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DNA Damage Products (5′*R*)- and (5′*S*)-8,5′-Cyclo-2′-deoxyadenosines as Potential Biomarkers in Human Urine for Atherosclerosis

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Supporting Information

ABSTRACT: We hypothesized that DNA damage products (5′*R*)-8,5′-cyclo-2′-deoxyadenosine (*R*-cdA) and (5′*S*)-8,5′-cyclo-2′-deoxyadenosine (*S*-cdA) may be well-suited biomarkers of risk and diagnosis for atherosclerosis. We tested this hypothesis by measuring the levels of *R*-cdA and *S*-cdA and another product, 8-hydroxy-2′-deoxyguanosine (8-OH-dG), in urine of atherosclerosis patients and healthy individuals using liquid chromatography−tandem mass spectrometry with isotope dilution. We showed the presence of these products at significantly greater concentrations in urine of atherosclerosis patients than in that of healthy individuals. Our data suggest that *R*-cdA and *S*-cdA can be accurately and reproducibly measured in human urine as potential biomarkers of risk and diagnosis for atherosclerosis.

Atherosclerosis remains a major health problem worldwide and is expected to be a dominant cause of death in the future, as well.[1] It is an inflammatory disease,[2] and oxidative stress is a risk factor.[3−5] Inflammation and oxidative stress produce oxygen- and nitrogen-derived species, including free radicals that may cause oxidatively induced DNA damage.[6] Free radicals, most notably highly reactive hydroxyl radical (•OH), are well-known to generate numerous products in DNA.[6] There is evidence of oxidatively induced DNA damage in patients with coronary artery disease.[7] DNA damage including mitochondrial damage has been found in atherosclerotic lesions and peripheral blood cells of atherosclerosis patients.[8] These patients also exhibited a higher micronucleus index than healthy individuals, which positively correlated with the severity of the disease, indicating an important role of genetic instability in atherosclerotic mechanisms.[9] High levels of 8-OH-dG have been observed in fragments of aorta from patients with severe atherosclerotic lesions, significantly correlating with the occurrence of atherogenic risk factors.[3] A strong correlation has been found between the level of 8-OH-dG in lymphocyte DNA and premature deaths from coronary heart disease.[10] DNA damage not only accompanies atherosclerosis but also is likely to play a causal role in both initiation and progression of the disease.[8,11] Defects in DNA repair capacity and/or failure of DNA repair promotes atherosclerosis and the metabolic syndrome.[8] Evidence of the role of DNA repair in atherosclerosis also comes from the acceleration of DNA repair by drugs such as statins that reduce the level of DNA damage and atherosclerosis in vivo.[12] Most of the oxidatively induced DNA lesions are repaired by base excision repair (BER). However, some lesions such as 8,5′-cyclopurine 2′-deoxynucleosides are subject to nucleotide excision repair (NER).[13,14] Following repair, DNA lesions may be excreted into blood and consequently appear in urine. Mounting evidence suggests that oxidatively induced DNA lesions in cellular DNA or in human urine may be used as potential biomarkers for disease risk assessment, monitoring the progression of disease and determining the efficacy of therapeutic drugs.[3] The measurement of DNA lesions in human urine offers a noninvasive approach for this purpose and may be readily applicable in research and clinical environments. Over the past two decades, 8-OH-dG and its free base 8-hydroxyguanine (8-OH-Gua) in urine have been mainly used as potential biomarkers.[15] Recently, we discovered the presence of *R*-cdA and *S*-cdA in human urine and described their accurate measurement.[16]

Because of the role of inflammation and oxidative stress in atherosclerosis as described above, we hypothesized that *R*-cdA and *S*-cdA may be well-suited biomarkers in human urine for this disease. To test this hypothesis, we measured these lesions in urine samples from patients with clinical atherosclerosis and from healthy individuals. The aim was to determine whether suitable biomarkers of oxidatively induced DNA damage other than 8-OH-dG and 8-OH-Gua can be found and accurately measured in urine with respect to atherosclerosis. Previously, 8-OH-dG and 8-OH-Gua have been mostly measured as possible biomarkers for this purpose.[15] The latter has been thought to be released into urine by its removal from DNA by the DNA glycosylase OGG1 in the first step of BER.[17,18] In contrast, it is not clear how BER would release the nucleoside 8-OH-dG, because DNA glycosylases excise DNA lesions as free bases rather then as nucleosides.[6] Because there is in vitro evidence that NER may play a role in repair of 8-OH-dG,[19] this pathway may be responsible for the excretion of 8-OH-dG into urine. On the other hand, no oligomers containing 8-OH-dG have

Received: December 22, 2011
Revised: January 12, 2012
Published: February 23, 2012

been found in urine.[20] The best-characterized enzyme that may be involved in the presence of 8-OH-dG in urine is the 8-hydroxy-2′-deoxyguanosine triphosphatase (8-OH-dGTPase activity of NUDT1; MutT homologue, MTH1), hydrolyzing 8-OH-dGTP to 8-OH-dGMP. It is possible that further processing, perhaps by 5′(3′)-nucleotidases, may give rise to 8-OH-dG, which can be removed from the cell, ultimately appearing in urine.[18] The excretion of DNA lesions into urine as a result of diet has been excluded.[17,18,21] In this context, it is not known at present whether diet or cell turnover contributes to the excretion of *R*-cdA and *S*-cdA into urine.

R-cdA and *S*-cdA are repaired by NER and not subject to BER.[13,14] Because of their extraordinary chemical stability and accurate measurement, we proposed that *R*-cdA and *S*-cdA can be used as biomarkers in human urine.[16] In this work, we used urine samples from 22 patients who then underwent carotid endarterectomy and from 22 healthy individuals without any symptoms of atherosclerosis. We simultaneously measured the concentrations of *R*-cdA, *S*-cdA, and 8-OH-dG by liquid chromatography−tandem mass spectrometry (LC−MS/MS) with isotope dilution and that of 8-OH-Gua by high-performance liquid chromatography coupled with gas chromatography and mass spectrometry (HPLC/GC−MS) with isotope dilution using ^{15}N-labeled analogues of these compounds as internal standards. The amount of creatinine was also measured. The concentrations of these analytes were normalized to that of creatinine. Figure 1 illustrates

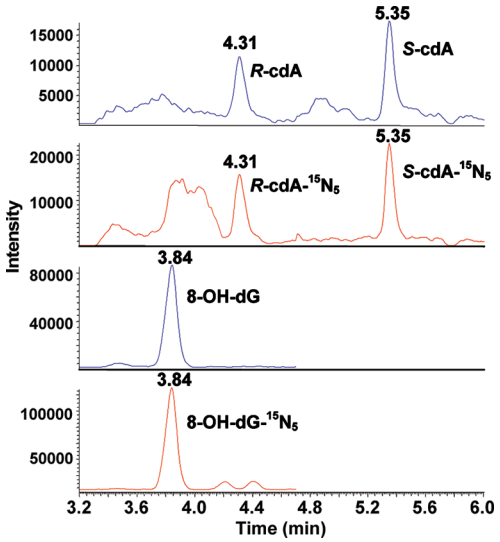

Figure 1. Ion−current profiles of the m/z 250 → 164 (*R*-cdA and *S*-cdA), m/z 255 → 169 (*R*-cdA-$^{15}N_5$ and *S*-cdA-$^{15}N_5$), m/z 284 → 168 (8-OH-dG), and m/z 289 → 173 (8-OH-dG-$^{15}N_5$) mass transitions.

representative ion−current profiles of the mass transitions for *R*-cdA, *S*-cdA, *R*-cdA-$^{15}N_5$, *S*-cdA-$^{15}N_5$, 8-OH-dG, and 8-OH-dG-$^{15}N_5$, which were recorded during the LC−MS/MS analysis of a urine sample from a patient. The concentrations of *R*-cdA and *S*-cdA in urine of healthy individuals and patients are shown in Figure 2. The scattered dot plots are shown. The thick horizontal lines in these plots represent the mean value of all measured data points in each group. The standard deviations are shown by the thin vertical lines. The data show that the concentrations of *R*-cdA and *S*-cdA were significantly greater in urine of patients than in controls. The difference between the mean values was 2.43-fold for *R*-cdA and 3.14-fold for *S*-cdA.

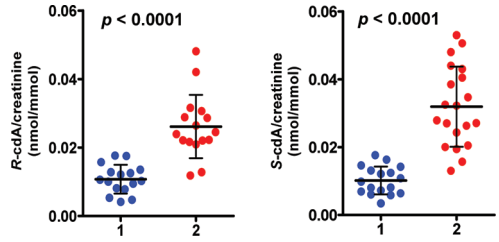

Figure 2. Levels of *R*-cdA and *S*-cdA in urine of controls (1) and patients (2). The uncertainties are standard deviations.

The concentration of 8-OH-dG in urine of patients was also significantly greater than that in controls (Figure 3). A 1.55-fold

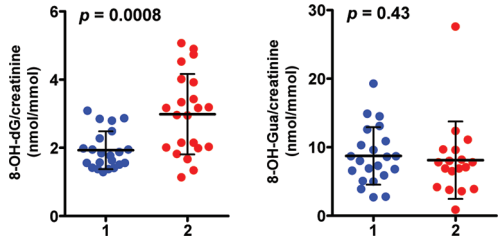

Figure 3. Levels of 8-OH-dG and 8-OH-Gua in urine of controls (1) and patients (2). The uncertainties are standard deviations.

difference between the mean values was found. No statistical significance was observed for the level of the free base 8-OH-Gua between patients and controls (Figure 3).

Our data show the presence of *R*-cdA and *S*-cdA in urine of atherosclerosis patients at significantly greater concentrations than in urine of healthy individuals. The statistical difference was highly significant ($p < 0.0001$). 8-OH-dG concentrations in urine of patients were also significantly higher than those in controls. The significance of the data for *R*-cdA and *S*-cdA was greater than that for 8-OH-dG. It should be pointed out that the concentrations of these three compounds found in controls in this work were quite similar to those we previously measured in urine of healthy individuals.[16] This fact points to the accuracy and reproducibility of our measurements. No statistically significant difference was observed between the concentrations of 8-OH-Gua in the two groups. This fact suggests that this compound may not be a reliable biomarker for atherosclerosis. High levels of *R*-cdA and *S*-cdA in urine of patients are strong evidence of the increased production of these compounds in DNA, most likely due to known augmented oxidative stress and inflammation, and consequently DNA damage in atherosclerosis.[3−5,7−11] The excretion of high levels of *R*-cdA and *S*-cdA into urine may indicate functional cellular repair of DNA damage in patients. The patients were on statins. However, it is not known whether these drugs affect NER of *R*-cdA and *S*-cdA or repair of any other lesions.

8,5′-Cyclopurine 2′-deoxynucleosides cause significant distortion in the DNA helix due to the 8,5′-covalent bond.[22] *S*-cdA blocks transcription and DNA polymerases, causes transcriptional mutagenesis and multiple nucleotide deletions, and may cause neuronal death in diseases with defective NER.[23] The analogous lesion, (5′*S*)-8,5′-cyclopurine 2′-deoxyguanosine, blocks replication and is highly mutagenic, leading to G → A and G → T mutations, and inefficiently repaired.[24] Elevated levels of 8,5′-cyclopurine 2′-deoxynucleosides in genomic DNA of patients with various diseases suggest a role for these DNA lesions in disease processes.[25] A highly significant increase in

dx.doi.org/10.1021/bi201912c | *Biochemistry* 2012, 51, 1822−1824

concentrations of *R*-cdA and *S*-cdA in urine of patients is strong evidence of the accumulation of these compounds in DNA of patients due to excessive oxidative stress, inflammation, and oxidative damage. It is conceivable, therefore, that the aforementioned adverse biological effects of *R*-cdA and *S*-cdA may play a causative role in the development of atherosclerosis. On the other hand, the question of whether NER, BER, or both play a role in pathogenesis of atherosclerosis arises. No detectable elevation of the level of 8-OH-Gua in contrast to the highly significant increase in the level of *R*-cdA and *S*-cdA in urine of atherosclerosis patients may suggest that NER rather than BER plays an important role in pathogenesis of atherosclerosis. In the same context, a recent work demonstrated the occurrence of accelerated atherosclerosis and features of metabolic syndrome by the haploinsufficiency of the DNA repair protein ATM (ataxia telangiectasia mutated) in mice.[8] The DNA damage response kinases ATM and ATR (ataxia telangiectasia mutated and Rad3-related) are stimulated by bulky adducts in DNA.[26] 8,5′-Cyclopurine 2′-deoxynucleosides are repaired by NER,[13,14] as other helix-distorting bulky lesions.[27] It is, therefore, likely that ATM and ATR play a role in repair of *R*-cdA and *S*-cdA. This fact, in turn, points to the possible involvement of these lesions in atherosclerosis.

In conclusion, we show that the typical $^{\bullet}$OH-induced products of DNA, *R*-cdA and *S*-cdA, are excreted into urine of atherosclerosis patients at significantly greater levels than into urine of healthy individuals. The accurate and reproducible measurement, extraordinary chemical stability, and clear origin of *R*-cdA and *S*-cdA suggest that these compounds may be used as potential biomarkers of atherosclerosis for early detection, testing of drugs, monitoring and outcome of the therapy, and epidemiological studies. The noninvasive nature of urine collection is a great advantage for large-scale basic research and clinical studies of atherosclerosis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary experimental methods, patient information, purification and measurement of urine samples by LC−MS/MS and HPLC/GC−MS, and statistical analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written by M.D. with contributions of P.J. and R.O. All authors approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Certain commercial equipment or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified is necessarily the best available for the purpose. Urine samples were collected at the Hospital of the Collegium Medicum, Nicolaus Copernicus University. The study was approved by the Medical Ethics Committee of the Collegium Medicum (in accordance with Good Clinical Practice, Warsaw 1998), and all the patients gave informed consent. The extracts of urine samples were sent by the Collegium Medicum, Nicolaus Copernicus University to the National Institute of Standards and Technology pursuant to a Materials Transfer Agreement between the two institutions.

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