Urinary Biopterin Levels and Blood Dihydropteridine Reductase Activities in Patients with Thyroid and Breast Disorders

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Abstract

Biopterin as a stable metabolite is produced by oxidation of 5,6,7,8-tetrahydrobiopterin (BH4). It is known that many diseases may cause changes in BH4 concentration and/or dihydropteridine reductase (DHPR) enzyme activity. There is only a limited number of studies correlating DHPR activity in malignancies. The main goal of the present study was to evaluate alterations in the DHPR activity and biopterin levels in patients with breast and thyroid cancers. The breast cancer patients (n=24) were compared to patients with benign breast diseases (n=19) and controls (n=30). In addition; the patients with thyroid cancer (n=17) were compared to patients with benign thyroid diseases (n=42) and the control group. We did not observe any significant difference between the benign disorders and the malignancies. Biopterin concentrations in patients with benign and malign thyroid and breast diseases were lower than the controls (all p <0.05). DHPR activities in thyroid diseases were significantly higher than the controls while insignificant decreases in DHPR activities in breast patients were detected. Our results suggested significant alterations in unconjugated pteridine pathway in thyroid and breast disorders.

Key words: biopterin, dihydrobiopterine reductase (DHPR), breast, thyroid

Introduction

5,6,7,8-Tetrahydrobiopterin (BH4) is an endogenously synthesized cofactor, required for various enzyme activities and for some less well-defined functions on the cellular level (1). BH4 is best recognized as the essential, non-protein cofactor for the aromatic amino acid monoxygenases, that rate limiting enzymes in phenylalanine, tyrosine, and tryptophan catabolism and in biosynthesis of the neurotransmitters dopamine and serotonin (2). Every cell or tissue of higher organisms possibly contains BH4, which additionally appears to be involved in other metabolic and cellular functions (2). BH4 is an essential cofactor for phenylalanine hydroxylases, glycercyl-ether monoxygenase, and three main nitric oxide synthase isoenzymes (3). Thus far, nitric oxide synthase is the only enzyme where biopterin radical formation is clearly linked to its catalytic properties (4). Regeneration of BH4 from its oxidized forms is particularly important in biological system (4). The enzymes, pterin-4a-carbinolamine dehydratase and dihydropteridine reductase (DHPR) are responsible for the regeneration of BH4 (7-9). As shown in Figure 1, the quinonoid form of dihydrobiopterin is mainly reduced by DHPR (4). It has been recognized that

be a major cause of the loss of antioxidants and the development of oxidative stress. Subnormal concentrations of antioxidants, like vitamin C and E, have been observed in diseases associated with immune activation and inflammation, and may also impair availability of BH4 in patients with malignancy (5, 6). These clinical conditions are known to be linked with inflammation and immune activation and with increased concentrations of immune activation markers such as serum soluble 75 kDa tumor necrosis factor-α (sTNF-R75) and neopterin (7, 8). On the other hand, it has been shown that BH4 enhances the proliferative activity of hemopoietic and leukemic cells (2).
many diseases and xenobiotics including drugs may cause a change in BH₄ concentration and/or DHPR activity (10-15).

Tetrahydropterins such as BH₄ are labile in solution and react with oxygen, superoxide, hydrogen peroxide, and peroxynitrite because this molecule has a core structure of two or three heterocyclic six-membered rings (4). This property has been suggested to act as a self-protection in order to protect cells against oxidative damage (4, 7, 8). It is also known that the role of pteridine derivatives in oxidative stress may differ according to the conditions (Figure 1) (16-21). For instance, neopterin derivatives may directly interfere with the intracellular redox balance as a potential function of neopterin and 7,8-dihydroneopterin in oxygen radical mediated process (20, 21). The role of inflammation is a debated subject in the area of cancer research and furthermore the role of inflammatory markers in the follow up of malignancy and the follow up of response to therapy is continuously investigated (22). In our previous studies we have focused on neopterin as a biomarker part of the pterin pathway in both of patients with thyroid and breast disorders (23, 24). Still there is only a limited number of studies on changes in BH₄ pathway and/or DHPR activity in cancer patients (25-33). Therefore, in the present study we evaluated the alteration in biopterin excretion and DHPR activity which would reflect impairment of BH₄ maintenance in patients with breast and thyroid disorders.

**Materials and Methods**

**Subjects**

Total one-hundred-two patients were included in the study and the demographics of the subjects are presented in Table 1. Forty-three of them were operated...
for breast disorders. Twenty-four of the patients had breast cancer while the others had benign breast tumor. The benign tumor group consisted of fibroadenoma and fibrocystic disease patients. The preoperative diagnosis of all breast cancer patients were confirmed by core biopsy and recurrent cases were excluded from the study. The exclusion criteria of the study were; obscure perioperative diagnosis, history of chemotherapy or radiotherapy, the patients with familial pattern of breast diseases, the patients who had a history of breast or other organ malignancies and presence of active infection at the time of sample collection. Fifty-nine of the patients were operated for various thyroid disorders. Seventeen of the thyroid patients were papillary thyroid cancer while the others were benign thyroid disorders (including thyroiditis and multinodular goiter). Preoperative malignancy was diagnosed by fine needle aspiration biopsy. The excluding criteria were; preoperative indetermined diagnosis of malignancy, history of chemotherapy or who are currently receiving antithyroid therapy, history of irradiation to the neck and history of prior thyroid surgery. All the patients were euthyroid at the time of operation. The control group consisted of thirty healthy subjects who work in the Faculty and were systemically healthy and had not received any medication during this period. The principles of the Ethical Committee according to the Helsinki Declaration were followed during the study.

Measurements of biopterin

Urine samples were collected early in the morning before the operation and kept from direct light. All samples were stored at -20°C until the assayed. Biopterin concentrations in each urine sample were analyzed by high performance liquid chromatography (HPLC, HP Agilent 1100, Vienna, Austria) without any oxidation step during sample preparation. A column (25 cm X 4.6 mm) containing octadodecyl silica gel C$_{18}$ (5 µm particle size; Hichrom), protected with a 4-cm guard column filled with the same material was used. Biopterin was isocratically eluted at a flow rate of 1 mL/min with 15 mM potassium dihydrogen phosphate buffer containing 2.5% methanol (v/v), pH 7.0 and quantified using a fluorescence detector ($\lambda_{ex}$: 353 nm, $\lambda_{em}$: 438 nm). Creatinine concentrations were determined simultaneously by using a fluorescence detector (HP Agilent 1100) at the wavelength of 235 nm. The biopterin levels were expressed as micromoles of biopterin per mole of creatinine.

DHPR measurements

Peripheral venous blood samples from the subjects were drawn and dropped on a filter paper. DHPR enzyme assay was performed on dry blood spots. After measuring the diameter of each blood spot, it was cut and extracted with 0.15 M cold KCl solution at 0 - 4 °C. This elute was used in the assay of DHPR enzyme activity. Enzyme activity was measured spectrophotometrically at 550 nm wavelength (Shimadzu UV160, Japan) by following the BH$_4$-dependent reduction of ferricytochrome C in the presence of NADH (14). The assay tube contained Tris-HCl buffer, pH 7.6, ferricytochrome C in Tris HCl containing KCl, pH 7.6, NADH in KOH, 6-methyl-tetrahydropteridine (Sigma, USA) in 0.01 M HCl and the enzyme extract, in a final volume of 2 ml. To correct for pterin and enzyme-independent reduction of ferricytochrome C, appropriate blanks were used. The enzyme activity was expressed as nanomoles of cytochrome C reduced per minute relative to the 6 mm diameter of blood spots.

Statistical analysis

All of the results are expressed as the mean ± standard error of mean (SEM). Because not all data sets showed normal distribution, non-parametric methods were used for their statistical analyses. The differences among the groups were evaluated with Kruskal-Wallis analysis of variance, and comparisons between two independent groups were made with the Mann-Whitney U-test. The correlations of the parameters were detected by Spearman non-parametric correlation test. Furthermore, the results were confirmed with a multivariate regression model. P <0.05 was considered statistically significant.

Results

The mean biopterin excretion was found as 93.9 ± 7.12 µmol/mol creatinine in the controls while the mean results were 32.8 ± 4.32 and 31.4 ± 5.38 µmol/mol creatinine in benign and malign breast tumors, respectively. As shown in Figure 2, biopterin concentrations in both benign and malign breast patients were statistically lower than in controls (both p <0.001). However, there was no significant difference between benign and malign breast disorders groups. The biopterin concentrations in benign and malign thyroid disorders were determined as 64.2 ± 6.26 and 82.6 ± 15.5 µmol/mol creatinine, respectively. There was no difference in biopterin concentrations between benign and malign thyroid groups (not significant), while there were significant differences between the controls and benign cases (p <0.001) or malign thyroid patients (p = 0.025).

The results of measurements of DHPR activity in the study groups were given in Figure 3. There were no significant differences in DHPR activities among the
Figure 2. Box plots of urinary biopterin concentrations of the patients groups (shown are medians = horizontal lines, interquartile ranges = boxes, and ranges = bars). MT, malign thyroid (n = 17); BT, benign thyroid (n = 42); MB, malign breast (n = 24); BB, benign breast diseases (n = 19), and C, control group (n = 30). ‡p < 0.05 vs. control group

Figure 3. Box plots of blood dihydropteridine reductase (DHPR) activities of the patients groups (shown are medians = horizontal lines, interquartile ranges = boxes, and ranges = bars). MT, malign thyroid (n = 17); BT, benign thyroid (n = 42); MB, malign breast (n = 24); BB, benign breast diseases (n = 19), and C, control group (n = 30). †p < 0.05 vs. control group
breast disorders groups and the control group (both not
significant). Conversely, the mean values of DHPR
activity in both of the benign and malignant thyroid
groups were significantly higher than the control results (both p <0.05). However, the difference
between benign and malignant thyroid patients was not
significant (not significant).

There were no correlations between the mean
biopterin concentrations and DHPR activities in the
study groups (all not significant).

The effects of age and gender on biopterin levels
and the enzyme activities were checked in control
healthy subjects. There were neither associations
between age and the measured biopterin concentra-
tions nor the DHPR activities (all not significant).
Furthermore, gender did not affect the results either
(all not significant).

Discussion

For the last 40 years, the evaluation of pteridines
has been focused on obtaining knowledge about the
unconjugated pteridine pathway (34). Due to the insta-
Bility of BH₄, the main metabolites neopterin and
biopterin are preferred to gain further information on
the pathway. A number of publications has document-
ed the anomalies in unconjugated pteridines metabo-
lim in patients suffering from malignant diseases, in
which there was a characteristically elevated urinary
excretion of neopterin, which is produced in human
monocytes/macrophages at the expense of BH₄ (15,
22-24, 35-37). The studies have potentiated the role of
neopterin as the biomarker of immune system stimula-
tion. Therefore in our previous studies we have
demonstrated the correlation of serum and urinary
neopterin concentrations with the malignant breast and
thyroid disorders (23, 24, 37). Furthermore, we have
also emphasized the correlation between neopterin concentra-
tions and the grade of the tumors (23, 24).

DHPR converts quinonoid dihydrobiopterin to BH₄
in a NADH-mediated reaction (38). The measurement
of pterins in different biological fluids is the most com-
mon method for the screening and differential diagno-
sis of inborn errors of BH₄ metabolism (39). In addi-
tion, biopterin accumulates upon oxidation of BH₄ and
is excreted by urine (34). Therefore we preferred to
detect the biopterin in the urine samples of the patients
in our study.

Thyroid is an oxidatively active organ and the
majority of thyroid tumors represent well differentia-
ted functional tumors (24). Thyrocytes produce con-
stantly moderate amounts of reactive oxygen species
(ROS), which are physiologically required for thyroid
hormone synthesis. To maintain cell integrity, several
protective systems against ROS are active in thyro-
cytes. Increased oxidative stress is not necessarily
lethal for goitrous cells but is associated with large cel-
ular destruction and inflammation in iodine-induced
thyroid involution (40). Our results pointed out
deceased urinary biopterin in both of the thyroid and
breast disorders in comparison with the control sub-
jects. This situation is not a surprising outcome since it
might originate from an increased DHPR activity. In
the present study we have found that the urinary
biopterin levels in malignant tumors of thyroid were
higher when compared to benign counterparts which
are suggestive of a better antioxidant protection in
patients with benign diseases. The increase in DHPR
activity may be an adaptive process to enhanced oxida-
tive stress which is not well compensated by other
antioxidant mechanisms.

Dhondt et al. (27) focused on a study in order to
evaluate DHPR activity in breast cancer patients and
they reported that large variations in breast malignan-
cy. Furthermore, they pointed out a significant correla-
tion between DHPR activity and hormonal depen-
dence, as measured by cytosolic estrogen receptor
sites. In another study performed by Sanchez-Urretia
et al. (26), it was reported that foetal tissues and neo-
plasm exhibit lower activities than the cognate adult
tissues. Despite the fact that the urinary biopterin le-
vels in thyroid patients were lower than the controls, in
the present study, higher biopterin concentrations were
observed in malignant thyroid disorders in comparison
with the benign thyroid group. Similar to thyroid
group, the mean biopterin concentrations in breast
patients were lower than the controls. Moreover, the
reduction in biopterin concentrations in patients with
breast disorders was considerably more than the
biopterins in thyroid disease. In patients with breast
disorders, DHPR activity as a related parameter to
biopterin pathway was close to the mean enzyme acti-
vity of controls while it was lower than the mean
DHPR activity in thyroid patients. The reduced DHPR
activity in breast tumors is believed to be related to the
characteristics of the tumor.

It can be concluded that analysis of these mecha-
nisms on the tissue level may be more explanatory, it
may reveal more informative results. Moreover, fur-
ther studies with different cancer types are needed to
answer and explain some points in maintenance of the
pathway.

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