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Cellular background level of 8-oxo-7,8-dihydro-2′-deoxyguanosine: an isotope based method to evaluate artefactual oxidation of DNA during its extraction and subsequent work-up

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The measurement of oxidative damage to cellular DNA is a challenging analytical problem requiring highly sensitive and specific methods. In addition, artefactual DNA oxidation during its extraction and subsequent work-up may give rise to overestimated levels of oxidized DNA bases. In the present study, we have used ^{18}O-labelled 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) as an internal standard to evaluate the extent of artefactual DNA oxidation during the critical steps preceding the measurement. The labelled oxidized purine nucleoside was specifically generated in cellular DNA using the recently available generator of ^{18}O-labelled singlet oxygen. Artefactual DNA oxidation that could take place during the work-up increases the level of 8-oxodGuo but not of the ^{18}O-oxidized nucleoside. Therefore, the ratio between the two compounds, as measured by high performance liquid chromatography coupled to tandem mass spectrometry, allows an unambiguous comparison of different methodologies. The comparison of different DNA extraction protocols led to the conclusion that artefactual DNA oxidation during the extraction step could be minimized if: (i) nuclei are isolated after cell lysis; (ii) desferrioxamine, a transition metal chelator is added to the different extraction buffers; and (iii) sodium iodide (or alternatively guanidine thiocyanate) is used for DNA precipitation. It was also demonstrated that sodium iodide does not decompose the targeted oxidized purine nucleoside. In addition, three different DNA digestion protocols were evaluated and they were found to give rise to similar results. Using the best-studied protocol, the steady-state cellular background level of 8-oxodGuo, in a lymphocyte cell line, was determined to be ~0.5 lesions/10^6 DNA nucleosides.

Introduction

The level of oxidized DNA damage in cells has been extensively used during the last two decades as an indicator of the occurrence of oxidative stress (1,2). In this respect, much attention was focused on the measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), a major and ubiquitous oxidation product of DNA (3). Several analytical methods (4) that imply two different strategies are available for this purpose (5). The direct approach is designated to single out the lesion individually using physico-chemical methods subsequent to DNA extraction and hydrolysis. These include, among others, high performance liquid chromatography (HPLC) coupled to electrochemical detection (HPLC-EC) (6–8), gas chromatography-mass spectrometry (GC-MS) (9,10) and more recently HPLC associated to tandem mass spectrometry (HPLC-MS/MS) (11–15). In the indirect approach, the whole DNA structure is preserved and the formation of the lesions is monitored *in situ*. The measurement could be performed either using antibodies (16) that generally exhibit low specificity or through the nicking activity of a specific DNA repair enzyme. The bacterial formamidopyrimidine DNA *N*-glycosylase is able to convert 8-oxodGuo into a strand break. Quantification of the number of strand breaks thus generated is assessed by applying the comet assay (17), the alkaline elution technique (18) or the unwinding method (19).

A survey of the literature indicates that, depending on the method used, significant differences, up to two or three orders of magnitude, were observed for the measured values of the cellular level of DNA lesions, especially 8-oxodGuo (20–22). Even if variations between cell types and organs could not be excluded, such large differences may be attributed, at least partly, to inaccurate experimental protocols that give rise to overestimated or underestimated levels of 8-oxodGuo (23,24). As a general trend, values obtained by the enzymatic assays (indirect approach) are significantly lower than those inferred from the more direct methods of measurement. Therefore, this indicates that either the indirect method underestimates the cellular level of 8-oxodGuo or application of the direct approach gives rise to an overestimation of this level (or both?).

For direct approaches, the measurement of the lesion requires in the first step isolation of DNA either from cells or tissues, followed by its hydrolysis that can be achieved either chemically or biochemically. Then, the released lesion has to be quantified using a sensitive detection technique at the output of a chromatographic column. In each of the individual steps, artefactual DNA oxidation may occur (25–27). Interestingly, the main drawbacks that are associated with the application of the available analytical techniques are now identified (22). For instance, it is now established that overestimated values obtained by the GC-MS assay were mostly due to the occurrence of artefactual DNA oxidation during the derivatization step (28,29) used to obtain volatile bases, prior to the gas chromatography analysis. Overestimated measurements may have led to inaccurate conclusions (30) such as, for example, the claimed *in vivo* pro-oxidant properties of vitamin C (31). Prepurification of the lesion to be measured before

Abbreviations: DHPN$^{18}O_2$, naphthalene derivative; EC, electrochemical detection; GC-MS, gas chromatography-mass spectrometry; GTC, guanidine thiocyanate; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine.

derivatization prevents the artefactual oxidation of the overwhelming normal bases occurring (32). Interestingly, application of the improved assay, that consists in the HPLC prepurification of the targeted lesion prior to GC-MS analysis, gives values similar to those obtained by HPLC-EC (32,33). Recently, similar levels of 8-oxodGuo were measured in DNA using the HPLC-EC, HPLC-MS/MS and HPLC-GC-MS assays (15,34,35). It should also be mentioned that the origin of the high levels of 8-oxodGuo obtained using the [^{32}P]post-labelling assay might be explained, at least partly, by the occurrence of an artefactual DNA oxidation due to the self-radiolysis process associated with the β-decay of ^{32}P atoms (5,36).

A still remaining major issue deals with the possible contribution of DNA extraction and hydrolysis to the induction of artefactual oxidation of the normal nucleosides (1). Recent methodological improvements, including the use of NaI to isolate DNA, have allowed measurement, using a direct approach, of 8-oxodGuo in cellular DNA of untreated cells at levels <1 lesion/10^6 DNA bases (3,37–40). However, the lowest value is not necessarily the best one. Thus, the possibility of 8-oxodGuo decomposition, for example in the presence of NaI, that could give rise to an underestimated value, was recently raised (1,3,40,41). Therefore, the comparison of different protocols aimed at isolating cellular DNA is still required to determine the most appropriate methodology.

In the present work we have used ^{18}O-labelled 8-oxodGuo that could be specifically produced in cellular DNA, as an internal standard in order to define an optimized protocol for DNA isolation and hydrolysis. Interestingly, a chemical source of ^{18}O-labelled singlet oxygen has recently become available through the synthesis of a labelled endoperoxide of a water-soluble naphthalene derivative (DHPN$^{18}O_2$) (42). Such a thermolabile endoperoxide is able to penetrate eukaryotic cells and to release singlet oxygen intracellularly. Incubation of cells with DHPN$^{18}O_2$ at 37°C was shown to specifically induce the formation of [^{18}O]8-oxodGuo (43), as measured using the HPLC-MS/MS assay (12,15). Therefore, the labelled oxidized nucleoside could be used as a reference compound to evaluate different protocols of DNA extraction and hydrolysis. Artefactual DNA oxidation that may take place during the work-up will increase the level of unlabelled 8-oxodGuo but not of the labelled oxidized nucleoside. On the other hand, a treatment that induces degradation of the oxidized purine base should lower the measured level of [^{18}O]8-oxodGuo.

Materials and methods

Chemicals and biochemicals

Nuclease P1, acid phosphatase (from sweet potatoes), phosphodiesterases I and II, RNase A, RNase T1, Triton X-100, NaI, $MgCl_2$, desferrioxamine mesylate, SDS, TEMPO and calf thymus DNA were obtained from Sigma Co. (St Louis, MO, USA). Alkaline phosphatase was obtained from Roche Diagnostic (Mannheim, Germany). Protease was a product from Qiagen Genomic (Hilden, Germany). RPMI-1640, FCS and PBS Dulbecco's were purchased from Life Technologies (Paisley, United Kingdom). The isotopically labelled internal standard [$^{15}N_5$]8-oxodGuo (15) and the ^{18}O-labelled endoperoxide of *N,N′*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide (DHPN$^{18}O_2$) (42) were synthesized as described previously. The isotopic purity of DHPN$^{18}O_2$ was 95%, as determined by MS analysis.

Cell line and culture

THP-1, a neoplastic monocytic cell line, was isolated from the peripheral blood of a young leukaemic donor. To obtain cells in exponential growth phase, 24 h prior to treatment they were suspended in a fresh RPMI-1640 medium supplemented with 10% decomplemented fetal calf serum and incubated in a 5% CO_2 atmosphere at 37°C.

DNA treatment with DHPN$^{18}O_2$

Calf thymus DNA was dissolved in deionized distilled water to a final concentration of 0.5 mg/ml. Then, 1 ml of the DNA solution was treated with 0, 10, 20 or 50 µl of 85 mM DHPN$^{18}O_2$ (42,44). After incubation for 1 h at 37°C, DNA was precipitated using 50 µl of 3 M ammonium acetate and 2.5 ml of EtOH. The tubes were inverted several times and precipitated DNA was recovered by centrifugation at 5000 *g* for 5 min. The pellets were washed twice with 70% cold EtOH. Thereafter, each treated DNA sample was dissolved into 1.2 ml of deionized distilled water and the resulting solution was divided into 12 identical fractions, prior to enzymatic digestion.

Cell treatment with DHPN$^{18}O_2$

Cells were recovered by centrifugation (300 *g*, 4 min) and washed twice with PBS. About 400 million cells were suspended into 800 µl of cold (4°C) PBS. Then, either 100 or 200 µl of 85 mM DHPN$^{18}O_2$ was added and the resulting suspension was stored at 4°C for 15 min. Subsequently, the solution was maintained for 1 h at 37°C in a water bath (43). Then, 25 ml of PBS was added and the cells were recovered by centrifugation. The cellular pellet was suspended into 24 ml of PBS and then divided into 12 identical fractions that contained ~20×10^6 cells. Cells were again recovered by centrifugation to remove the PBS prior to DNA extraction.

DNA digestion

Protocol Dig-1. To 100 µl of the DNA solution was added 5 µl of nuclease P1 (5 U), 1 µl of phosphodiesterase II (0.004 U) together with 10 µl of buffer P1 10× (200 mM succinic acid, 100 mM $CaCl_2$, pH 6.0). The resulting solution was incubated for 2 h at 37°C. Thereafter, 10 µl of alkaline phosphatase buffer 10× (500 mM Tris, 1 mM EDTA, pH 8) was added together with 0.2 µl (0.003 U) of phosphodiesterase I and 0.25 µl (5 U) of alkaline phosphatase. The samples were then incubated at 37°C for 2 h. After enzymatic digestion, the solution was neutralized by addition of 10 µl of 0.1 M HCl. Then, the samples were centrifuged for 5 min at 5000 *g* prior to HPLC-MS/MS analysis.

Protocol Dig-2. To the DNA solution was added 10 µl of 1 U/µl nuclease P1 dissolved in 300 mM sodium acetate and 1 mM $ZnSO_4$ aqueous solution, pH 5.3. Then, the samples were incubated at 37°C for 2 h. Thereafter, 10 µl of 10× alkaline phosphatase buffer (500 mM Tris, 1 mM EDTA, pH 8) together with 0.2 µl of alkaline phosphatase was added and the incubation was pursued at 37°C for 2 h. Subsequently, 10 µl of 0.1 M HCl was added to neutralize the solution.

Protocol Dig-3. To the DNA solution was added 10 µl of 1 U/µl nuclease P1 dissolved in 300 mM sodium acetate, 1 mM $ZnSO_4$ aqueous solution, pH 5.3 together with 2 µl (0.36 U) of acid phosphatase. The solution was then incubated for 2 h at 37°C prior to HPLC-MS/MS analysis.

DNA extraction

An overview of the major differences between the evaluated protocols for DNA isolation is given in Table I.

Protocol A. The 'chaotropic' method was used (39,45,46) with the following modifications. To the cellular pellet was added 1.5 ml of lysis buffer A (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris, 0.1 mM desferrioxamine pH 7.5, 1% Triton X-100). After a vigorous agitation, the nuclei were collected by centrifugation at 1500 *g* for 10 min at 4°C and washed with 1.5 ml of buffer A. To the nuclear pellet, obtained after centrifugation (1500 *g* for 10 min at 4°C) was added 600 µl of buffer B (10 mM Tris, 5 mM EDTA-Na_2, 0.15 mM desferrioxamine, pH 8.0) and 35 µl of SDS 10%. A vigorous agitation was performed to allow lysis of the nuclear membrane. Thereafter, 30 µl of RNase A (1 mg/ml) in RNase buffer (10 mM Tris, 1 mM EDTA, 2.5 mM desferrioxamine, pH 7.4) and 8 µl of RNase T1 (1 U/µl in RNase buffer) was added and the samples were incubated for 15 min at 50°C. Then, 30 µl of Qiagen protease (20 mg/ml in H_2O) was added prior to incubation at 37°C for 1 h. Subsequently, 1.2 ml of the NaI solution (7.6 M NaI, 40 mM Tris, 20 mM EDTA-Na_2, 0.3 mM desferrioxamine, pH 8.0) and 2 ml of 2-propanol was added. DNA precipitation was achieved by gently inverting the tube several times. Then, DNA was recovered by centrifugation at 5000 *g* for 15 min at 4°C and washed with 1 ml of 40% 2-propanol. After appropriate centrifugation (5000 *g* for 15 min), DNA was washed again using 1 ml of 70% EtOH. Finally, DNA was recovered by centrifugation and dissolved into 0.1 mM desferrioxamine prior to DNA digestion.

Protocol B. The same procedure as described for protocol A was used, except for DNA precipitation. DNA was precipitated using 1.2 ml of 5 M NaCl, 40 mM Tris, 20 mM EDTA-Na_2, 0.3 mM desferrioxamine, pH 8.0 and 2 ml of 2-propanol. DNA was collected by centrifugation and washed twice as described for protocol A and finally dissolved into 0.1 mM desferrioxamine.

Protocol C

The same procedure described for the protocol A was used but desferrioxamine was absent in all the different buffers used to extract DNA.

Table I. Major differences between the evaluated protocols for DNA isolation

Protocol	Nuclei isolation	Antioxidant	Incubation	Final DNA isolation	Approximate duration
A	+	Desferrioxamine	15 min 50°C, 60 min 37°C	NaI precipitation	2 h 30 min
B	+	Desferrioxamine	15 min 50°C, 60 min 37°C	NaCl precipitation	2 h 30 min
C	−	None	15 min 50°C, 60 min 37°C	NaI precipitation	2 h 30 min
D	−	Desferrioxamine	2×60 min 37°C	NaCl precipitation	3 h
E	+	?[a]	15 min 37°C	Precipitation ?[a]	1 h 30 min
F	+	None	Always on ice	GTC precipitation	3 h 30 min
G	+	Desferrioxamine	15 min 50°C, 60 min 37°C	NaI Precipitation	2 h 30 min
G-	+	Desferrioxamine	60 min 37°C	NaI precipitation	2 h 15 min
H	+	Desferrioxamine during nuclei isolation	Always on ice	GTC precipitation	3 h
I	+	Desferrioxamine after nuclei isolation	15 min 50°C, 60 min 37°C	NaI precipitation	3 h
J	+	TEMPO	60 min 37°C, 30 min 37°C	Purification: anion exchange columns	6 h

[a]For protocol E, DNA was extracted using a commercial kit, and it was not possible to obtain from the company the composition of the reagents.

Protocol D. To the cellular pellet was added 3 ml of the extraction buffer (20 mM NaCl, 20 mM Tris, 20 mM EDTA, 5 mM desferrioxamine, pH 8) together with 200 µl of 10% SDS. After a vigorous stirring, 100 µl of 1 mg/ml proteinase was added and the resulting solution was incubated for 1 h at 37°C. Thereafter, 0.5 ml of dichloromethane was added and the samples were agitated. After centrifugation (5000 *g*, 5 min), 300 µl of 4 M NaCl was added to the collected aqueous (upper) phase. Then, 7.5 ml of cold EtOH was added and the samples gently shaken until complete precipitation of the nucleic acids. Then, the samples were centrifuged at 3000 *g* for 10 min and the pellets were rinsed using 500 µl of cold 70% EtOH. The resulting nucleic acid pellets were solubilized into 1 ml RNase buffer (10 mM Tris, 1 mM EDTA, 2.5 mM desferrioxamine, pH 7.4) to which 100 µl of RNase A (5 mg/ml in RNase buffer) and 10 µl of RNase T1 (1000 U/ml in RNase buffer) were added. The samples were then incubated at 37°C for 1 h. Subsequently, 100 µl of 4 M NaCl aqueous solution and 2.5 ml of cold EtOH were added. The samples were gently shaken and then centrifuged at 5000 *g* for 5 min. The supernatant was discarded and the DNA pellet was rinsed with 500 µl of 70% EtOH. Then, the DNA pellet was solubilized into 100 µl of 0.1 mM desferrioxamine prior to enzymatic digestion.

Protocol E. DNA was extracted using the GenomicPrep® 'Cells and Tissue DNA Isolation Kit' obtained from Amersham Pharmacia Biotech (Roosendaal, Nederland). Typically, 1.8 ml of nuclei extraction buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris, 0.1 mM desferrioxamine, 2% Triton X-100, pH 7.4) was added to the cellular pellet. The resulting solutions were agitated for 15 s and then centrifuged for 10 min at 1500 *g*. The supernatant was removed with a Pasteur pipette and 20 µl of 0.9% NaCl was added to resuspend the nuclear pellet. Then, 900 µl of the kit 'Cell lysis solution' was added and homogenization was performed using a pipette (10 times). Thereafter, 3 µl of the kit 'RNase A', was added and the tubes were mixed 25 times by inversion and the resulting solution was incubated at 37°C for 15 min. Then, tubes were allowed to cool down to ambient temperature (10 min) and 300 µl of the kit 'Protein precipitation solution' was added. Samples were agitated for 20 s and centrifuged at 13 000 *g* for 3 min. The supernatant was transferred to a microtube that contained 900 µl 2-propanol. Tubes were mixed 50 times by inversion until DNA condensation and then centrifuged at 13 000 g for 1 min. The supernatant was discarded and 600 µl of 70% ethanol was added. After agitation by inversion 10 times, precipitated DNA was recovered by centrifugation at 13 000 *g* for 1 min. The supernatant was again discarded and the tubes were inverted open on a filter paper for 15 min. Then, 0.1 mM desferrioxamine was added prior to enzymatic digestion.

Protocol F. DNA was extracted using the so-called 'cold GTC method', which recently became available (47). All aqueous solutions were treated with the chelex resin, as described previously (47). Cells were homogenized with a pipette in 1 ml 20 mM Tris, pH 7.5. Then, 4.5 ml 20 mM Tris, pH 7.5 was added and samples were centrifuged at 1000 *g* for 10 min. The supernatant was carefully removed and the nuclear pellet dissolved into 1 ml 0.5% Tween 20, 20 mM Tris, pH 7.5 using a 1 ml pipette. Then, 3.5 ml buffer was added and samples were kept on ice for 5 min. Thereafter, samples were centrifuged at 1000 *g* for 10 min, the supernatant was discarded and the recovered nuclei were washed a second time. Then, the supernatant was carefully removed and 1.7 ml of guanidine thiocyanate (GTC) was added and nuclei were dissolved by pipetting 10 times. The samples were left on ice and again mixed by pipetting 20 times. Then, 850 µl of the solution was transferred to two pre-spun 2.0 ml PLG tube. Tubes were filled with 850 µl sevag, shaken and centrifuged at 13 000 *g* for 5 min. After transferring the upper DNA-containing phase to a new 2.0 ml tube, 850 µl 2-propanol was added to precipitate DNA. This was achieved at –20°C for 15 min. Then, DNA was collected by centrifugation at 20 800 *g* for 10 min, and washed with 1.8 ml of 70% ethanol. After centrifugation (20 800 *g* for 3 min), the supernatant was carefully removed with a pipette and DNA dissolved into 0.1 mM desferrioxamine prior to HPLC-MS/MS analysis.

Protocol G. DNA was isolated under conditions similar to those used for protocol A, with the following modifications. Cell pellet was obtained in a 1.5 ml tube by centrifuging at 2000 *g* for 20 s. PBS was removed and the tube flicked to disperse the pellet. Then, 0.5 ml lysis solution (buffer A of protocol A) was added and the samples were vortex mixed for ~30 s. The samples were pulse centrifuged at top speed (16 000 *g*) for 20 s in a microcentrifuge. Then, the supernatant was removed and the tube flicked again. This step was repeated a second time using 1 ml of lysis solution (buffer A, protocol A). Then, 0.2 ml buffer B (protocol A) that contained (1% w/v final) sarcosyl was added and samples were vortex mixed for ~30 s. Thereafter, 10 µl of RNase A (1 mg/ml) in RNase buffer (10 mM Tris, 1 mM EDTA, 2.5 mM desferrioxamine, pH 7.4) and 3 µl of RNase T1 (1 U/µl in RNase buffer) were added and the resulting samples were incubated for 15 min at 50°C. Then, 10 µl of Qiagen protease (20 mg/ml in H_2O) was added and the samples were further incubated for 1 h at 37°C. To precipitate DNA, 0.4 ml of NaI solution (protocol A) was added and the solution was gently mixed by inversion. Then, 0.5 ml ice cold 100% 2-propanol was added and the resulting solutions were gently mixed by inversion. Samples were pulse centrifuged at top speed for 20 s, and the supernatant was removed. Subsequently, 1 ml of 40% (v/v) ice cold 2-propanol was added and the resulting samples were gently mixed by inversion and centrifugation was repeated. The supernatant was removed and 1 ml of 70% (v/v) ice-cold ethanol was added and gently mixed by inversion. After centrifugation, the supernatant was removed and DNA was resuspended into 0.1 mM desferrioxamine prior to its digestion.

Protocol G–. This protocol was similar to protocol G except that the RNase treatment was omitted.

Protocol H. This was a combination of protocols F and G. Nuclei were isolated according to protocol G and then, DNA was isolated from the nuclei using the second part of protocol F. Typically, isolation of nuclei was performed following protocol G (using buffer A). Then, DNA was isolated by adding the GTC solution as described for protocol F.

Protocol I. Again, this was a combination of the protocols F and G. Nuclei were isolated using protocol F. Then, instead of using the GTC solution for DNA precipitation, DNA was precipitated using sodium iodide following protocol G.

Protocol J. DNA was extracted using the Qiagen kit as described previously (8). Nuclei were isolated using 10 mM Tris, 140 mM KCl, 10 mM Na-EDTA, 5 mM TEMPO aqueous solution, pH 7.4. Then, 4 ml of 50 mM MOPS, TEMPO 5mM aqueous solution, pH 7.0, 0.5 ml of 10% SDS and 250 µl of proteinase K (20 mg/ml) were added to the nuclear pellet and the digestion was carried out for 1 h at 37°C. This was followed by the addition of 1.5 ml of 50 mM Tris–HCl and 10 mM EDTA aqueous solution, pH 8.0 that contained RNase A (100 µg/ml). The resulting homogenate was further digested for 30 min at 37°C. The DNA purification from the homogenate was achieved using Qiagen anion exchange columns according to the specifications provided by the manufacturer. However, 5 mM TEMPO, final concentration, was added to the different extraction buffers (except to the QBT buffer which was used

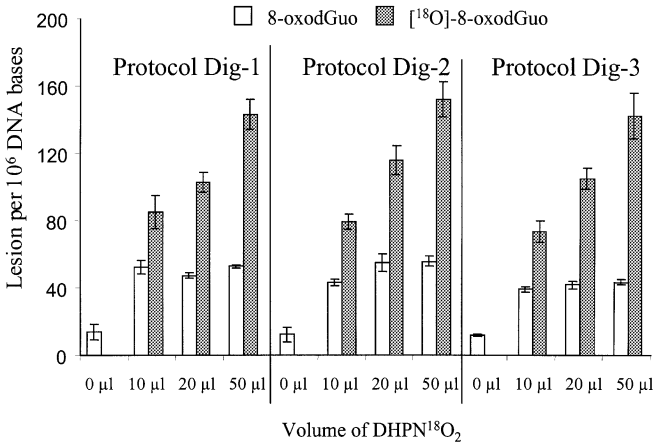

Fig. 1. Effect of different enzymatic digestion protocols on the measured levels of 8-oxodGuo and [^{18}O]8-oxodGuo. Calf thymus DNA was treated with various amounts of DHPN$^{18}O_2$ and subsequently digested by either protocols Dig-1, Dig-2 or Dig-3 (for details see Materials and methods), prior to HPLC-MS/MS measurement. Values, expressed as the number of lesions per 10^6 DNA bases, represent the average and standard deviation of four independent determinations.

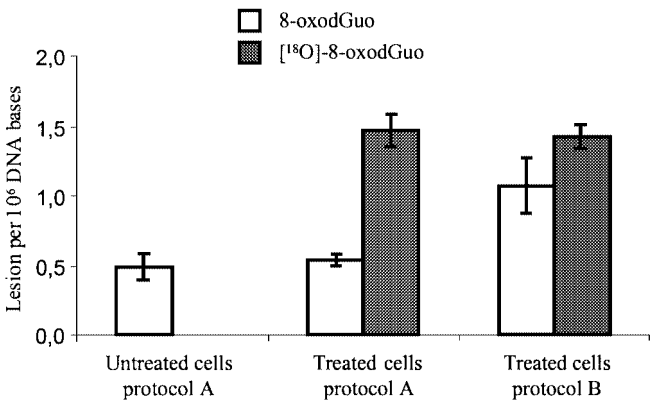

Fig. 2. Effect of NaI precipitation on the measured levels of 8-oxodGuo and [^{18}O]8-oxodGuo in cellular DNA. DNA was extracted from either untreated cells (using protocol A) or from DHPN$^{18}O_2$ treated cells extracted by protocols A and B. In this experiment, cells were treated with 200 µl of 85 mM DHPN$^{18}O_2$. Results, representing the average and standard deviation of four independent determinations, are expressed as the number of lesions per 10^6 DNA bases.

to equilibrate the anion exchange columns). The DNA pellet was solubilized into 0.1 mM desferrioxamine prior to enzymatic digestion.

HPLC-MS/MS analysis

The HPLC-MS/MS system used to measure 8-oxodGuo, as well as the corresponding ^{18}O- and $^{15}N_5$-labelled derivatives has been described in detailed elsewhere (12,15,43). In addition to the MS detector, a UV detector set at 260 nm was used to quantify the amount of DNA using the area of the peak of 2′-deoxyguanosine (dGuo) and external calibration (15). In addition, the amount of contaminating RNA was determined using the area of the peak of guanosine (Guo).

Results

DNA digestion

Calf thymus DNA was incubated in the presence of DHPN$^{18}O_2$ to induce the formation of [^{18}O]8-oxodGuo (44). It should be noticed that such a treatment also induces a significant formation of unlabelled 8-oxodGuo (Figure 1). Work is in progress to determine the origin of unlabelled 8-oxodGuo that cannot be due to the contaminating unlabelled DHPNO$_2$. Then, DNA was digested using either nuclease P1 and subsequently alkaline phosphatase (protocol Dig-2), a mixture of nuclease P1 and acid phosphatase (protocol Dig-3) or a cocktail of endo- and exo-nucleases (protocol Dig-1). The levels of the oxidized purine, determined by the HPLC-MS/MS assay, according to the different protocols are reported in Figure 1. No significant differences were observed between the levels of 8-oxodGuo on one hand and those of [^{18}O]8-oxodGuo on the other hand, following enzymatic digestion by the three above-mentioned protocols. In contrast to previous observations (48–50), our data indicate that no significant decomposition of 8-oxodGuo occurs under the slightly alkaline conditions required by the alkaline phosphatase hydrolysis, nor that DNA oxidation takes place in the presence of acid phosphatase. Therefore, for the measurement of 8-oxodGuo, DNA digestion could be performed by any of the three enzymatic procedures.

DNA extraction

To compare the different protocols of DNA extraction, cells were initially treated with the chemical generator of labelled singlet oxygen. As described previously (43), such a treatment induces the formation of [^{18}O]8-oxodGuo in cellular DNA (Figure 2). It should be noticed that the amount of induced [^{18}O]8-oxodGuo could vary between experiments (Figures 2 and 3) due to the difficulty to treat a similar number of cells under identical conditions. Then, in a first series of experiments, evaluation of the chaotropic protocol was achieved varying one parameter at a time (Table I). First, an experiment was performed to evaluate the effect of DNA precipitation using NaCl (protocol B) instead of NaI (protocol A) on the steady-state level of 8-oxodGuo. The amounts of 8-oxodGuo and [^{18}O]8-oxodGuo measured in cellular DNA upon treatment with DHPN$^{18}O_2$, using the two different protocols are reported in Figure 2. The results indicate that the measured level of [^{18}O]8-oxodGuo is similar for both protocols, therefore this strongly suggests that the lowest values obtained using the chaotropic NaI method for DNA precipitation, are not due to 8-oxodGuo degradation. In addition, the level of 8-oxodGuo measured following NaI precipitation is lower compared with that determined after NaCl precipitation. Such a result indicates that DNA oxidation had occurred during NaCl precipitation and that the use of NaI suppresses, or at least significantly reduces, the artefactual oxidation of the guanine base. In addition, to determine the putative protective role of desferrioxamine, DNA extraction was performed using buffers that contained (protocol A) or not (protocol C) desferrioxamine. In parallel, a protocol that does not involve the initial isolation of nuclei was used for DNA extraction (protocol D). For the three different studied protocols, the measured levels of [^{18}O]8-oxodGuo are similar (Figure 3). Indeed, the measured level of 8-oxodGuo is lower when the extraction is performed using desferrioxamine-containing buffers. In addition to the higher level of 8-oxodGuo, a concomitant larger variation in the measured values is observed in the absence of desferrioxamine, indicating the importance of desferrioxamine in order to obtain reliable results (51). This demonstrates the protective role played by the transition metal chelator. Moreover, higher levels of 8-oxodGuo are obtained if DNA is isolated with protocol D compared with protocol A (Figure 3). It is important to note that the different buffers used to extract DNA following protocol D also contained desferrioxamine. The major difference between the two latter protocols concerns the cell lysis procedure. Using the chaotropic method, the cellular membrane

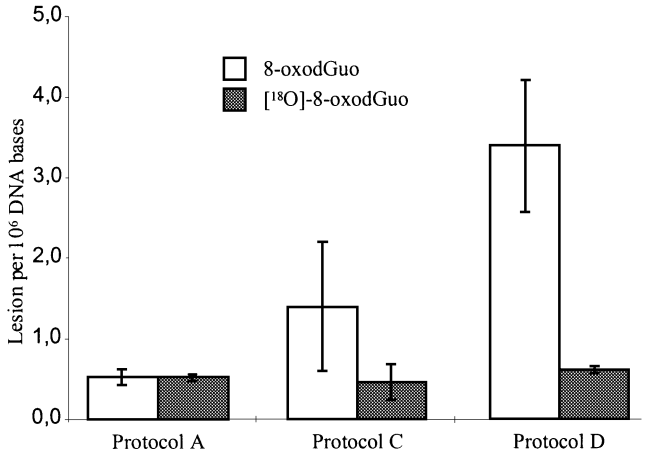

Fig. 3. Effect of different DNA extraction protocols on the measured levels of 8-oxodGuo and [^{18}O]8-oxodGuo. The lesions were measured by the HPLC-MS/MS assay in the DNA of cells treated with 100 µl of 85 mM DHPN$^{18}O_2$. The DNA was extracted from the cells following protocols A, C or D. Results, representing the average and standard deviation of four independent determinations, are expressed as the number of lesion per 10^6 DNA bases.

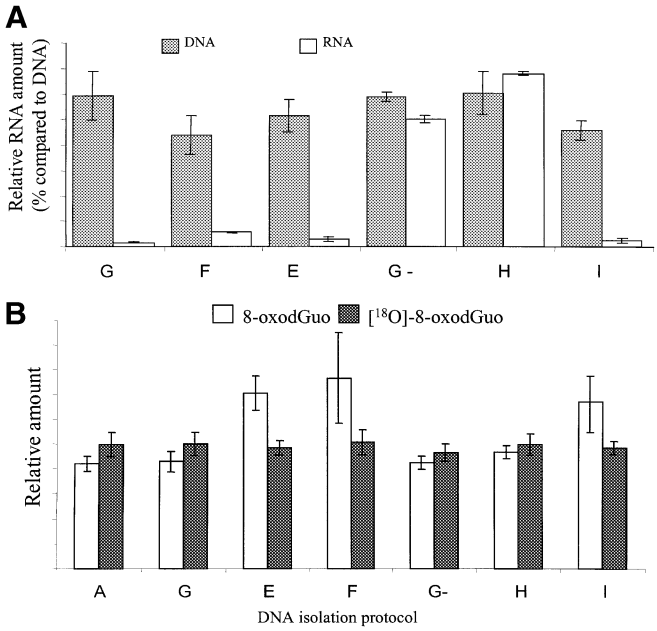

Fig. 4. Relative amounts of DNA and RNA (**A**) as well as the measured levels of 8-oxodGuo and [^{18}O]8-oxodGuo (**B**) in the samples extracted with the different studied protocols. The data represent the average and standard deviation of three independent determinations.

is selectively lysed and nuclei are subsequently isolated, whereas, using protocol D, nuclear and cellular membranes are lysed simultaneously and no NaI was added. In addition, it should be noted that two nucleic acid precipitations are performed when DNA is extracted using protocol D. Therefore, results reported in Figure 3 indicate that the transient isolation of the nuclei is preferable in order to minimize spurious DNA oxidation during the work-up. It should be added that the amount of DNA isolated was similar for A, B, C and D protocols (data not shown).

In a third experiment, several protocols (G–I) were evaluated and compared with protocol A. For all protocols, DNA was extracted from THP1 cells simultaneously, in the same laboratory, just after cell treatment with DHPN$^{18}O_2$. The amount of DNA isolated from the same number of cells is reported in Figure 4. In addition, the relative amounts of unlabelled 8-oxodGuo and [^{18}O]8-oxodGuo determined by HPLC-MS/MS following digestion of the DNA isolated according to the evaluated protocols are also reported in Figure 4. Similar amounts of DNA were obtained irrespective of the applied method. However, a lower DNA recovery was observed for protocols F, E and I. RNA contamination was observed in the DNA samples isolated with protocols G– and H. Concerning the oxidized DNA bases, results indicated that almost similar ratios are obtained for the different protocols; however, slightly higher values were obtained using protocols F, E and I. Therefore, in all the tested conditions, it seems that specific decomposition of 8-oxodGuo, that could give rise to an underestimated value does not occur. The amounts of [^{18}O]8-oxodGuo (Figure 4) were almost similar for all the evaluated protocols. However, some interesting observations could be made. First, for protocols F and E, ~20% higher amount of 8-oxodGuo is measured, suggesting that DNA was partly oxidized during the work-up. In addition, for protocol F, the recovery of DNA was lower (Figure 4). It is worth noting that for protocol F, the samples were stored on ice during all the DNA isolation procedure (Table I). In addition, no RNase treatment was performed and, as indicated in Figure 4, RNA was almost completely removed. Using the latter method, as well as protocols A, G and E, nuclei are first isolated (Table I). However, for A, G and E protocols, an RNase treatment is performed to eliminate contaminating RNA. If such a treatment is omitted, high amounts of RNA are isolated together with DNA as shown from the results of protocol G– (Figure 4). However, as demonstrated previously (52), the presence of RNA did not appear to compromise the determination of 8-oxodGuo (Figure 4).

Similar results were obtained for protocols A and G, which are based on the use of the chaotropic method. However, lower volumes of buffers are used for protocol G. These results suggest that the volumes of buffers used to isolate DNA with protocol A could be significantly reduced without prejudice. A combination of protocols G and F was performed to evaluate the efficiency of the two major parts (i.e. nuclei isolation and DNA precipitation) of the protocols for eliminating RNA and for avoiding DNA oxidation. In protocol I, nuclei were isolated using protocol F (cell lysis under hypotonic conditions) and they were subsequently treated with protocol G (NaI precipitation). In addition, for protocol H, nuclei were first isolated with protocol G (specific lysis of the cytoplasmic membrane) and subsequently treated with protocol F (DNA precipitation using GTC salts). Concerning the recovery of DNA, obtained results (Figure 4) indicate that a lower recovery was obtained for protocol I (and F) compared with protocols G and H. However, DNA isolated with protocol H was contaminated with high amounts of RNA. Such results indicate that RNA is eliminated during the first part of protocol F (cell lysis under hypotonic conditions). However, at the same time, a loss of DNA (representing ~15%) is observed during that step. Such a loss is not very important, but is accompanied by an increase in the level of 8-oxodGuo (Figure 4). According to the results obtained using protocol I, it may be concluded that oxidation of DNA takes place during the first part of protocol F.

A fourth experiment was performed to compare protocols A and J. For this purpose, DNA was isolated using either

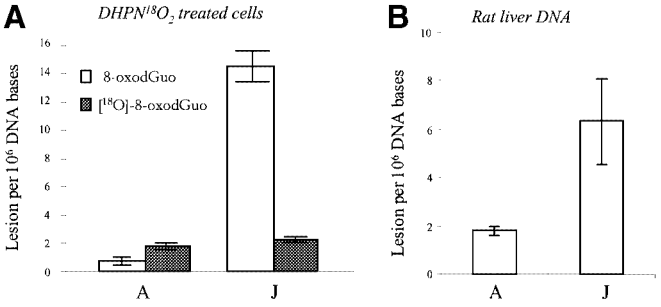

Fig. 5. Levels of 8-oxodGuo and [^{18}O]8-oxodGuo in DHPN$^{18}O_2$ treated cells (**A**) and level of 8-oxodGuo in rat liver DNA (**B**) measured following DNA extraction using either protocol A and J. Results, expressed as the number of 8-oxodGuo per 10^6 bases represent the average and standard deviation of three independent determinations.

protocols A and J either from DHPN$^{18}O_2$ treated cells (THP1) or from rat liver. The level of 8-oxodGuo and [^{18}O]8-oxodGuo in the treated cells or 8-oxodGuo in the rat liver DNA, as measured by HPLC-MS/MS are reported in Figure 5. The results clearly indicated that relatively higher levels of 8-oxodGuo were measured when DNA was extracted using protocol J. According to the results reported in Figure 5, significant DNA oxidation takes place using protocol J, mainly when DNA is isolated from cultured cells, and, to a minor extent, from rat liver DNA.

Discussion

Measuring oxidative damage to cellular DNA is a challenging analytical problem, not only because the sensitivity should allow the detection of <1 modification/10^6 DNA bases within a few micrograms DNA, but mainly because of the possibility of artefactual DNA oxidation during the work-up (1,23,53). Although significant progress has been made during the last decade to accurately measure oxidative DNA lesions (4), still a wide range of values is reported concerning the cellular background level of 8-oxodGuo. The pending question that could be still raised for the measurement of lesions in cellular DNA concerns the possibility of artefactual oxidation during DNA extraction and subsequent hydrolysis. Do the values obtained represent the true levels of DNA lesions in cellular DNA (1) or are they overestimated due to the occurrence of DNA oxidation during the work-up? What is in fact the cellular background level of 8-oxodGuo? In this respect, it should be mentioned that low background levels of 8-oxodGuo in cellular DNA (39,40,54) representing <1 modification/10^6 nucleosides were recently measured using direct approaches requiring the initial DNA extraction step. However, a lower value does not necessarily represent an improvement of the protocol but might be possibly attributed to an underestimation of the level of the measured lesion.

Therefore, experiments that could allow a rapid and unambiguous comparison of different protocols for both DNA extraction and digestion were still required. In this respect, our strategy allows us to determine simultaneously both the possibility of DNA oxidation during the work-up and also the recovery of the modified DNA bases, as [^{18}O]8-oxodGuo could not be produced as an artefact. Concerning DNA digestion, no significant differences were observed between the levels of 8-oxodGuo, following enzymatic digestion according to the three evaluated protocols (Figure 1). However, care has to be taken for the measurement of DNA lesions other than 8-oxodGuo since some of them might not be quantitatively cleaved by nuclease P1 (55). For instance, the measurement of the *cis* and *trans* diastereomers of thymidine glycols, DNA lesions that are not efficiently cleaved by nuclease P1, requires the use of a cocktail of endo- and exo-nucleases (protocol Dig-1) (15). Since the HPLC-MS/MS assay could enable the simultaneous determination of several DNA lesions during the same analysis, it is therefore preferable to use protocol Dig-1 for DNA digestion. This will allow the accurate measurement of lesions that are not quantitatively released by the single use of nuclease P1.

Concerning DNA extraction, previous work has shown that the use of NaI decreases the measured level of 8-oxodGuo in cellular DNA (39,45). Such a decrease may be attributed to either a reduction of spurious DNA oxidation during precipitation, or, as previously postulated (1,40,41,45) to a degradation of 8-oxodGuo. Decomposition of [^{18}O]8-oxodGuo was observed in none of the studied protocols (Figure 4). This indicates that the proposed NaI-mediated decomposition of 8-oxodGuo, which is very sensitive to further oxidation, does not take place during extraction, at least using the studied protocols. Therefore, the lower levels of 8-oxodGuo recently measured upon DNA extraction using the chaotropic method could be attributed to a decrease in the artefactual DNA oxidation of the guanine base during the work-up.

Altogether, the data indicate that, among the different evaluated protocols, the use of protocols A or G (chaotropic method) leads to a minimization of DNA oxidation during its isolation. In addition, it may be concluded that, to prevent such oxidation reaction, three main conditions have to be fulfilled: (i) Initial nuclei isolation after specific lysis of the cellular membrane (ii) presence of desferrioxamine in the different extraction buffers; and (iii) precipitation of DNA using sodium iodide (or the GCT salts). It may be pointed out that protocols F (cold GTC) and E (Amersham kit) give slightly higher levels of 8-oxodGuo, but both possess advantages. Protocol E is very fast (Table I) and using protocol F, samples are stored at 4°C during the extraction. Concerning the latter protocol, we have shown that DNA oxidation takes place during the first step that consists of the isolation of the nuclei (*vide supra*). Work is in progress in order to optimize this step. As none of the buffers in the Amersham kit (protocol E) currently contains desferrioxamine (information from the company), the addition of this chelatant could reduce artifactual oxidation; further optimization is, however, almost impossible since the exact composition of reagents is not disclosed. As a striking observation, according to the results presented in Figures 4 and 6, we cannot recommend the use of protocol D and J, which were shown to significantly induce DNA oxidation during the work-up. Another interesting result was obtained when DNA was isolated with protocol G–, which consisted of the chaotropic method (protocol G) with the exception that the RNase treatment was not performed. As expected, using protocol G–, a high amount of RNA was isolated together with DNA (Figure 4) but similar levels of 8-oxodGuo were measured compared with protocol A. Therefore, protocol G– would be appropriate for the simultaneous measurement of lesions in both DNA and RNA.

The cellular background level of 8-oxodGuo determined in the monocyte cell line, in the absence of any induced stress, using the chaotropic method (protocols A and G) was determined to be ~0.48 ± 0.11 lesions/10^6 DNA base (Figure 2).

Such a background level of 8-oxodGuo is similar to that obtained by other groups using similar protocols (40,45). It should be noted that significantly higher levels of 8-oxodGuo, have been reported in the literature during the last 20 years. Therefore, significant improvements have been recently made to reduce artefactual DNA oxidation during extraction and also during the measurement of the lesion (*vide supra*). Even if variation between cell lines and tissues could not be excluded, the measurement of cellular background levels of 8-oxodGuo significantly >1 lesion/million nucleosides may reflect a notable artefactual DNA oxidation during the work-up. However, the background level obtained in the present work is close, but still about three to five times higher than that estimated using either the comet assay (56,57) or the alkaline elution technique (58) associated with specific DNA repair enzymes. Therefore, the pending question concerns the origin of the discrepancies between the two approaches. Does the improved extraction protocol (chatropic method) still induce a significant DNA oxidation during the work-up? Does the comet assay underestimate the level of 8-oxodGuo? Further work is required in order to answer these questions.

In conclusion, in the present work, an experiment was designed in order to achieve an unambiguous comparison of different protocols for either DNA extraction or digestion that minimize DNA oxidation during the work-up. We have demonstrated that the lower levels of 8-oxodGuo measured using the chaotropic method, compared, for example, to phenol extraction (26,40), are not due to a side-decomposition of 8-oxodGuo [as recently postulated (1,40,41,45)] but to a minimization of spurious oxidation of DNA during the work-up. The described strategy could be used to compare other protocols for DNA hydrolysis and extraction. In that respect, it would be interesting to evaluate the recently proposed method (38) that has allowed the measurement of levels of 8-oxodGuo in cellular DNA similar to those obtained by the indirect approaches. Using our strategy, it would be possible to determine if the reduced level of 8-oxodGuo obtained in the latter work is effectively due, as proposed by the authors, to a minimization of DNA oxidation obtained by the specific hydrolysis of 8-oxo-7-8-dihydroguanine (8-oxoGua) by the Fpg protein, or to a lower recovery for 8-oxoGua due to a non-quantitative release of the lesion by the DNA repair enzyme. Such an incomplete release of DNA modifications by specific repair enzymes may also explain the lower values obtained by indirect approaches such as the comet assay and the alkaline elution technique. Using the possibility of specifically generating [^{18}O]8-oxodGuo in cellular DNA, work is in progress to determine the quantitative aspect of such indirect approaches, by a direct comparison with the results obtained by the HPLC-MS/MS assay. Our data, together with recent work strongly suggest that the cellular background level of 8-oxodGuo, in untreated cells, is not >1 lesion/10^6 nucleosides. Higher measured levels may reflect the occurrence of an artefactual DNA oxidation during the work-up.

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