



Original Contribution

Urinary excretion of biomarkers of oxidatively damaged DNA and RNA in hereditary hemochromatosis

Kasper Broedbaek^{a,*}, Henrik E. Poulsen^a, Allan Weimann^a, Ghainsom D. Kom^b, Edzard Schwedhelm^b, Peter Nielsen^c, Rainer H. Böger^b^a Laboratory of Clinical Pharmacology Q7642, Rigshospitalet, DK-2200 Copenhagen, Denmark^b Clinical Pharmacology Unit, Institute of Experimental and Clinical Pharmacology and Toxicology, University Medical Center Hamburg–Eppendorf, D-20246 Hamburg, Germany^c Department of Biochemistry and Molecular Biology II, University Medical Center Hamburg–Eppendorf, D-20246 Hamburg, Germany

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ABSTRACT

Oxidatively generated damage to nucleic acids is considered to play a significant role in carcinogenesis, and it has been shown that people with hereditary hemochromatosis are at increased risk of cancer. In this study we used a new refined liquid chromatography–tandem mass spectrometry method to measure the urinary excretion of oxidatively generated 8-oxo-7,8-dihydroguanine and related 2'-deoxyribonucleoside and ribonucleoside derivatives in hereditary hemochromatosis patients, and we investigated the effect of treatment on the levels of these modifications. The study was carried out as a classical case–control study of 21 newly diagnosed, never treated hereditary hemochromatosis patients and 21 matched controls. We found that at baseline the urinary excretion of the RNA oxidation product 8-oxo-7,8-dihydroguanosine (8-oxoGuo) was 2.5-fold increased in patients compared with controls, and after phlebotomy treatment the excretion of the RNA oxidation product 8-oxoGuo returned to control values and the excretion of the DNA product 8-oxo-7,8-dihydro-2'-deoxyguanosine was reduced by 30%. In patients with hereditary hemochromatosis oxidative stress on nucleic acids is an important feature of the iron overload seen in this disease. By this mechanism cellular damage resulting in end organ damage, typically seen in the liver of such patients, may be mediated.

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Iron overload in hereditary hemochromatosis was first suggested by J.L. Sullivan as the major mechanism for the manifestations of the disease. A cysteine-to-tyrosine mutation (C282Y) in the autosomal hemochromatosis gene (HFE), which regulates the amount of iron absorbed from food, is present in the majority of hereditary hemochromatosis patients [1,2]. An increase in intestinal iron absorption leads to increased deposition of iron in parenchymal cells. The disease manifestations are particularly related to liver, pancreas, heart, and pituitary and include liver cirrhosis, diabetes mellitus, cardiomyopathy, and hypogonadism. These manifestations have mostly been observed in homozygotes, and their relevance in heterozygotes remains controversial. Untreated, the disease carries increased risk of premature death owing to cardiac failure, hepatocellular failure, portal hypertension, or hepatocellular carcinoma, and there are suggestions of increased risk of cardiovascular disease as well [3].

Abbreviations: Guo, guanosine; Hb, hemoglobin; 8-oxodGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; 8-isoPGF_{2α}, 8-isoprostaglandin F_{2α}.

* Corresponding author. Fax: +45 3545 2745.

E-mail address: kasper.broedbaek@rh.regionh.dk (K. Broedbaek).

Oxidative stress is a condition defined as an imbalance in the cells between oxidants formed by various processes and the antioxidative mechanisms [4]. Among the macromolecules that can be oxidatively modified, nucleic acids and particularly DNA are regarded as crucial targets. Because the nuclear genome exists in one copy only in each cell and because it carries the genetic information, oxidatively generated modifications of DNA carry a potential for permanent and transmissible changes in the molecule. Whereas other macromolecules can be replaced by de novo synthesis, the integrity of DNA relies on repair mechanisms [5]. Numerous oxidatively generated modifications of DNA have been identified in vitro [6], though only a minor portion has been verified to occur in vivo in humans. Most of the latter modifications are promutagenic [7] and have long been considered to be related to the development of cancer. Animals with deficiencies in DNA repair enzymes have an increased risk of cancer. Recently, increased oxidative stress on DNA has been shown as a risk factor for lung cancer in nonsmokers [8].

The Fenton reaction, i.e., the reaction between hydrogen peroxide and divalent iron, was first described in 1894 and produces the hydroxyl radical, which readily leads to 8-hydroxylation of 2'-deoxyguanosine, the resulting product often abbreviated as 8-oxodGuo¹. The corresponding oxidized base is termed 8-oxoGua,

Table 1
Oxidative stress on nucleic acids in hereditary hemochromatosis patients and controls

	Patients at baseline		Controls at baseline		<i>P</i> vs controls ^a
	Median	IQR	Median	IQR	
8-oxoGua	115.6 (<i>n</i> = 16)	51.8–152.5	107.2 (<i>n</i> = 18)	32.8–200	0.70
8-oxoGuo	40.4 (<i>n</i> = 21)	19.9–78.7	14.8 (<i>n</i> = 20)	8–34.8	0.006
8-oxodGuo	12.3 (<i>n</i> = 21)	10.1–15.7	9.0 (<i>n</i> = 20)	4.2–23	0.31

Values are nmol/g creatinine, *n* is given in parentheses. IQR, interquartile range.

^a Statistics were computed using the Mann-Whitney test.

and the RNA ribonucleoside counterpart 8-oxoGuo. These products can be measured in urine and are estimates of the combined DNA and RNA oxidation in an intact organism [9].

Iron is utilized in a variety of processes in the cells, e.g., cytochrome P450-mediated oxidation and oxygen transport in red blood cells, but it also carries potential detrimental effects from increased oxidative stress. Other transition metals possess the same detrimental potential. The iron overload in hereditary hemochromatosis has recently been demonstrated to inflict oxidative stress, measured as high levels of the lipid peroxidation product 8-isoprostaglandin F_{2α} (8-isoPGF_{2α}) in urine [1], and exposure to transition metals in ambient particulate matter in urban air increases oxidative stress in human volunteers [10]. In this paper we investigate whether hereditary hemochromatosis presents increased oxidative stress to nucleic acids and whether phlebotomy treatment has any effect on the oxidative stress to nucleic acids.

Material and methods

The study design has been described in detail elsewhere [1]. In brief, urine samples were collected from 21 newly diagnosed untreated cases with hereditary hemochromatosis and 21 controls, and blood samples were secured for analysis of iron status. Liver iron concentration and non-transferrin-bound iron were quantified in patients only [1]. All patients were HFE C282Y homozygotes. Spot urine samples were collected from controls and from the patients before the latter underwent treatment with phlebotomy, i.e., removal of 400–500 ml blood weekly (13–84 times), until they had slight anemia (Hb <11.5 g/dl) and until serum ferritin was below 30 μg/L. After this initial therapeutic goal was achieved, maintenance therapy consisted of 2–5 phlebotomies per year. Follow-up urine and blood samples were collected immediately before one of the routine phlebotomies during maintenance therapy.

Approval from the local ethics committee for studies in humans was obtained before the beginning of the study, and all participants gave their written informed consent to the study.

The chemical analyses of blood and urine are described in detail elsewhere [1] and urine was assayed for oxidatively modified guanine nucleobases and nucleosides by liquid chromatography–tandem mass spectrometry [9]. After the samples were thawed, they were heated to 37 °C for 10 min to dissolve the precipitate in the samples [11]. The

Table 2
Oxidative stress in patients with hemochromatosis before and after phlebotomy treatment

	Median	IQR	<i>P</i> vs baseline ^a
8-oxoGua (<i>n</i> = 12)	115.6	51.8–164.7	
After treatment	112.1	66.1–134.2	0.24
8-oxo-Guo (<i>n</i> = 19)	40.7	19.9–85.5	
After treatment	15	12.5–26.8	<0.001
8-oxodGuo (<i>n</i> = 19)	13.2	10.1–16.3	
After treatment	9.6	7.5–10.8	0.002

Values are nmol/g creatinine ± SEM, *n* is given in parentheses. IQR, interquartile range.

^a Statistics were computed using the Wilcoxon test.

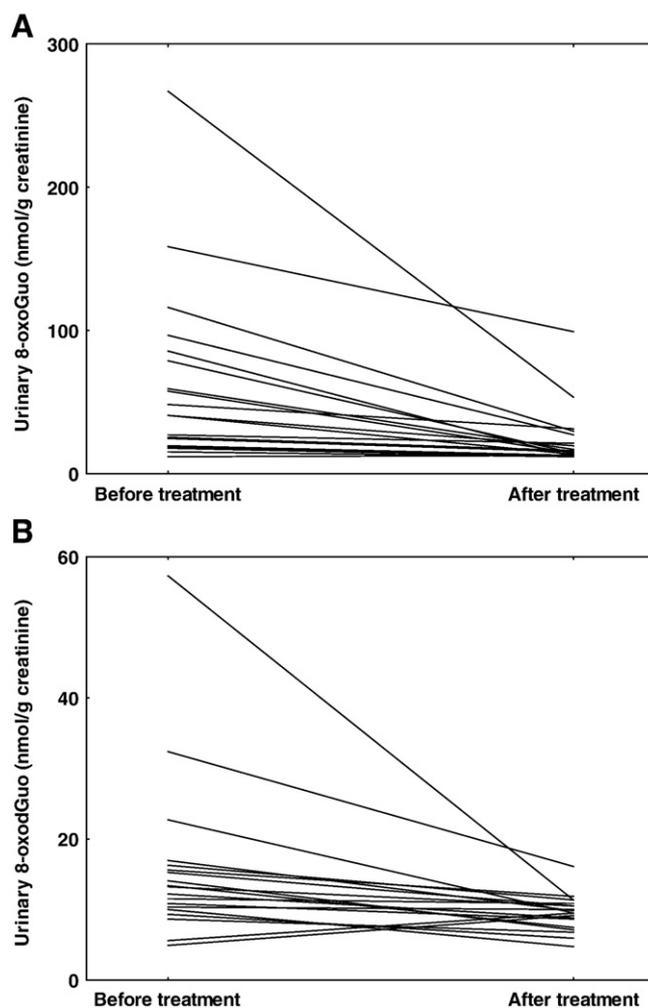


Fig. 1. (A) Urinary excretion of 8-oxoGuo (nmol/g creatinine) before and after phlebotomy. (B) Urinary excretion of 8-oxodGuo (nmol/g creatinine) before and after phlebotomy.

urine samples were then filtered through a VectaSpin 10K MWCO polysulfone centrifuge filter (Whatman, Maidstone, England). This step was introduced as it has been shown that some urine samples are unstable during storage in the autosampler, giving increasing concentrations of dGuo and guanosine (Guo) with increasing storage

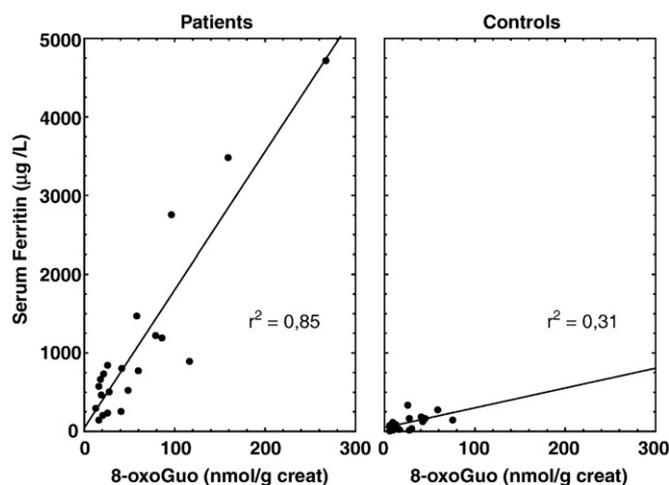


Fig. 2. Correlation between 8-oxoGuo and ferritin.

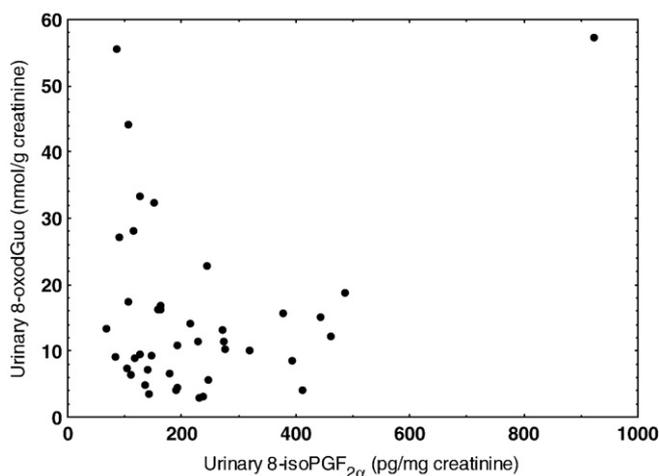


Fig. 3. Correlation between urinary 8-isoPGF_{2α} and 8-oxodGuo.

times at 4 °C. Apparently oligonucleosides in the urine are hydrolyzed to the unmodified nucleosides upon storage [12]. After ultrafiltration the samples are stable during storage in the autosampler.

The analysis of nucleosides makes it possible to interpret the origin as DNA or RNA. It is assumed that contributions from the nucleotide pool must be negligible owing to the small pool size compared to total DNA or total RNA [13,14]. Thus an increased 24-h urinary excretion of 8-oxodGuo and 8-oxoGuo will reflect a general and increased rate of oxidation of DNA and RNA.

In the process of hydroxylation of guanine the transient 8-hydroxy-7,8-dihydroguanyl radical is formed. Under reducing conditions a competitive reduction of this intermediate has been shown to be efficient in cells, which leads to the formation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine [15]. To what extent the 8-hydroxy-7,8-dihydroguanyl radical is reduced to 2,6-diamino-4-hydroxy-5-formamidopyrimidine is not estimated in this project owing to inadequate methodology at the time of analysis.

Results

At baseline the urinary excretion of the RNA oxidation product 8-oxoGuo was 2.5-fold increased in patients with hereditary hemochromatosis ($P=0.006$) compared with controls (Table 1). There were no significant differences in the excretion of the DNA oxidation product (8-oxodGuo) or the oxidized base (8-oxoGua).

After treatment of the patients with phlebotomy, the excretion of both the DNA and the RNA oxidation products was reduced significantly (Table 2). The excretion of the RNA oxidation product 8-oxoGuo returned to control values ($P<0.001$) and the excretion of the DNA product 8-oxodGuo was reduced by 30% compared to baseline values ($P=0.002$). Figs. 1A and B depict the individual values of DNA and RNA oxidation before and after phlebotomy.

Serum ferritin was linearly correlated with 8-oxo-7,8-dihydroguanosine in hemochromatosis patients, $r^2=0.85$, $P<0.001$, as well as in controls $r^2=0.31$, $P=0.01$. However, the plots show quite different slopes (Fig. 2). There were no consistent correlations between the other measures of iron status and the excretion of the oxidized guanine species (data not shown).

There was no significant correlation between the change in excretion of 8-oxoGuo or 8-oxodGuo and 8-isoPGF_{2α} from before to after treatment (Figs. 3 and 4).

Discussion

In this study we show a 2.5-fold increase in RNA oxidation, measured as excretion of 8-oxoGuo in HFE C282Y homozygous

patients compared with controls. Interestingly, the DNA oxidation measured as excretion of 8-oxodGuo did not differ between hemochromatosis patients and controls. However, after treatment of the hemochromatosis patients until normalization of their iron parameters, both RNA and DNA oxidation were reduced—the DNA oxidation by 30% and the RNA oxidation to one-third of the values in the untreated state.

There are rather few studies on oxidative stress in HFE C282Y homozygous patients and on the effects of treatment on oxidative stress. It was recently reported [1] that lipid peroxidation measured by 8-isoPGF_{2α} urinary excretion was increased in hereditary hemochromatosis patients compared with control subjects, and subsequently reduced after the treatment that normalized iron status. Taken together, these data provide clear evidence of severe oxidative stress in this disease.

The major feature of hemochromatosis is iron overload. Iron is an essential element whose deficiency leads to severe anemia and cellular malfunctions. Iron plays a crucial role in cellular biochemical processes such as oxygen transport, mitochondrial respiration, and metabolism of xenobiotics. An important feature of iron is its favorable redox potential that can switch between ferrous and ferric states. Under nonpathological conditions iron is carefully controlled and bound to various structures and molecules, which prevents the hazards of free iron in biological systems [16]. Iron per se is not regarded as a carcinogen; however, HFE C282Y heterozygotes show an increased prevalence of hepatocellular carcinoma [17] and are also suggested to have an increased risk of breast cancer due to low-level-radiation sensitivity [18]. Also, in a mouse model of hepatic porphyria with iron overload, increases in hepatic lipid peroxides and 8-oxodGuo were found [19]. Thus, there is sufficient evidence to conclude that oxidative stress on lipids and nucleic acids is a consequence of the iron overload in homozygous HFE C282Y patients. The iron overload and subsequent nucleic acid oxidation also seem to be involved in the high risk for hepatocellular carcinoma in carriers of the HFE C282Y mutation.

In a small study of 6 patients with end-stage hemochromatosis who underwent orthotopic liver transplantation, liver levels of 8-oxodGuo were similar to levels in control livers [20]. This is in agreement with the present finding of similar excretion of 8-oxodGuo in urine in our larger number of hemochromatosis patients. In their investigation, Carmichael et al. did not have the chance to do a similar follow-up. In our study there was a 30% reduction in the excretion of 8-oxodGuo after treatment (from 13.2 to 9.6 nmol/g creatinine). Earlier, a 20% reduction in oxidative stress on DNA after smoking cessation was demonstrated [21]. In view of that effect the 30% reduction is indeed considerable.

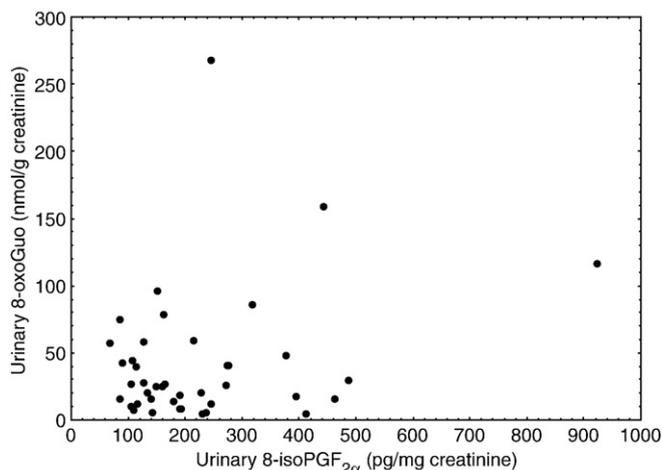


Fig. 4. Correlation between urinary 8-isoPGF_{2α} and 8-oxoGuo.

The oxidation of the RNA-derived guanosine was 2.5 times increased in hereditary hemochromatosis patients. We have shown earlier that RNA oxidation is more frequent than DNA oxidation [9], and RNA oxidation has been demonstrated to be a prominent feature of other conditions such as degenerative CNS diseases [22]. The present finding of high oxidation of RNA in hemochromatosis patients and the reduction from about 40 to 15 nmol/g creatinine, the same as in controls, is firm evidence of the oxidative stress from iron overload.

Serum ferritin originates as an intracellular apoferritin protein with iron bound. The plasma level reflects the total iron depot in the body. It is high in conditions with increased iron stores. The very strong correlation between ferritin levels and 8-oxo-7,8-dihydroguanosine and the fact that the regression line passes the graph origin are strong indicators of iron overload being the mechanism for the increased RNA oxidation. This supports the notion that iron, at least in patients with iron overload, induces severe oxidative stress.

The lack of correlation between changes in 8-isoPGF_{2α} excretion and changes in 8-oxo-7,8-dihydroguanosine and 8-oxo-7,8-dihydro-2'-deoxyguanosine excretion could reflect the fact that these molecules exist in different compartments. Naturally, excretion of all three molecules declines with decreased oxidative stress, but because of the cellular compartmentalization and possible differences in the kinetics of their resynthesis, the correlation is nonsignificant. The lack of correlation also suggests (1) that a more detailed organ, site, and maybe spatial view on "oxidative stress" is needed and (2) differential effects on various biomarkers might prove an opportunity for more detailed insight into oxidative stress in vivo.

In conclusion, we have demonstrated that oxidative stress is an important feature of the iron overload seen in hereditary hemochromatosis resulting in considerable and reversible RNA oxidation, which correlates strongly and linearly with ferritin levels. The treatment known to improve the normalized survival rate and lower lipid peroxidation also results in reduced RNA and DNA oxidation and is a plausible explanation for the amelioration of the increased liver cancer risk seen in treated patients. Currently, it is unknown which type of RNA is oxidized, and the biological consequences are also obscure. Oxidation of mRNA, and maybe siRNA and microRNA, has the potential for impairment of important cellular functions and differentiation.

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