B-HYDROXYDEOXYGUANOSINE AS A URINARY BIOMARKER OF OXIDATIVE DNA DAMAGE

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Living organisms are continuously exposed to pro-oxidant oxygen species as a consequence of biochemical reactions as well as external factors. Oxidative DNA damage has been implicated in aging, cancer progression and other degenerative diseases. The urinary excretion of the DNA repair product B-hydroxydeoxyguanosine (BHGMC) has been proposed as a non-invasive biomarker of oxidant DNA damage in humans. In 1980, we have developed a one-dimensional HPLC analysis with electrophoretic detection for the analysis of BHGMC in urine and studied the effect of the consumption of 83 healthy humans in various laboratory settings, including day, night, and weekend. Previously, other workers have used comparable HPLC methods or gas chromatography-mass spectrometry with triphenyl tetrazolium for measuring the excretion of BHGMC in humans, rats, mice, and monkeys. In the 169 humans studied so far, the average BHGMC excretion was 200-300 pmol per 24 hr with a semiconstant range, and the coefficient of variation was 30-45%. This variation corresponds 140-200% of the variation of a given population per cell per day. For two studies from our study population exceeded 80% (45-95% confidence interval), more BHGMC than 5% (5% 95%) was detected. This indicates a 50% increased rate of oxidative DNA damage from smoking, working in the other well-known factors, and smoking. The urinary biological half-life of BHGMC is unknown, but may be related to renal concentrations including as generating reactive oxygen species and/or consuming exopolis and/or the well-known enhancing effect of smoking on the renal rate. In this study, healthy subjects the BHGMC excretion correlated with body composition. Thus, lean and elderly males excreted more than obese, and/or female subjects, possibly related to differences in metabolic rate. In conclusion, the excretion of BHGMC decreased after cardiac allograft, which causes a blunting of the metabolic rate. Among the investigated species, humans, dogs, rats, and mice, the excretion of BHGMC correlates with the specific metabolic rate, confirming data from other species on humans, monkeys, and mice. The excretion of BHGMC decreases with age in parallel with the decrease in metabolic rate with advancing age. The excretion of BHGMC reflects the formation and repair of only one of approximately 20 described oxidative DNA modifications. 50% excreted methods are not available as a reliable determination of the corresponding major products, except BHGMC and aminoguanidine, which are also. Moreover, in terms of measurement, particularly reactive urinary supernatants and urine samples, it is necessary to determine BHGMC in DNA. In addition, only one scavenger system enzyme complexes for BHGMC has been demonstrated, but whether the main product is BHGMC or the base is yet unknown. In addition, BHGMC may arise from DNA during treatment of radioactive and with a reflux cirrhotic patients.

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INTRODUCTION

In the living organism, reactive oxygen species (ROS) are formed continuously as a consequence of biochemical reactions as well as external factors. Inadequate physiological antioxidant defense systems may thus lead to oxidative damage (Halliwell and Catterall, 1989). ROS are proposed as important in aging as well as a number of age-related degenerative diseases, including cancer, heart disease and other inflammatory conditions, and cataract formation (Ames and Salloum, 1986; Spector and Williams, 1981; Ames, 1989; Frazer et al., 1980; Luttrell, 1990; Reilly et al., 1991; Greenwald, 1991; Ames and Gold, 1991).

During mitochondrial respiration a substantial part (~1-3%) of the consumed oxygen escapes the simultaneous transfer of four electrons and undergoes stepwise single-electron transfer, generating ROS, the superoxide anion radical, hydrogen peroxide, and the hydroxyl radical (Farreras and Bowman, 1980; Reilly et al., 1991). The first line of defense against the superoxide radical is superoxide dismutase, which forms hydrogen peroxide. This may in turn be converted to water by glutatione peroxidase or catalase. Hydrogen peroxide has limited reactivity and crosses membranes easily. In the presence of transition metals hydrogen peroxide is reduced to hydroxyl radicals, the most reactive ROS. Superoxide radicals and hydrogen peroxide, which in turn may give rise to hydroxyl radicals, are generated by many other mechanisms, for example, via hypoxia/myeloperoxidase action by the sputum oxidase system during postischemic reperfusion, by circulatation during the metabolism of xenobiotics and aromatic acids, and in phagocytes (Reilly et al., 1991). Nitric oxides also generate hydroxyl radicals, a very reactive free radical known from household bleach, during their respiratory burst by means of myeloperoxidase and chloride ions (White, 1989). Ionizing radiation generates superoxide and hydroxyl radicals (Crawford, 1987). Single oxygen, also an important ROS, is generated by illumination of photosensitizers and in minor amounts by cyclotron and other exotic reactions (Ree, 1991).

ROS can attack any cellular structure or molecule. However, with respect to aging and cancer, DNA is considered an important target (Amos and Gold, 1991). Thus, ROS may cause DNA protein cross-links and DNA damage as well as specific chemical modifications of the purine and pyrimidine bases (Dizdaroglu, 1991). Oxidation of the sugar moiety induces base release and strand breaks whereas oxidative base modifications result in mutations (Kochino et al., 1987; Shibutani et al., 1991; Cheng et al., 1992). One of the most abundant oxidative damages to DNA bases is the C8-hydroxylation of guanine (Shigemura et al., 1989; Floyd et al., 1986; Reilly et al., 1991; Dizdaroglu, 1991; Floyd, 1990). Whereas, hydroxyl radicals generate multiple products from all four bases, singlet oxygen preferentially modifies guanine, particularly by 8-hydroxylation (Dizdaroglu, 1991; Eguchi, 1991). The occurrence of 8-hydroxyguanine (8-OHdG) in DNA, usually measured as a ratio to thymine guanine after enzymatic digestion of DNA, has been used extensively to study damaging effects on DNA of ROS generated by all the mechanisms mentioned above as well as the protective or promotive effects of antioxidants (Kadi and Mishima, 1984; Kawai et al., 1986; Floyd et al., 1986; Leanderson and Tagesson, 1990, 1992; Kiyosawa et al., 1990; Faga et al., 1991; Fischer-Nielsen et al., 1992).

In vivo, damaged DNA is repaired by exonucleases or glycosylases liberating deoxynucleotides or bases, respectively. These repair products are water-soluble and mostly excreted into the urine without further metabolism. Nermberg et al., 1978; Shigemura et al., 1989, 1989. Exogenous DNA from the diet contributes only to the excretion of oxidized bases but not to that of oxidized deoxynucleotides (Shigemura et al., 1989; Park et al., 1992; Bengt and Simic, 1991). Thus, the urinary excretion of oxidized deoxynucleotides, such as 8-OHdG, and/or thymine glycol, has been proposed as biomarker of the current oxidative DNA-damage and repair (Cathcart et al., 1984; Shigemura et al., 1989, 1992, 1998; Shigemura and Arase, 1991). At present most data are available regarding 8-OHdG.

METHODS FOR ANALYSIS OF 8-OHdG IN URINE

A number of analytical methods have been developed for the measurement of 8-OHdG in urine. The group of Amos and co-workers used multiple solid-phase extractions on mini-columns before HPLC gradient separation, but more recently the extractions have been accomplished by immunoaffinity columns with specific antibodies to 8-OHdG and BOHG, requiring only isocratic elution (Shigemura et al., 1989; Degan et al., 1992; Park et al., 1992). In both methods detection is electrochemically and indolactam 8-OHdG is used as internal standard. A Swedish group has developed an isocratic coupled columns HPLC method also with electrochemical detection (Toppo et al., 1992).

In our laboratory an automated three-dimensional HPLC method with isocratic separation and electrochemical detection has been developed for the analysis of 8-OHdG in urine (Loff et al., 1992). A flow path diagram is shown in Figure 1. By this method, urine is chromatographed under alkali conditions on a 15-cm C18 2.5-um column. A 1 ml fraction of the effluent containing the 8-OHdG is brought on a 2-cm cation-exchange column via a 5-um automatic valve. With a switch of the valve this column is then flushed with an acidic eluent (pH 2.2), laving the column
onto a 25-cm C18 3-μm column. The effluent of this column is monitored by an ESA ColumnChrom II electrochemical detector set at 120 mV to detect 1 and 300 mV for electrode 2. For quantification, samples are run with and without addition of genuine 8OHdG and the concentrations calculated from the peak heights. The method includes an interassay coefficient of variation of 10% and a limit of detection of 0.2 nM as compared to concentrations ranging from 1 to 70 nM in human urine. Moreover, the method is reasonably simple and automated, allowing the handling of larger number of samples.

By means of gas chromatography-mass spectrometry with selected-ion monitoring, several oxidatively modified deoxynucleosides, including 8OHdG and thymidine glycol, may be simultaneously measured in urine (Bergdolt et al., 1990). Indeed, this method may potentially allow determination of urinary excretion of repair products of the many oxidative DNA modifications demonstrated in human tissue, as well as in many in vitro model systems (Dizdaroglu, 1991; Malins and Haimanot, 1991; Halliwell and Dizdaroglu, 1992; Olinski et al., 1992). So far, however, limited data have emerged from this highly specific and sensitive method.

FACTORS DETERMINING 8OHdG EXCRETION

In humans the excretion of 8OHdG is normally distributed with a 7- to 10-fold range and the coefficient of variation is 30-40% (Table 1) (Park et al., 1992; Bergdolt and Simic, 1991; Loit et al., 1992). The intraindividual variation appears to be somewhat less. Thus, the urinary excretion of 8OHdG measured on two occasions 10 d apart in 20 individuals correlated closely (r = 0.53) (Park et al., 1992). This is in agreement with data obtained in one subject studied repetitively for 1 yr (Simic and Bergdolt, 1991) and our own unpublished experience. The excretion of 8OHdG has been shown to be associated with a number of factors which by inference are thought to modify oxidative DNA damage.

Species

Across species, including humans, monkeys, rats, and mice, linear correlations have been reported between the specific metabolic rate and the excretion of 8OHdG and thymine and thymidine glycol, other repair products of oxidative DNA damage (Adelman et al., 1988; Shigenaga et al., 1989; Bergdolt and Simic, 1991). Supposedly, the high mitochondrial oxygen metabolism in small animals, such as rodents, allows generation of more ROS as the most important source of oxidative DNA damage. We have investigated the correlation between 8OHdG excretion and table values (Spector, 1956) of the specific metabolic rate among humans (Loft et al., 1992), pigs, dogs and rats. Urine was collected for 4 h under anesthesia by means of suprapubic bladder catheters in 7 pigs aged 8 wk, for 3 h by means of bladder catheterization in 1 beagle dog aged 24 d and for 24 h by means of metabolic cages in 12 male rats aged 7 wk and 10 rats aged 15 wk (Fig. 2; unpublished data). Within these species we found a linear correlation although the slope was steeper than in the study by Ames and co-workers (Shigenaga et al., 1989). As shown in Table 1, the data on human BOHG excretion agree between the laboratories, whereas the excretion in our rats was more than twice the value reported by Ames and co-workers (Fraga et al., 1990; Park et al., 1992). Moreover, in mice a 15 times higher
rate of 8OHdG excretion has been reported by Bergfeld and Simic (1991) as compared to the value published by Ames and co-workers (Table 1) (Shigenaga et al., 1989). The reason for these substantial interlaboratory differences with respect to rodent data is unknown and interlaboratory exchange of samples has not been reported. However, they are less (i.e., two-fold) with respect to thymidine glycol excretion in mice (Adelman et al., 1988; Bergfeld and Simic, 1991).

Age
In a human study population with a narrow age range there was no correlation between age and 8OHdG excretion (Lott et al., 1992). However, in rats the excretion of 8OHdG has been shown to decrease with age while the modified base accumulates in tissue DNA (Fraga et al., 1990). Similarly, 8OHdG has been reported to accumulate in the mitochondrial DNA of the human diaphragm muscle cells (Hayakawa et al., 1991). The accumulation may be a result of slight reduction of repair efficiency (Fraga et al., 1990; Hayakawa et al., 1991). From the rate of 8OHdG excretion and accumulation it has been estimated that less than 1 in 50 damaged bases are left unpaired in the kidney and even less in other organs (Fraga et al., 1990). If the age-related decrease in 8OHdG excretion resulted from failing repair, a much more massive accumulation of oxo-naturally modified bases would be expected. Moreover, as shown in Figure 2, the decrease in 8OHdG excretion may be explained by the decrease in metabolic rate with age.

Smoking
In 83 healthy subjects the most important factor determining 8OHdG excretion was smoking. Thus, smokers excreted 50% (31-69%; 95% confidence interval) more 8OHdG than nonsmokers (Fig. 2 and Table 1) (Lott et al., 1992). In agreement, a 30% higher ratio between 8OHdG and creatinine was found in spot urine collected from 10 smokers as compared to 24 nonsmokers (Tagesse et al., 1992).

In vitro, tobacco smoke and several of its constituents, such as hydroquinone and catechol, have been shown to generate ROS and to induce oxidative damage to isolated DNA as well as to produce 8OHdG in cell cultures (Leanderson and Tagesse, 1990, 1992). The volatile phase of tobacco smoke induces lipid peroxidation and lipoprotein oxidation in human plasma in vitro (Frie et al., 1991). In the plasma of smokers low concentrations of the important antioxidants, ascorbic acid and β-carotene, have been found, suggesting increased consumption and reduced antioxidant capacity (Ziegler, 1989; Biemers et al., 1991; Frie et al., 1991). Moreover, in smokers leukocyte DNA has been found to contain approxima tely 50% more 8OHdG than in nonsmokers (Kiyoashi et al., 1990). The finding of increased urinary 8OHdG excretion in smokers is the first verification of these suggestions in vivo in humans. The apparent increasing effect of tobacco smoking on oxidative DNA damage may also be related to its well-documented enhancing effect on the basic metabolic rate (Mollatt and Owens, 1991).

Body Composition
In 83 healthy subjects the significant predictors of the 8OHdG excretion were, except smoking, either the body mass index (BMI: body weight divided by the square of the height) and gender or the fat-free body fraction measured by body impedance (Lott et al., 1992). There was an inverse relationship between relative 8OHdG excretion and BMI that may relate to the fact that lean persons have a higher metabolic rate than obese (Sibat et al., 1980). By the same token, men have a higher basic metabolic rate than women (Meijer et al., 1992), which may provide an explanation of a higher 8OHdG excretion in men (Lott et al., 1992). Moreover, the sedentary lifestyle expenditure has been shown to correlate closely with fat-free body mass and BMI (Webb and Sangal, 1991). Accordingly, concurrent measurements of 8OHdG excretion and metabolic rate in subjects with different body compositions are warranted. Nevertheless, the data suggest that, ideally, the 8OHdG excretion should be corrected for lean body mass for comparison of individuals with respect to external factors, such as smoking.

Diet
Reduction of the calorie intake by 40-50% reduced the excretion of 8OHdG and thymidine glycol by an even larger fraction in two subjects
(Simic and Bergtold, 1991). Although not measured, this effect may have been related to a decrease in the metabolic rate due to the calorie restriction. In rats calorie restriction decreased the content of BOHdG in tissue DNA (Chung et al., 1992).

In two subjects a high intake of fruit and vegetables as compared to an isocaloric diet with none of these components reduced the excretion of thyminic glycol substantially, supposedly due to differences in antioxidant content (Simic and Bergtold, 1991). Moreover, a high intake of vitamin C has been reported to protect against BOHdG formation in human seminal DNA (Fraga et al., 1991). By contrast, in a study population of 83 healthy subjects the intakes of vitamin A, C, and E did not correlate to the BOHdG excretion (Loff et al., 1992), although as antioxidants they could have been expected to have a protective effect. However, the generally modest intake, which may not be accurately recorded from the diaries, may have less or no effect on BOHdG formation. In fact, the antioxidant effect of vitamin C may be outbalanced by reactions involving thiols (Borg and Schiaich, 1989; Fischer-Nielsen et al., 1992).

In women at high risk of breast cancer a low-fat diet decreases excretion of the DNA damage in nucleated peripheral blood cells as measured by the levels of oxidized thymine (Djuric et al., 1991). In another human study population, however, the relationship between dietary fat and the excretion of BOHdG was negative—d—any (Loff et al., 1992).

Ionizing Radiation

Ionizing radiation generates hydroxyl as well as superoxide radicals, resulting in oxidative DNA damage. Thus, the content of BOHdG increases in DNA from tissues and cell cultures after radiation (Kasai et al., 1984, 1986). In two patients receiving 1.8 Gy of radiation therapy for breast and colon cancer, respectively, i.e., a limited radiation field, the excretion of BOHdG and thyminic glycol was fourfold increased (Bergtold et al., 1990). Moreover, in a single subject total body irradiation with 10 Gy was followed by a short 20-fold increase in BOHdG excretion, which returned to almost normal within 16 h (Bouzit et al., 1991). In a comparison of irradiated mice and humans the relative increase in thyminic glycol excretion was similar in the two species and directly proportional with the radiation dose (Simic and Bergtold, 1991). This is in agreement with linear dose-response curves concerning radiation-induced oxidative damage in isolated DNA, including the formation of BOHdG (Dizdaroglu, 1991).

Conclusion

Except for the effects of ionizing radiation, the variation in BOHdG excretion within and between subjects and species may at least partly be explained by differences in metabolic rates. In support, mitochondrial DNA, which is closer to the source of ROS generated from the electron transport chain, has a 16-fold higher content of BOHdG than nuclear DNA, although some of this difference may be related to a more efficient repair in the latter (Richter et al., 1988). A protective effect of dietary antioxidants towards oxidative DNA damage has been suggested by observations in two subjects but needs to be confirmed in large-scale intervention studies, preferably including smokers.

LIMITATIONS OF BOHdG AS A URINARY BIOMARKER

The excretion of BOHdG reflects only the (potential) formation and repair of one oxidative DNA modification. Thus, in several in vitro systems with ROS generation as well as in human tissue a whole series of DNA modifications, including ring-opened base products, has been described (Dizdaroglu, 1991; Malins and Haimanot, 1991; Olinski et al., 1992). However, the determination of most of these DNA reactions requires a different methodology, and for most of these modifications, including 8-OHdG and thyminic glycol, in urine, and factors possibly associated with or affecting their excretion have not been studied. Moreover, the consequences of oxidative DNA modifications other than BOHdG (see later discussion) and pyrimidine dimers have not been investigated as thoroughly as BOHdG.

The exact sources of urinary BOHdG have yet to be determined. So far, the FPG (formamidopyrimidine-DNA glycosylase) enzyme of Escherichia coli has shown to repair BOHdG by liberating the base initially (Tchou et al., 1991; Boiteux et al., 1992). In mammalian cells a similar enzyme (hOGG1) has been shown to repair by excision at a different site, the proximal and distal guanine residues at the 5' and 3' side of lesion, respectively (Yamamoto et al., 1992). However, whether the base is liberated initially or the end product is 8-OHdG is yet unknown. In addition, BOHdG will be liberated from DNA during turnover of mitochondria and from nuclear DNA at apoptosis and other types of cell turnover. Mitochondrial DNA contains relatively more BOHdG due to its proximity to the respiratory chain, but the amounts are small in absolute terms compared with nuclear DNA (Richter et al., 1988). Other potential sources of urinary BOHdG include oxidation in the deoxyguanosine, and deoxyuridineside pools that provide building blocks for DNA. No matter which of these is most important, the excretion of BOHdG will reflect the general average risk of promutagenic oxidative adducts in DNA of all cells and organs.

CONSEQUENCES OF BOHdG FORMATION IN DNA

The available data regarding the urinary excretion of BOHdG and thymine and thyminic glycol suggest that oxidative damage to DNA is extensive in humans, approximately 106 base oxidations per cell per day (Cathcart et al., 1984; Adelman et al., 1988; Bergtold et al., 1988;
CONCLUSION AND PERSPECTIVES REGARDING BOHIG AND AS A URINARY BIOMARKER

ROS and related DNA damage have been implicated in the pathogene-
sis of a number of important diseases and aging. By means of simple noninva-
sive measures of oxidative DNA damage, such as urinary excretion of
BOHIG, the numerous hypotheses of the importance of ROS in aging,
degenerative and inflammatory diseases, and carcinogenesis may be chal-
lenged. Use of BOHIG and similar biomarkers provides unique opportuni-
ties for investigation of the related health effects in prospective studies or
in nested case-control studies with urine from biological banks. The postulated
beneficial effects of antioxidant supplementation and other dietary in-
tervention also represent unchallenged hypotheses to be tested. So far,
BOHIG and thymidine glycol are the only available noninvasive bio-
markers for oxidative DNA damage. In the future, other similar repair prod-
ucts, such as adenine adducts and ring-opened bases, may be determined
by gas chromatography-mass spectrometry and used as urinary biomarkers.
Potentially, such a series of biomarkers may allow determination of the kind
and source of ROS involved and the contribution from various target
organ, as well as a better indication of the related health risks.

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