Thioredoxin Reductase Activity in Hermansky-Pudlak Syndrome: A Method for Identification of Putative Heterozygotes

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Recent studies indicate that membrane-associated thioredoxin reductase (TR) is a possible regulator of melanin biosynthesis via the inhibition of tyrosinase by reduced thioredoxin. In normal individuals, the levels of TR activity in skin correlate linearly to the Fitzpatrick classification of skin type, being lowest in type I skin and highest in skin type VI. In this study, TR was measured in 3-mm skin biopsies in Hermansky-Pudlak syndrome (HPS) patients and their relatives. Forty-five individuals from seven Puerto Rican kindreds were tested, including 12 homozygotes, nine obligate heterozygotes, and 24 unclassified individuals. In addition, seven separate nonkindred HPS patients were tested. With one exception, TR activity was markedly decreased in 18 homozygotes. TR activity was decreased in eight obligate heterozygotes and in 12 unclassified kindred members, whereas 10 subjects had normal TR activity when compared to the expected activity of their skin type. Four individuals were excluded from the analysis because of inadequate controls for their age group or immunosupressive treatment for kidney transplant. The results indicate that decreased TR activity assayed in 3-mm skin punch biopsies is a useful method for detecting carriers of the HPS gene. *(J Invest Dermatol 90:372–377, 1988)*

Hermansky-Pudlak syndrome (HPS) [1] is a triad of tyrosinase-positive oculocutaneous albinism, a mild bleeding diathesis resulting from storage pool deficient platelets and accumulation of ceroid material in tissues [2]. Some individual components of the HPS triad are variable. Phenotypically, a few HPS patients have resembled tyrosinase-negative oculocutaneous albinos, but most have appeared like tyrosinase-positive oculocutaneous albinos, and some have had deeply pigmented skin and hair so that they resemble ocular albinos [3]. While ceroid storage has been associated with the development of restrictive lung disease, granulomatous colitis, and kidney failure, not all HPS patients develop these disorders. Ceroid in bone marrow or urine may be absent in infants and children and even in some young adults, which suggests that accumulation of ceroid is age-dependent [2,4]. The most consistent and diagnostically accurate abnormality in HPS is a platelet-dense body deficiency [5]. Platelet-dense bodies are the storage organelles for calcium, nonmetabolic adenine nucleotides (ADP and ATP), and serotonin.

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Abbreviations:

HPS: Hermansky-Pudlak syndrome

M/F: Male-female ratio

NADPH: reduced nicotinamide adenine dinucleotide phosphate

TR: thioredoxin reductase

Thioredoxin reductase (TR) is an ubiquitous flavoprotein found in almost all living cells; it occurs as a dimer with a MW of 70,000 in *Escherichia coli,* while in eukaryotic cells it is a monomer with a MW of 58,000 [6]. The natural electron acceptor for TR is the small protein thioredoxin, which has a MW of 12,000. The electron donor for TR is reduced nicotinamide adenine nucleotide phosphate (NADPH). To date, seven functions have been described for this enzyme system: *(a)* as electron donor to ribonucleotide reductases of bacteria, yeast, mammalian cells, and plants; *(b)* as antioxidant in the reduction of methionine sulfoxide to methionine; *(c)* in the reduction of sulfate to sulfite; *(d)* in the reduction of disulfide linkages in proteins; *(e)* in insulin degradation [7]; *(f)* as a partial requirement for filamentous phage assembly [8]; and, recently, *(g)* membrane associated TR was found to be a free radical scavenger at the cell surface of live epidermis [9]. We have purified TR from the membranes of human keratinocytes and from membranes of human melanoma tissue [10,11] (Schallreuter, unpublished data).

TR activity in the epidermis of normal subjects was found to vary by skin type as classified by Pathak et al [12]. The mean values of TR units were the following: type I, 6.1; type II, 14.8; types III and IV, 20.4; type V, 25.0; and type VI, 30.6 [9]. TR was found to be reduced in a number of hypopigmented disorders [10,13]. On the basis of these observations, and of studies of TR in cultured keratinocytes and melanocytes [10,11], a hypothesis was developed proposing that TR/thioredoxin was a regulator of tyrosinase in melanin biosynthesis (Fig 1) [9]. When the skin is exposed to sunlight, UV-generated free radicals are trapped by TR at the cell surface, leaving intracellular thioredoxin in an oxidized state, thus preventing the inhibition of tyrosinase by this thioprotein; whereas in the absence of UV-light, TR reduces thioredoxin, which can subsequently inhibit tyrosinase [9,13]. Previous studies have shown that oxidized thioredoxin has no effect on tyrosinase activity, while reduced thioredoxin strongly inhibits the enzyme by forming a *bis*-cysteinyl complex with one of the active site copper atoms [14].

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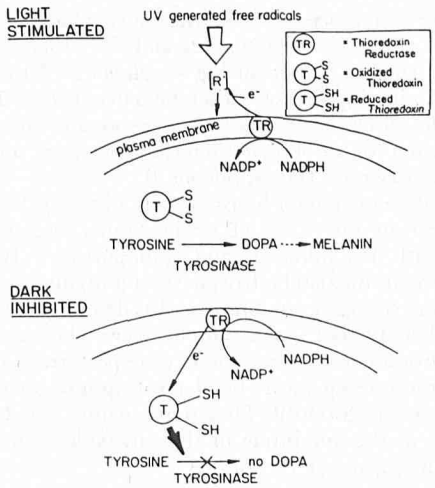

Figure 1. A molecular model for the defense against free radical attack and control of melanin biosynthesis by the thioredoxin reductase-thioredoxin system. Light Reaction: Electrons from central metabolism (*i.e.*, NADPH) flow primarily in the direction of free radical reduction at the surface of the plasma membrane (i.e., $2O_2^{\cdot-} + 3\ NADPH + 3H^+ \xrightarrow{TR} 2H_2O + H_2O_2$) [9]. This causes an increase in concentration of oxidized thioredoxin promoting melanin biosynthesis. Dark Reaction: Electrons from central metabolism primarily flow in the direction of reduction of oxidized thioredoxin in the cytosol (i.e., $T\begin{smallmatrix}S\\|\\S\end{smallmatrix} + NADPH + H^+ \rightarrow T\begin{smallmatrix}SH\\|\\SH\end{smallmatrix} + NADP^+$.)

The investigation of TR activity in the skin of 18 Puerto Rican patients with HPS and 33 relatives showed that TR activity was reduced in HPS patients as predicted by the model and that reduced TR activity assayed in 3-mm skin biopsies can detect HPS heterozygotes.

METHODS

Patients This study is part of a longitudinal investigation of HPS among Puerto Ricans [4]. A family pedigree chart was made for each propositus (Fig 2). A medical history was obtained from the propositi and their albino relatives. Albinos were examined clinically for features of albinism, hypopigmentation of skin, hair, and eyes, and the presence of nystagmus, photophobia, iris transillumination, fundal hypopigmentation, and foveal hypoplasia. Skin color was determined by visually matching skin unexposed to sunlight to a color chart, with values ranging from 1 (white) to 10 (dark brown). Hair color was determined by visually matching scalp hair to a color chart, with values ranging from 1 (white) to 14 (black). A 24-h urine sample was assayed for autofluorescent ceroid granules in sediment and urinary dolichols were measured [4]. The diagnosis of HPS was made on the basis of a deficiency of platelet-dense bodies in samples of platelet-rich plasma examined by electron microscope [4,5].

Forty-five members of seven Puerto Rican kindreds with 12 HPS patients (Fig 2) and seven nonkindred HPS patients were evaluated for TR activity. Nine individuals were obligate heterozygotes and 24 subjects were unclassified. Nonalbino patients were classified by skin type by the method of Pathak et al [12], and, with one exception, had skin types IV through VI, corresponding to skin types of the general Puerto Rican population. All non-HPS patients were in good health. HPS patients had moderate-to-severe solar changes consisting of freckles, lentigenes, pachydermia, keratoses, cheilitis, and (in two patients) lesions clinically suggestive of squamous cell carcinoma. Biopsies were taken only when the subjects of this study were on no medication for at least 6 days before examination. Four subjects were eliminated from the study. Patient 2-III-2 had a kidney transplant and was on immunosuppressive therapy. Patient 3-I-1, an obligate heterozygote, and patients 1-I-2 and 2-I-2 (un-

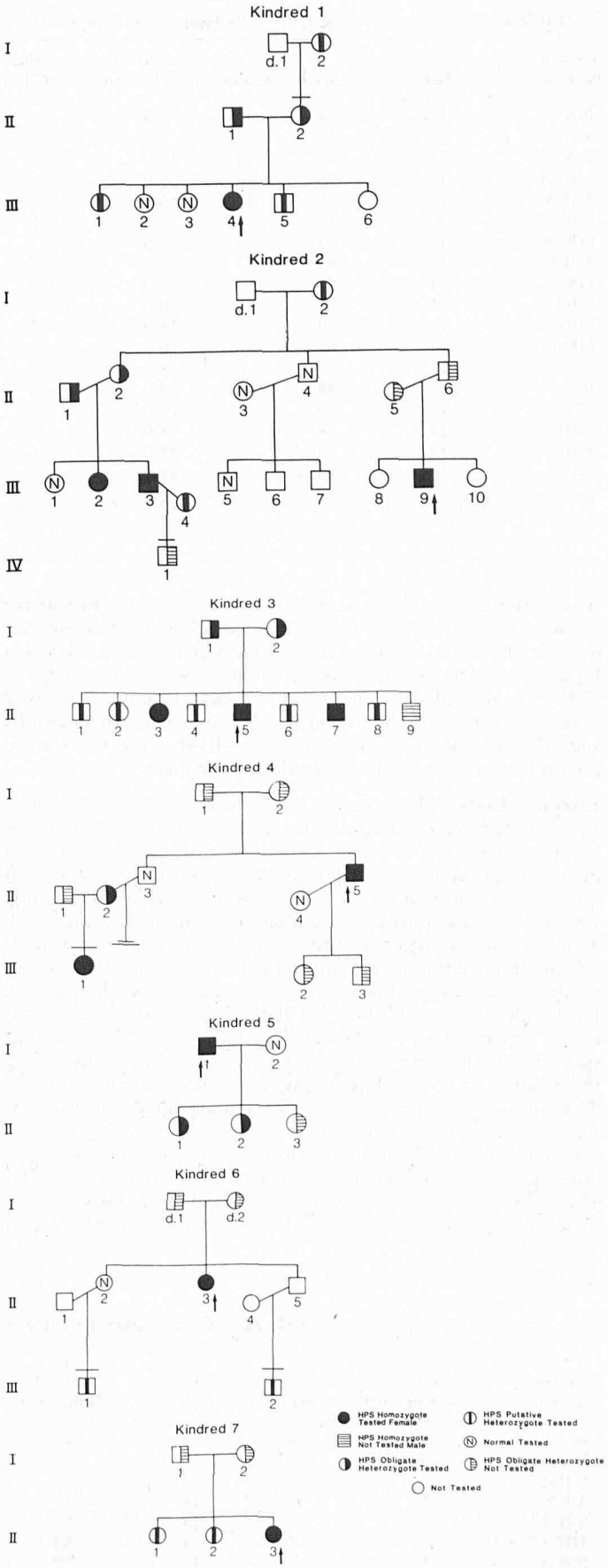

Figure 2. The seven pedigrees indicating the status of the kindred members.

Table I. Thioredoxin Reductase in HPS Homozygotes ($n = 18$)

Patient Number	Sex	Age	Hair Color	Patient's TR Units	Skin Color
NK-1	F	17	3	3.8	1
4-III-1	F	14	3	8.5	1
2-III-9	M	22	1	12.0	1
1-III-4	F	16	11	9.0	2
3-II-3	F	18	13	16.5	2
NK-2	F	17	11	14.0	2
NK-3	F	13	11	8.5	2
4-II-5	M	40	11	10.5	2
NK-4	M	26	3	17.0	2
5-I-1	M	38	5	10.8	2
NK-5	F	29	2	14.0	2
NK-6	M	11	6	16.5	3
7-II-3	F	27	3	8.5	3
6-II-3	F	44	11	17.0	3
NK-7	F	38	3	12.0	3
2-III-3	M	28	6	18.0	4
3-II-7	M	13	12	11.0	5
3-II-5	M	16	13	24.0	7
$\bar{X}$	M/F = 8:10			12.9 ± 4.7	2.6

classified) were eliminated because they were over 65 years of age and so older than the age range of the control population. Subjects resided in Puerto Rico, except those of Kindred 2. Patient 4-II-4 was Equadorian. The members of Kindred 2, with one exception (2-II-9), have resided continuously in Chicago, Illinois, for up to 32 years, with brief visits to Puerto Rico. All subjects in generations III and IV were born in Chicago except 2-III-9, who was born in Puerto Rico and resides in Milwaukee, Wisconsin.

Enzyme Assays Thioredoxin Reductase The most important aspect in studying extracellular free radical reduction resides in the selection of a radical substrate showing the incapability of plasma membrane penetration. An ideal substrate is one isoelectronic with OH· radical and with cationic surfactant properties. Accordingly, we synthesized a spin-labeled quaternary ammonium compound, which is a simple analogue of the common preservative trimethylbenzalkonium chloride. Preliminary experiments showed that this substrate is incapable of penetrating outer plasma membranes [15]. Furthermore, we established that the reduction rate for nitroxide radical depends on the surface area of skin biopsies [13]. Identical specific activities were obtained for reactions performed by adding the substrate in vivo (before biopsy) or in vitro (after biopsy) [13]. The following standard procedure was found to give reliable results relative to individuals skin type.

Individuals with TR values within the range expected for their skin types were considered as unaffected noncarriers of the HPS gene. The differences between their expected mean TR values for their skin type and their observed TR values were plotted in Fig 4, *column 1*. The differences between the expected mean TR values by skin type of obligate heterozygotes and the observed TR values were calculated and plotted in Fig 4, *column 2*. Those remaining unclassified individuals whose differences in expected TR values by skin type and observed values that were greater than the lowest value calculated for an obligate heterozygote were subclassified as putative heterozygotes (Fig 4, *column 3*).

Three-millimeter punch biopsies were obtained from clinically normal appearing skin of the left or right inner forearm under local anesthesia with 1% lidocaine and epinephrine 1:100,000. The biopsies were immediately frozen in liquid nitrogen, stored at −70°C before the assay, and measured as described by Schallreuter and co-workers [9,10,15]. All samples were taken in the patient's normal environment. Measurements were performed in quartz tissue cells at room temperature by electron spin resonance spectroscopy in a Bruker D 200 EPR. TR activity in units was determined as the decrease of the amplitude of the nitroxide radical signal per 3-mm punch biopsy per 10 minutes.

RESULTS

The kindreds are presented in Fig 2, and the results are summarized in Tables I to V and in Figs 3 and 4. The mean TR activity for all HPS patients (Table I) was 12.9 units; this is considerably less than the values expected in a normally pigmented Puerto Rican population with skin types IV, V, and VI ($\bar{X}$ = 20.4, 25.0, and 30.6 TR units, respectively). Further, they were significantly decreased compared to their unaffected pigmented relatives ($\bar{X}$ = 25.5 units, $P < 0.001$, Table V).

Table II shows a mean activity of 17.1 TR units for obligate heterozygotes, with an expected mean activity of 26.0 TR units based upon their skin types, which ranged from types IV to VI. Their observed TR activity as well as their activity adjusted for skin type was significantly lower than those of the unaffected subjects ($P < 0.001$, Table V).

Table III are those patients classified as putative heterozygotes. The difference between their observed and expected TR activity by skin type exceeds the lowest difference shown by an obligate heterozygote (Fig 4). The mean TR activity for putative heterozygotes was 14.6 units compared with an expected mean value of 27.0 TR units based upon their skin types.

Table IV shows that unaffected noncarriers had a mean TR activity of 25.5 TR units, being almost identical to the mean of 25.2 TR units expected for their skin type.

The results plotted in Fig 3 summarize the raw data on TR activity. The difference between the expected TR values by skin type and observed values are plotted in Fig 4.

Three individuals over 65 years of age 1-I-2, 2-I-2, and 3-I-1 were excluded from the tables, because adequate data from a control population for this age group are unavailable. However, a tentative interpretation of the TR activity of these three patients indicates that they had lower activity than the few controls tested to date.

Table II. Thioredoxin Reductase in HPS Obligate Heterozygotes ($n = 8$)

Patient Number	Sex	Age	Hair Color	Patient's Skin Type	Expected TR Units By Skin Type	Observed TR Units	Expected Minus Observed TR Units	Skin Color
4-II-2	F	36	13	IV	20.4	12.0	8.4	5
2-II-2	F	48	13	IV	20.4	16.0	4.4	7
1-II-2	M	42	14	V	25.0	12.0	13.0	8
3-I-2	F	43	14	V	25.0	15.0	10.0	6
5-II-1	F	11	14	V	25.0	14.0	11.0	6
1-II-1	F	40	14	VI	30.6	23.0	7.6	5
2-II-1	M	51	14	VI	30.6	23.5	7.1	7
5-II-2	F	10	14	VI	30.6	21.0	9.6	5
$\bar{X}$	M/F = 2:6				26.0	17.1 ± 4.8	8.9 ± 2.6	6.1

Table III. Thioredoxin Reductase in HPS Putative Heterozygotes ($n = 12$)

Patient Number	Sex	Age	Hair Color	Patient's Skin Type	Expected TR Units By Skin Type	Observed TR Units	Expected Minus Observed TR Units	Skin Color
3-II-2	F	19	14	IV	20.4	14.8	5.6	5
6-III-1	M	22	13	IV	20.4	8.5	11.9	3
3-II-6	M	14	14	V	25.0	11.0	14.0	8
3-II-8	M	10	13	V	25.0	18.5	6.5	3
2-III-4	F	28	14	V	25.0	13.5	11.5	8
6-III-2	M	14	13	V	25.0	20.3	4.7	5
1-III-5	M	18	14	VI	30.6	15.0	15.6	8
1-III-1	F	21	14	VI	30.6	8.0	22.6	4
3-II-1	M	21	14	VI	30.6	17.0	13.6	7
3-II-4	M	17	13	VI	30.6	12.0	18.6	7
7-II-1	F	35	14	VI	30.6	14.5	16.2	5
7-II-2	F	46	14	VI	30.6	21.5	9.1	5
$\bar{X}$	M/F = 7:5				27.0	14.6 ± 4.3	12.5 ± 5.4	5.7

Table IV. Thioredoxin Reductase in Unaffected Family Members ($n = 10$)

Patient Number	Sex	Age	Hair Color	Patient's Skin Type	Expected TR Units By Skin Type	Observed TR Units	Expected Minus Observed TR Units	Skin Color
5-I-2	F	38	14	III	20.4	23.0	−2.6	5
4-II-5	F	39	14	IV	20.4	22.5	−2.1	4
2-II-3	F	44	14	V	25.0	27.5	−2.5	7
2-III-5	M	22	13	V	25.0	25.5	−0.5	4
2-II-4	M	51	14	V	25.0	25.5	−0.5	7
6-II-2	F	56	13	V	25.0	28.5	−3.5	4
4-II-3	M	39	13	V	25.0	28.0	−3.0	5
2-III-1	F	26	14	V	25.0	21.0	4.0	7
1-III-3	F	16	14	VI	30.6	26.8	3.8	5
1-III-2	F	20	14	VI	30.6	27.0	3.6	5
$\bar{X}$	M/F = 3:7				25.2	25.5 ± 2.6	−0.3 ± 3.0	5.3

DISCUSSION

The specificity for the reduction of the spin-labeled quaternary ammonium compound by membrane-associated TR at the surface of human skin has been supported by the following evidence.

(I) Thioredoxin reductase has been purified from membranes of human keratinocytes [10] and from membranes of human metastatic melanoma tissue [11] (Schallreuter, unpublished data). These pure enzymes from human sources have identical properties to the well-characterized protein from rat liver [18] (i.e., 58,000 daltons, containing one bound FAD molecule as determined by fluorescence emission spectra).

(II) The thiolate active site of TR has a low enough potential (−271 mV) to reduce the spin-labeled quaternary ammonium compound to a secondary amine. [9].

(III) TR is inhibited by its own oxidized coenzyme $NADP^+$ [10], by Ca^{++} [16], and by azelaic acid [11]. Effective inhibition has been demonstrated for these three substances at the surface of the skin, on human keratinocytes, melanocytes, and on pure enzymes isolated from *E. coli,* rat liver, human keratinocytes, and human metastatic melanoma. Other free radical traps, such as glutathione reductase, superoxide dismutase, and catalase, do not react with the spin-labeled quaternary ammonium substrate or with the above inhibitors [9].

(IV) Thioredoxin is a specific electron-accepting protein for TR [7].

(V) While TR activity is low in HPS, glutathione reductase activity in platelets and leukocytes has been found to be normal.

The choice of Puerto Rican HPS subjects from this study has an advantage over selecting other albinos primarily because controls in this population have high TR activities based on skin types IV to VI. [13]. This provided a clear separation in activities between HPS homozygotes and normal family members.

The initial purpose of this study was to test the proposed connection between TR and skin pigmentation. It was anticipated that in a tyrosinase-positive form of oculocutaneous albinism with low tyrosinase activities, such as HPS, the HPS homozygotes should express low TR activity with a possible feedback inhibition on tyrosinase. The data from 18 HPS homozygotes revealed that all but one subject has TR activities well below those expected for normal Puerto

Table V. Means, Standard Deviations, and Comparisons of Observed TR Values and Expected Minus Observed TR Values by Skin Type

Subjects	Observed TR Units	Expected Minus Observed TR Units by Skin Type
	n, mean + SD	
Normal	10, 25.5 ± 2.6	10, −0.3 ± 3.0
HPS	18, 12.9 ± 4.7	—[a]
Obligate heterozygotes	8, 17.1 ± 4.8	8, 8.9 ± 2.6
Putative heterozygotes	12, 14.6 ± 4.3	12, 12.5 ± 5.4
Normal vs. HPS	$t = 7.79$, $p < 0.001$	—[a]
Normal vs. obligate heterozygotes	$t = 4.85$, $p < 0.001$	$t = -6.82$, $p < .001$
Normal vs. putative heterozygotes	—[b]	—[b]
Obligate vs. putative heterozygotes	—[b]	—[b]

[a] What the expected skin type of HPS patients would have been in the absence of albinism is unknown. Thus these values cannot be calculated.

[b] As putative heterozygotes were defined by TR values less than the highest value shown by an obligate heterozygote, a statistical comparison is invalid. See text discussion of Table V.

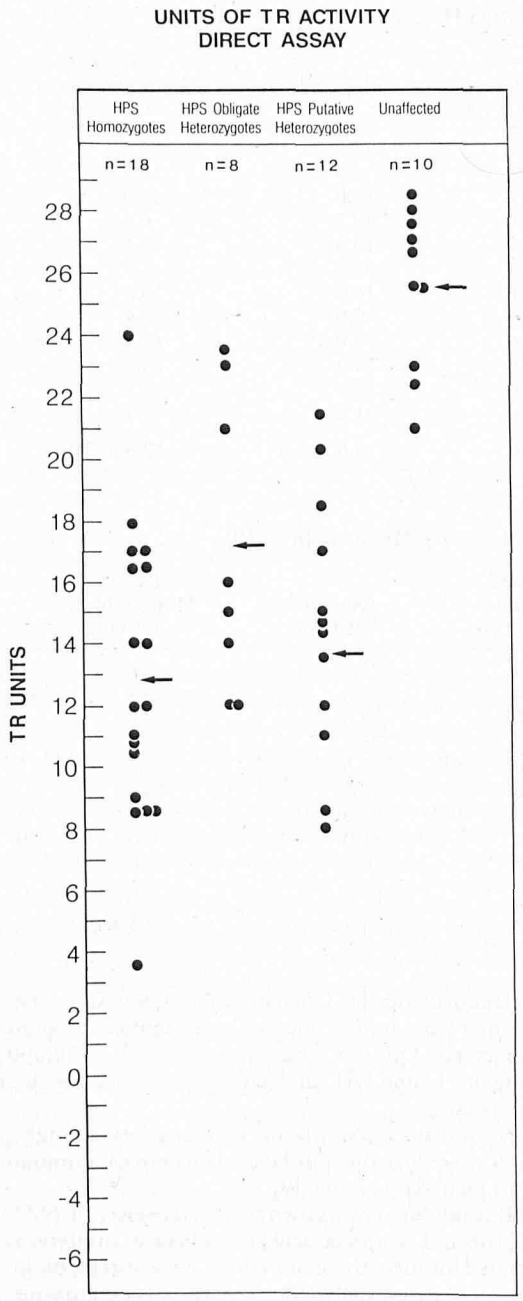

Figure 3. Distribution of the directly assayed individual values of TR activity by patient category. Arrows indicate means.

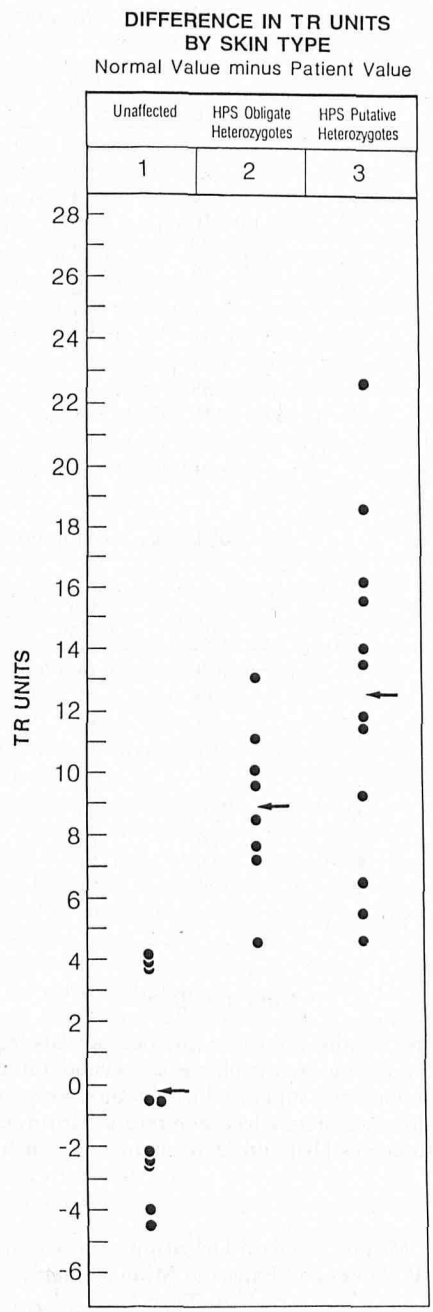

Figure 4. The distribution of the differences between the patients expected TR activity by skin type and patients observed activity. Arrows indicate means.

Rican skin types, which supports the thesis of a possible regulatory connection between free radical defense in the epidermis (TR) and melanin biosynthesis. Only one patient, 3-II-5 (Table I), who was phenotypically an ocular albino with medium brown skin color and dark brown hair, had normal TR activity.

The unexpected results in this study were the low TR activities in obligate heterozygotes, which allowed the identification of the putative heterozygotes among the initially unclassified patients. Figure 3 shows that the TR activities of the obligate heterozygotes ($\bar{X} = 17.1$ units) are significantly lower ($P < 0.001$, Table V) than the activities of the unaffected subjects ($\bar{X} = 25.5$ units), while the range of the two groups overlap. This raw comparison does not take into consideration that TR activity varies by skin type. Upon subtraction of the patient's observed TR activity from the expected value for their skin type, there is a good separation of groups (Fig 4). If this test is used to detect unknown heterozygotes, only a few individuals would be misclassified.

Table V does not show a statistical comparison of observed and expected minus observed TR activities based upon skin type of normals versus putative heterozygotes nor of obligate versus putative heterozygotes, as putative heterozygotes were defined as those with TR activities lower than the lowest difference between expected and observed TR activity of an obligate heterozygote. Thus, though both TR activity values were significantly different when normals were compared with putative heterozygotes ($P < 0.0001$), and were not when comparing obligate with putative heterozygotes, these are, nevertheless, invalid statistical comparisons.

All individuals on the kindred charts (Fig 2) are genetically compatible with their tested genotypes, except, possibly, for 6-III-1. His TR values indicate that he is a carrier, but the TR value of his mother, 6-II-2, indicates that she is an unaffected noncarrier. Unfortunately, his father, 6-II-1, was unavailable for testing. As the prevalence of HPS in the community in which his family resides is

approximately one in 946 persons (67 HPS in a total population of 63,350), the father would have an a priori chance of approximately one in 15 of being a carrier. Patient 6-II-5 was not biopsied, as he was a severe diabetic.

TR activity at the surface of human skin is driven by the electron donor NADPH, generated intracellularly from central metabolism. Therefore the same levels of NADPH are expected in all skin types. According to the model (Fig 1), for type I skin most of the electrons from NADPH would flow in the direction towards the reduction of thioredoxin (T), changing the $T_{ox} \rightleftharpoons T_{red}$ equilibrium and leading to the inhibition of tyrosinase through direct complexation with T_{red}. [14]. For type VI skin most of the electrons from NADPH would be used to reduce free radicals, leaving more oxidized thioredoxin to stimulate tyrosinase, thus producing more melanin. However, we have shown that TR is regulated by Ca^{++}, and therefore fine control of pigmentation may depend on extracellular and intracellular Ca^{++} concentrations.

The dilemma remains: Why are HPS carriers phenotypically normal despite low TR activities? This is an important issue because TR may be central to free radical defense and pigmentation. The answer to this question may reside in the regulation of the TR/thioredoxin/tyrosinase cascade by calcium [13,16]. Recent studies in our laboratory with cell cultures of human keratinocytes established from HPS homozygotes showed an increased inhibition by calcium [17]. This effect on cultured keratinocytes is even greater than that reported for patients with vitiligo [10], in which TR was proved to be regulated at the enzyme rather than the gene level, but the allosteric effect of calcium on TR could be under genetic control of the plasma membrane Ca^{++}-ATPase [10,16,17].

According to our earlier results [10,16], increased extracellular Ca^{++} has been shown to affect TR directly by allosteric inhibition of extracellular free radical reduction using intracellular NADPH as electron donor [16,17]. However, this reaction with Ca^{++} outside the cell does not affect the transfer of electrons from NADPH through TR to thioredoxin in the cytosol, an intracellular reaction that is controlled by the internal Ca^{++} concentration. As discussed before, HPS homozygotes have low extracellular TR activity indicative of an increased inhibition of free radical reduction by extracellular Ca^{++}. This may be the principal reason for increased susceptibility to UV-light causing the observed solar skin damage. Since these patients may have low levels of Ca^{++} in the cytosol because of a defective Ca^{++}-ATPase uptake system, electron transfer to thioredoxin would be expected to increase the concentrations of reduced thioredoxin and, as a consequence, inhibit tyrosinase. It is well established that both the obligate and putative heterozygotes in HPS have normal platelet-dense bodies, thus maintaining normal higher intracellular Ca^{++} concentrations that may decrease thioredoxin reduction. This would explain normal pigmentation in these carriers, even though they exhibit low extracellular TR activity.

In summary, we conclude that local concentrations of Ca^{++} on the external surface of the plasma membrane and in the cytosol are expected to have a similar effect on the equilibrium between oxidized and reduced thioredoxin, inside the cell, to give a result close to that proposed for the light to dark regulation presented in Fig 1. Clearly a regulatory connection between Ca^{++} and the TR/thioredoxin/tyrosinase cascade is supported by this study. Furthermore, the extent of solar damage observed on the 19 homozygous HPS subjects highlights the importance of TR activity in controlling the defense against UV-generated free radicals.

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