 2). In the presence of 440 μM homocysteine, a concentration similar to that of the patients' total plasma homocysteine prior to treatment, tyrosinase activity was reduced by 40% in comparison with control values

Copper sulfate reversed homocyst(e)ine-induced inhibition of tyrosinase (table 3). The activity of tyrosinase extracted from MNT-1 cells that proliferated in the presence of 440 μM homocysteine without the addition of copper sulfate was 53 pmol/h/μg protein. Tyrosinase activity increased with elevation of copper sulfate concentration present in the proliferation media to a maximum activity of 507 pmol/h/μg protein. Tyrosinase activity was not altered when MNT-1 cells were proliferated in the presence of elevated betaine or pyridoxine or low cystine (fig. 2).

Effects on Holo-Tyrosinase Activity

Homocyst(e)ine inhibited enzyme activity when it was added to the assay reaction mixture containing holo-tyrosinase. In the presence of 440 μM homocysteine concentration in the reaction mixture, tyrosinase activity was reduced by 66.5% in comparison with control (fig. 3).

Copper sulfate had a preventive effect against tyrosinase inhibition produced by the addition of homocysteine to the reaction mixture. When holo-tyrosinase was

Table 2

Tyrosinase Activity in MNT-1 Cells Proliferated in the Presence of Homocysteine

Homocysteine (μM)[a]	Tyrosinase Activity pmol/h/μg Protein (mean[b] ± SD)	Inhibition[c] (%)
0	326 ± 6	0
28	334 ± 11	0
55	361 ± 42	0
110	265 ± 33	18.6
220	213 ± 10	34.6
440	196 ± 4	40
880	169 ± 2	48

[a] MNT-1 cells proliferated in experimental media for 7 d followed by 17 d of proliferation in the presence of 0–880 μM DL-homocysteine. At the end of the proliferation period, tyrosinase was extracted and activity was determined.

[b] Mean of triplicate assay.

[c] Inhibition of tyrosinase activity in comparison with control value.

incubated with homocysteine and copper sulfate in a constant ratio of 1:1.25, no inhibition of tyrosinase was detected (fig. 3).

Discussion

Melanin is produced by melanocytes located in the skin, the hair follicle, and the eye. Tyrosinase plays a critical role in melanin synthesis and is involved in the type and the amount of melanin that is ultimately formed (Cleffmann 1963; King et al. 1995). Mutations of the tyrosinase gene that lead to partial loss of activity are associated primarily with pheomelanin synthesis (Witkop 1985; King et al. 1995).

Acquired hypopigmentation in homocystinuria has been well documented (Barber and Spaeth 1969; Shelley et al. 1972; Witkop 1985; Mudd et al. 1995), but reversal of this with increasing pigmentation (melanotrichia) following successful treatment was previously described in only two patients (Barber and Spaeth 1969; Shelley et al. 1972) with additional scattered notes in the literature (Wilcken et al. 1983). In investigation of the pathogenesis of melanotrichia, Barber and Spaeth (1969) analyzed the amino acid content of hair from individuals with homocystinuria before and after treatment and compared it to new and old control hair. They found more cystine in the new hair in both groups, but the control subjects showed smaller differences in cystine content. The authors suggested that the increase in hair cystine in the treated homocystinuric individuals, although not statistically significant, correlated with increased pigmentation and may have provided the minimal supply of this amino acid needed for hair pigmentation.

Our studies addressed the hypothesis that homocyst(e)ine may inhibit tyrosinase in cutaneous melanocytes, resulting in hypopigmentation (Barber and Spaeth 1969; Shelley et al. 1972). Our clinical observation showed that treatment associated with the correction of the biochemical abnormality in two patients with CBS-deficient homocystinuria was associated with an increase in the synthesis of cutaneous (hair follicle) melanin; however, we could not study this with these patients, and we therefore carried out a series of in vitro experiments that could shed light on this process. A decrease in activity of tyrosinase that had been translated in the presence of homocysteine was noted. At plasma homocysteine concentrations found in the patients prior to treatment (440 μM), tyrosinase activity was inhibited by 40%. Even higher inhibition (66.5%) was documented when homocysteine was added to the assay reaction mixture of holo-tyrosinase, showing that the inhibition was not related to a general cell insult that may have resulted in impaired production of tyrosinase.

The experimental media used for MNT-1 cell proliferation in our studies included low cystine and methionine to avoid possible secondary increases in cysteine that might result from the addition of homocysteine. We demonstrated that cystine deficiency in proportions similar to those found in the patients prior to treatment (30%–60% of the normal) did not cause inhibition of tyrosinase in MNT-1 cell cultures. We also showed that high concentrations of pyridoxine, in contrary to previous speculations (Shelley et al. 1972), or betaine did not affect tyrosinase activity (fig. 2).

Table 3

Restoration of Tyrosinase Activity in MNT-1 Cells by the Addition of Copper Sulfate

Copper Sulfate (nM)[a]	Tyrosinase Activity (pmol/h/μg protein; mean[b] ± SD)
0	53 ± 28
9.31	53 ± 9
27.9	60 ± 14
83.8	101 ± 4
251	134 ± 35
754	228 ± 57
2.3×10^3	322 ± 188
6.8×10^3	192 ± 52
20.4×10^3	264 ± 7
61.1×10^3	455 ± 146
183×10^3	393 ± 30
550×10^3	507 ± 132

[a] MNT-1 cells were cultured for 17 d in experimental media that contained 440 μM of homocysteine and different concentrations of copper sulfate. The ratio of copper sulfate to homocysteine varied from 0 to a maximum ratio of 1.25 mol of copper sulfate per 1 mol of homocysteine. At the end of the proliferation period, tyrosinase was extracted and the enzyme activity was determined.

[b] Mean of triplicate assay.

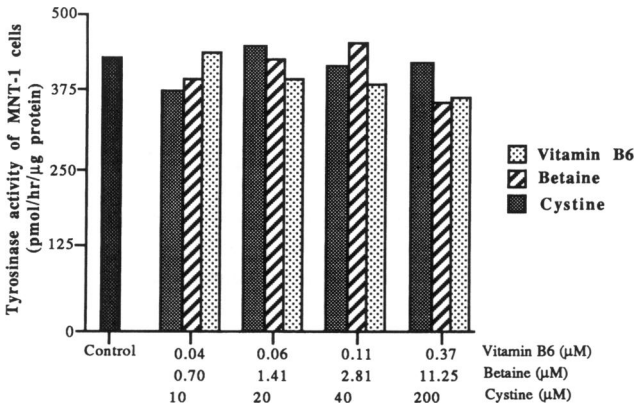

Figure 2 Effect of pyridoxine, betaine or cystine on tyrosinase activity in MNT-1 cells. MNT-1 cells proliferated in experimental media for 7 d followed by 17 d of proliferation in experimental media containing variably elevated concentration of pyridoxine or betaine. These compounds were added in the same proportion as was used for the patients' treatment (250 mg pyridoxine, 6 g betaine). Control media contained 0.03 µM betaine as choline and 0.02 µM vitamin B6 as pyridoxal hydrochloride. Cystine effect was determined by proliferation of the cells in the presence of decreasing cystine concentration. At the end of the proliferation period, tyrosinase was extracted and activity was determined.

Methionine levels in patient 2 decreased to normal values in response to treatment with pyridoxine and folic acid, while patient 1, who was additionally treated with betaine, developed a marked increase in methionine levels attributable to betaine-induced remethylation of homocysteine to methionine. In spite of the contrast in methionine levels after treatment, both patients manifested melanotrichia, indicating that methionine does not play a role in altered pigmentation in homocystinuria.

Tyrosinase is a copper-containing enzyme, and it has been suggested that a lack of copper might be responsible for the deficiency of hair color in homocystinuria (Barber and Spaeth 1969; Shelley et al. 1972). However, analysis of plasma copper from homocystinuric patients failed to confirm this hypothesis (Dudman and Wilcken 1983). When blood copper–containing proteins and total plasma copper were measured in 15 patients with homocystinuria and compared to matched controls, increases in both total plasma copper and ceruloplasmin were found in homocystinuric patients, although the levels were unrelated to total plasma homocysteine. Yoshida et al. (1992) also studied the potential role of copper and showed that administration of homocysteine to methylmercury-treated rats resulted in a large amount of methylmercury excretion in the urine, suggesting that chelation and excretion of metals by homocysteine could lead to reduced total body metals and copper. They also measured metal concentrations in hair and plasma in two homocystinuric patients and found that they were higher than those of normal controls. They concluded that extracellular copper might be elevated in homocystinuria secondary to the elevated plasma homocysteine and that this is associated with intracellular copper depletion.

We demonstrated in this study that the addition of copper sulfate to homocyst(e)ine-inhibited tyrosinase in cultured cells restored its activity and that the addition of copper sulfate and homocysteine to holo-tyrosinase did not alter enzymatic activity. Thus, copper has a protective effect against homocyst(e)ine-induced tyrosinase inhibition. Our results are in agreement with Lerner's experiments (Lerner et al. 1950) which showed that thiol groups chelate copper ions, leading to inhibition of tyrosinase. We suggest that homocysteine which contains a free sulfhydryl group, chelates copper from the active site of tyrosinase leading to its inactivation. This can be reversed by adding copper sulfate or by decreasing the homocysteine concentration. Chelation of copper by homocysteine will explain the inaccessibility of this metal ion to the tyrosinase-active site and is consistent with the elevated plasma copper and homocysteine levels that were found in homocystinuric patients (Yoshida et al. 1992).

In addition to being fair, the hair of homocystinuric individuals is characteristically thin and brittle (Carson et al. 1965). Patient 1 reported a dramatic improvement in her hair consistency and softness in response to treatment. The mechanism for this is not clear (Mudd et al.

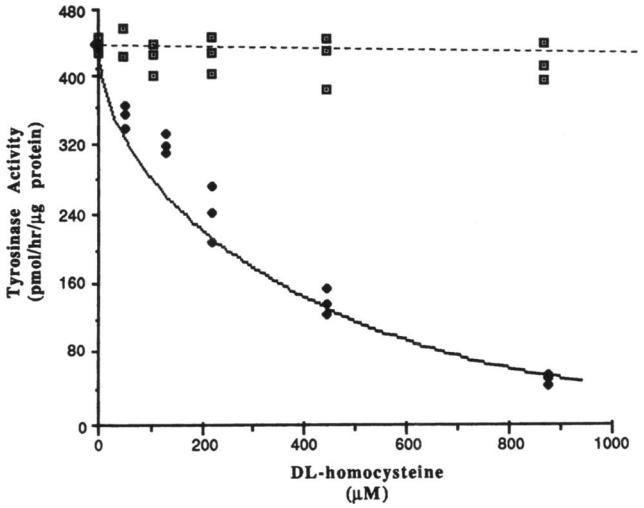

Figure 3 Prevention of homocyst(e)ine-inhibition of tyrosinase by 25% molar excess of copper sulfate. Holo-tyrosinase (enzyme extracted from MNT-1 cells that proliferated in a media with no homocysteine) was incubated for 2 h in a reaction mixture with copper sulfate and homocysteine together in ratio of 1.25 mol of copper sulfate per 1 mol of homocysteine (shown as squares [■]). Holo-tyrosinase activity assayed similarly in the presence of homocysteine without copper sulfate is shown as diamonds (◆).

1995). Copper is necessary for keratinization (Marston 1952; Gubler 1956), and collagen fibril formation. Kang and Trelstad (1973) suggested a mechanism involving reduced cross-linking of collagen, by which homocysteine could bind to allysine residues of the forming collagen fibril and prevent the cross-linking of collagen.

Another potential mechanism is a reduction in the activity of lysyl oxidase (Barber and Spaeth 1969), a copper-dependent enzyme that catalyzes the formation of cross-linking aldehydes in collagen. The interaction of homocysteine with copper, as suggested by our studies of tyrosinase inhibition by homocyst(e)ine, may alter the availability of copper in lysyl oxidase and lead to the collagen defects seen in patients with homocystinuria. Lindberg et al. (1976) showed an inhibition of lysyl oxidase activity in the presence of homocysteine in vitro, and a similar effect was demonstrated by Levene et al. (1992). In vivo studies of chick embryo lysyl oxidase by Levene et al., however, failed to show any decreased enzymatic activity in the presence of homocysteine. It seems that lysyl oxidase activity in homocystinuric patients needs further evaluation to assist determination of the copper-metabolism role in the pathogenesis of homocystinuria.

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