Free Radical Research, Volume 38 Number 11 (November 2004), pp. 1201–1205

Taylor & Francis
healthsciences

Diet is Not Responsible for the Presence of Several Oxidatively Damaged DNA Lesions in Mouse Urine

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Accepted by Professor J. Cadet

(Received 1 September 2004; In revised form 5 October 2004)

Ryszard Olinski dedicated this work to the memory of Professor Jerzy Popinigis

In order to eliminate the possibility that diet may influence urinary oxidative DNA lesion levels, in our experiments we used a recently developed technique involving HPLC pre-purification followed by gas chromatography with isotope dilution mass spectrometric detection. This methodology was applied for the determination of the lesions: 8-oxo-7,8-dihydroguanine (8-oxoGua), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and 5-(hydroxymethyl)uracil (5HMUra) in the urine of mice fed with nucleic acid free diet and normal, unrestricted diet. The mean levels of 8-oxoGua, 8-oxodGuo and 5HMUra of the animals fed the normal diet reached the mean values of 15.6 ± 3.5, 2.0 ± 0.53 and 16.8 ± 10.4 nmol/kg/24 h. After feeding the mice for 12 days with nucleic acid free diet the respective values were 18.8 ± 4.6, 1.6 ± 0.3 and 25.4 ± 10.5 nmol/kg/24 h, respectively. The results clearly demonstrate that irrespective of the diet, the excretion rates were not statistically different during the course of feeding. The respective *p* values for the differences between lesions in the two types of diets were: 0.13 (8-oxoGua), 0.16 (8-oxodGuo), 0.18 (5-HMUra). Our results clearly indicate that diet does not contribute to urinary excretion of the lesions in mouse model.

Keywords: Diet; 8-oxoGua; Oxidative DNA damage; Urine

INTRODUCTION

Cellular DNA is continuously exposed to reactive oxygen species (ROS) generated as by-products of normal metabolism or derived from exposure to environmental insult such as ionizing or ultraviolet radiation and certain chemicals. Although complex enzymatic and nonenzymatic systems exist that protect the cell against ROS some of them may escape the defenses and damage cellular biomolecules including DNA. Oxidative DNA damage escaping repair may be a major cause of various age-related diseases such as cancer. Numerous DNA repair pathways exist to prevent the persistence of damage, and are integral to the maintenance of genome stability, and hence prevention of disease. Excised lesions arising from repair may ultimately appear in the urine,[1] offering the potential to assess DNA damage and repair non-invasively.

The major argument that is used against the assumption that the excretion of the modifications reflects DNA repair is literature report that diet may contribute to urinary levels of the lesion.[2] In the aforementioned study it was shown that diet can influence the level of 8-oxo-7,8-dihydroguanine (8-oxoGua) in rat urine. On the contrary, our experiments clearly show that diet has no influence on the level of 8-oxoGua and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) in human urine.[3] A different methodological approach may be a reason for this inconsistency. Park *et al.* [2] used immuno-affinity pre-purification (using an antibody which recognised 8-oxodGuo and related adducts) and

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760400017350

HPLC with EC detection for the determination of modified base and the nucleoside. However, in their subsequent paper the authors stated that "8-oxoGua in urine presents particular difficulties, and a modified method for analysis of this species is required".[4] In order to eliminate the possibility that diet may influence urinary lesion levels, in our experiments we used a recently developed technique involving HPLC pre-purification followed by gas chromatography with isotope dilution mass spectrometric detection for the detection of oxidative DNA lesions: 8-oxodGuo, 8-oxoGua and 5-(hydroxymethyl)uracil (5-HMUra) in the urine of mice fed with nucleic acid (NA) free diet and normal, unrestricted diet. In addition to unequivocal identification of the analysed compounds and high sensitivity, isotopically labelled internal standards used in this approach allowed compensation for eventual losses of the analysed products.

MATERIALS AND METHODS

Animals

Male C57Bl/6J mice were housed under standard conditions in metabolic cages. Experiments were carried out with groups of 2–5 mice per cage. All cages comprise 29 individual animals. Urine was collected every 24 h from each cage. The body weight (in gram) of mice was in the range between 29 and 38. Mice were fed Rat and Mouse nr 1 maintenance diet which contained among other components 12.92% digestible crude protein and 2.47% digestible crude oil (special diet service (SDS); Product code 801002) or alternatively nucleic acids free basal PD 1/2 diet (synthetic testdiet; product code 7024).

Urine Sample Preparation

A measure of 0.5 nmol of [$^{15}N_3$, ^{13}C] 8-oxoGua, 0.05 nmol of [$^{15}N_5$] 8-oxodGuo, 0.05 nmol of [$^{13}C_2$, $^{2}H_2$] 5-HMUra and 10 μl of acetic acid (Sigma, HPLC grade) were added to 2 ml of mice urine. Isotopic purity of the applied standards were 97.65, 95 and 98%, respectively. Isotopically labelled internal standards of 8-oxoGua and 5-HMUra were the generous gift of Dr M. Dizdaroglu from the National Institute of Standards and Technology, Gaithersburg, USA. The standard of 8-oxodGuo was prepared from [$^{15}N_5$] dGTP (Silantes GmbH, Germany) according to the procedure described by Bialkowski[5] with the addition of alkaline phosphatase digestion step.

After centrifugation (2000 × *g*, 10 min) supernatant was filtered through a Millipore GV13 0.22 μm syringe filters and 500 μl of this solution was injected onto HPLC system.

HPLC Purification and GC/MS Analysis

Urine HPLC purification of 8-oxoGua, 8-oxodGuo and 5-HMUra was performed according to the method described by Ravanat *et al.* [6] with some modifications. Briefly, the HPLC system consisted of P580 gradient pump, Gina 50 autosampler (both from Dionex), SPD M10 AVP diode array detector from Shimadzu and Foxy 200 fraction collector from Isco Inc. Urine samples enriched in labelled compounds were injected onto Supelcosil LC 18 column (250 × 10 mm) equipped with Supelguard LC18 guard column (20 × 4.6 mm), both from Supelco. A 30 min linear gradient elution was performed (0.5% acetic acid at start to 0.5% acetic acid and 10% of acetonitrile), at a flow rate of 3 ml/min. After this time the column was washed with 7.0% of acetonitrile for 10 min and equilibrated with 0.5% acetic acid for 20 min prior to a further injection. The effluent was monitored with UV detector at 220–360 nm.

Using standards for 8-oxoGua, 8-oxodGuo, 5-HMUra, Gua, dGuo and thymine we were able to avoid an overlapping of the peaks containing the modified and unmodified base/nucleoside. The collected fractions were dried by evaporation under reduced pressure in a Speed–Vac system. The 8-oxodGuo was treated with 400 μl of 60% formic acid (Sigma) for 30 min at 130°C. Subsequently, samples were prepared for GC/MS analysis. GC/MS analysis was performed according to the method described by M. Dizdaroglu,[7] adapted for additional [$^{15}N_5$] 8-oxoGua analyses (m/z 445 and 460 ions were monitored).

Statistical Analysis

All results are expressed as means ± SD. The STATISTICA (version 6.0) computer software (StatSoft, Inc, Tulsa, USA) was used for the statistical analysis. Student-*t* test was carried out to compare the means of variables with normal distribution. Statistical significance was considered at $p < 0.05$.

RESULTS

Using recently developed methodology which involved HPLC pre-purification followed by gas chromatography with isotope dilution mass spectrometric detection,[6] we found that urinary excretion of 8-oxoGua and 8-oxodGuo does not depend on diet in the case of humans. With this methodology, in the present work we wanted to discover whether a similar phenomenon could be seen in mice. Animals used in this study were fed either with normal unrestricted diet or, for 12 days with nucleic acid (NA) free diet (see, "Materials and Methods" section)

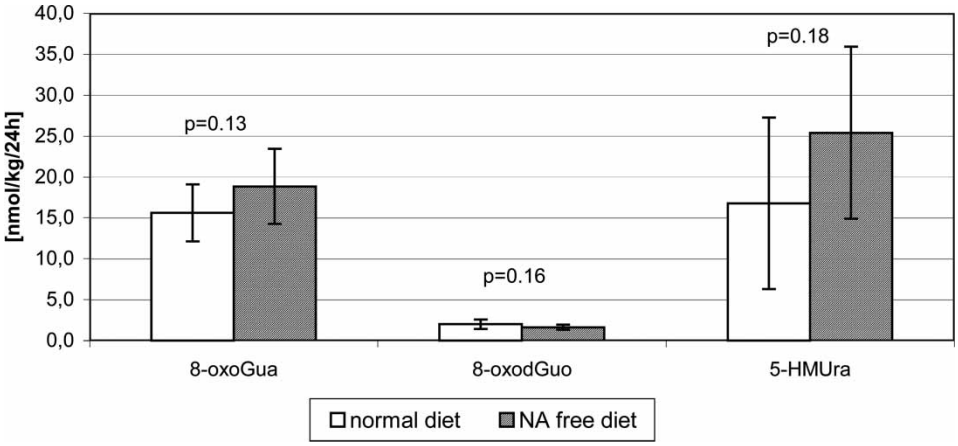

FIGURE 1 Urinary excretion of 8-oxoGua, 8-oxodGuo and 5-HMUra of wild type strain mice fed normal diet and mice fed for 12 days NA free diet.

and urine samples (24 h output) were collected for each of the dietary approach. The mean levels of 8-oxoGua, 8-oxodGuo and 5HMUra for animals fed the normal diet reached the mean values of 15.6 ± 3.5, 2.0 ± 0.53 and 16.8 ± 10.4 nmol/kg/24 h, respectively. After feeding the mice for 12 days with NA free diet the respective values were 18.8 ± 4.6, 1.6 ± 0.3 and 25.4 ± 10.5 nmol/kg/24 h (the values obtained for the urine samples collected during days 1–11 are very similar to those aforementioned). The mean values are obtained from an average of 9 cages and were calculated assuming the number and body weight of animals in every cage (see, "Materials and Methods" section). The results presented in Fig. 1 clearly show that irrespective of the diet, the excretion rates were not statistically different during the course of feeding.

The reported values of the urinary excretion products expressed in nmol/kg/24 h enables measurement of the number of the repaired lesions per, day per cell.[4] The mean values for 8-oxoGua, 8-oxodGuo and 5HMUra for animals fed the normal diet are presented in Table I.

DISCUSSION

8-Oxo-7,8-dihydroguanine (8-oxoGua) and its 2′-deoxynucleoside equivalent, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) are the most abundant and the most extensively studied oxidative DNA lesions and they are considered to be potential repair products. Several enzyme activities can account for the production of these lesions.[1,8] Excised lesions from repair may ultimately appear in the urine.

It has been generally accepted that the presence of the modified nucleoside (8-oxodGuo) in urine represents the primary repair product of the oxidative DNA damage *in vivo* and that this compound may mirror involvement of nucleotide excision repair pathway (NER).[9] Alternatively, 8-oxodGuo in urine could derive from MutT sanitization of the cellular nucleotide pool by mut t homolog (MTH) directed pathway.[10] However, it should be remembered that the products of NER and MTH both require further processing to result in 8-oxodGuo. Moreover, there is little evidence that 8-oxodGuo is a product of DNA repair itself (for a detailed discussion of this issue see Ref. [1]).

On the other hand there is no doubt that the modified base (8-oxoGua) is the major product of BER pathway. Moreover, several glycosylases, which specifically recognize and remove 8-oxoGua in cells, have recently been described.[11–15]

5-HMUra is a thymine derivative,[16] its presence in urine should reflect oxidative DNA damage. Moreover, in humans and other vertebrates, an interesting glycosylase has been described that specifically excised 5-HMUra from DNA. It appears to be unique to higher eukaryotes and has been reported to be absent from prokaryotes.[17]

From the earliest studies examining urinary 8-oxoGua in rats fed with nucleic acid-free diets, it appears that 8-oxoGua level is affected by diet[2] and as a consequence, it would not be a suitable marker of oxidative stress, or DNA repair. In contrast with studies in rats Gackowski *et al.*[3] showed no difference in urinary 8-oxoGua levels in the study involving humans fed nucleic acid-free diets compared to those with a normal diet, suggesting that urinary 8-oxoGua is independent of diet in human urine. Similar studies do not, as yet, appear to have

TABLE I The estimated number of oxidative adducts per cell, per day; mouse vs humans

Species	8-oxoGua	8-oxodGuo	5-HMUra
Humans*	1202 ± 176	286 ± 34	1312 ± 269
Mouse	13 310 ± 2986	1706 ± 512	14 334 ± 8959

*Data from Reference [19]

been performed for other oxidized nucleobases like 5-HMUra. To clarify a controversial issue concerning diet influence on urinary excretion of DNA lesions, experimental animals used in this study were fed with normal and nucleic acid free diet (see, "Materials and Methods" section) and a new technique was applied which allowed for simultaneous determination of several urinary oxidative DNA products in the same urine sample.[6]

Our results clearly pointed out that irrespective of the diet the excretion rates were similar. Similarly, our previous experiments clearly show that diet has no influence on the level of 8-oxoGua in human urine.[3] Our analyses support the suggestion that lesions other than 8-oxoGua and 8-oxodGuo may also be suitable urinary markers of oxidative DNA damage.

Summing up, our results clearly indicate that diet does not contribute to urinary 8-oxo-Gua, 8-oxodGuo or 5-HMUra in mouse model. Our previous work demonstrated that it is also the case in humans. We propose that these findings combined exclude urinary 8-oxoGua and 8-oxodGuo and 5-HMUra levels from influence of diet. Furthermore, reports examining the effects of cytotoxic agents upon urinary 8-oxodGuo attribute the presence of this lesion to repair, rather than to release from dead cells.[1] In the absence of these confounding factors, urinary measurements may be attributed entirely to DNA damage and repair what in turn suggests they possible usefulness for studying associations between DNA repair and disease.

Expression of the urinary excretion rates in nmol/kg/24 h enables measurement of the extent of the repaired lesions per day per cell.[4] Interestingly, urinary level of 8-oxoGua, found in our study accounted for 13310 repaired events per average cell of the mouse per day. This value is lower than that estimated by Hamilton and co-workers who calculated that the DNA of the liver cell in mouse is exposed to about 47000 8-oxoGua lesions in a 24 h period.[18] However, this inconsistency may be explained taking into consideration that the liver is a high metabolic rate organ and that our values are an average for the whole organism. Corresponding values for 8-oxodGuo and 5-HMUra, were respectively: 1706 and 14334 repaired events per average cell. In contrast, our recently obtained results[19] indicated that the number of such events in humans accounts for about 1202, 286 and 1312 for 8-oxoGua, 8-oxodGuo and 5-HMUra, respectively. Therefore, estimates of the analyzed lesions in urine from mouse presented in this work are several times higher than those obtained for humans.[19] This difference almost exactly fit the difference in oxygen consumption between mouse and humans.[20] It is therefore possible that high metabolic rate in mouse may be responsible for severe everyday oxidative DNA insult that may be reflected in high excretion rate of the modifications.

Acknowledgements

Rafal Rozalski, Daniel Gackowski, Ryszard Olinski were supported by a Foundation for Polish Science fellowships. The authors are greatly indebted to K.B. Iversen (The National Hospital in Oslo, Norway) for technical assistance in this work.

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