Hydrogen peroxide induces DNA single- and double-strand breaks in thyroid cells and is therefore a potential mutagen for this organ

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Abstract

DNA double-strand breaks (DSBs) are considered as one of the primary causes of cancer but their induction by hydrogen peroxide (H_2O_2) is still controversial. In this work, we studied whether the high levels of H_2O_2 produced in the thyroid to oxidize iodide could induce DNA modifications. Scores of DNA damage, in terms of strand breaks, were obtained by comet assay (alkaline condition for single-strand breaks (SSBs) and neutral condition for DSBs). We demonstrated that in a rat thyroid cell line (PCCl3), non-lethal concentrations of H_2O_2 (0.1–0.5 mmol/l) as well as irradiation (1–10 Gy) provoked a large number of SSBs (~2–3 times control DNA damage values) but also high levels of DSBs (1.2–2.3 times control DNA damage values). We confirmed the generation of DSBs in this cell line and also in human thyroid in primary culture and in pig thyroid slices by measuring phosphorylation of histone H2AX. L-Buthionine-sulfoximine, an agent that depletes cells of glutathione, decreased the threshold to observe H_2O_2-induced DNA damage. Moreover, we showed that DNA breaks induced by H_2O_2 were more slowly repaired than those induced by irradiation. In conclusion, H_2O_2 causes SSBs and DSBs in thyroid cells. DSBs are produced in amounts comparable with those observed after irradiation but with a slower repair. These data support the hypothesis that the generation of H_2O_2 in thyroid could also play a role in mutagenesis particularly in the case of antioxidant defense deficiency.

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Introduction

Thyroid nodules are common and constitute an important clinical problem. They may occur in up to 50% of a population above 60 years old (Ross 2002). Among these nodules, 5% are cancerous, mainly papillary cancers (PTC). While the prevalence of clinically significant PTC remains relatively low, the prevalence of papillary microcarcinoma is clearly higher and reaches 13% in some series of autopsies (Nasir *et al*. 2000). There is no clear explanation for this high frequency of thyroid tumors. Iodine deficiency increases the prevalence of hot nodules and multinodular goiters but does not modify the global incidence of thyroid cancers even though it raises the relative proportion of follicular carcinoma (Krohn & Paschke 2002). Irradiation is the only environmental risk factor clearly implicated in thyroid cancer pathogenesis (Ron *et al*. 1995).

The initial event in the majority of PTC consists of an activation of the RAS/RAF/MEK/MAP kinase pathway, either directly by mutation of BRAF and more rarely of RAS, or indirectly by constitutive activation of tyrosine kinase receptors resulting from chromosomal rearrangements (RET/PTC, TRK; Lacroix *et al*. 2005). After irradiation, carcinogenic processes are mainly attributed to the formation of

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DNA double-strand breaks (DSBs; Sarasin *et al*. 1999). This damage results from a direct effect of irradiation but also from the generation of reactive oxygen species (ROS) formed during water radiolysis (Mikkelsen & Wardman 2003). However, the precise molecular mechanisms of most radiation-induced cancers are largely unknown. Nevertheless, irradiation is certainly not responsible for the majority of thyroid tumors.

Therefore, since 1990, we hypothesized that the elevated frequency of thyroid tumors (benign or malignant) could be partially explained by the prominent mutagenic environment present in the thyroid, resulting from its metabolism producing large amounts of hydrogen peroxide (H_2O_2; Song *et al*. 2007). Indeed, in thyroid, H_2O_2 generated by the DUOX enzymes (Dupuy *et al*. 1999, De Deken *et al*. 2000) is required and is the limiting co-factor for iodide oxidation by thyroperoxidase (TPO) and thyroid hormone synthesis (Nunez & Pommier 1982). Quantitatively, a stimulated thyrocyte generates almost as much H_2O_2 as a stimulated leukocyte (Corvilain *et al*. 1994). However, while the leukocyte dies soon after activation, the thyrocyte life is much longer (maximum 7 divisions during adulthood; Coclet *et al*. 1989) allowing mutations to accumulate. H_2O_2 could facilitate a mutagenic process and lead to tumorigenesis by altering the DNA (oxidation of bases, DNA single-strand breaks (SSBs) and DSBs). H_2O_2 could also enhance cell proliferation through various mechanisms (Stone & Yang 2006). Arguments to support the involvement of H_2O_2 in mutagenesis and etiopathogenesis of thyroid nodules were recently reviewed by our group and others (Maier *et al*. 2006, Song *et al*. 2007). The existence of a more mutagenic environment in the thyroid than other organs are suggested: 1) the spontaneous mutation rate is around 10 times higher in the thyroid than in the liver, 2) the comet assay detects increased levels of oxidized pyrimidine and purine in thyroid compared with other organs, and 3) immunohistochemistry methods reveal higher levels of 8-oxoguanine in the thyroid (Maier *et al*. 2006). A gene expression signature reflecting the differences in cellular response to γ-radiation and H_2O_2 could distinguish radiation-induced (from the Chernobyl Tissue Bank) and spontaneous PTCs (from French patients with no history of radiation exposure; Detours *et al*. 2007). These data reinforce our hypothesis suggesting that in thyroid, H_2O_2 could provoke DNA damage and mutations. Nevertheless, harmful effects caused by H_2O_2 are tightly controlled in thyroid, thanks to the restricted apical localization of its production and the presence of various intracellular H_2O_2 detoxifying enzymes, like seleno-dependent glutathione peroxidase (GPx; Kohrle *et al*. 2005). In epidemiological studies, it has been shown that the modification of antioxidant capacities in the diet can modulate cancer risk (Bertram *et al*. 1987, Ip *et al*. 1994, Vogt *et al*. 2003). Antioxidant status of cells may also play a role in the mutagenic potency of irradiation and oxidative stress (Kim *et al*. 2000, Neumann *et al*. 2003, Wang *et al*. 2003).

While carcinogenic DNA DSBs are clearly induced by irradiation, their induction by H_2O_2 is still controversial. Therefore, we analyzed in this work the capacity of H_2O_2 to generate DNA SSBs and especially DSBs and compared this with the effects observed with a well-known carcinogenic factor (irradiation) to determine if H_2O_2, like irradiation, could therefore be implicated in the pathogenesis of thyroid tumors.

Materials and methods

Cell lines and culture conditions

PCCl3 cells, a rat thyroid cell line, were cultured as previously described (Rigutto *et al*. 2007). Non-transformed rat fibroblasts F208 were grown in DMEM with 10% v/v fetal bovine serum, 1% v/v sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone. Pig thyroids were obtained from freshly killed animals in accordance to ethical guidelines. Thyroids were cut into thin slices (0.3 mm) of ∼50 mg wet weight. Slices were preincubated at 37 °C for 1 h in 2 ml Krebs-Ringer–Hepes (KRH) medium and then transferred to fresh KRH before treatment (Corvilain *et al*. 2000). Primary cultures of human thyroid cells were prepared and incubated as previously described; the experiments were performed, four days after seeding the thyroid follicles, on differentiated thyrocytes (Roger *et al*. 1988). Human thyroid tissues were obtained from patients undergoing thyroidectomy for multinodular goiter with approval from the institutional ethic committee.

Cell treatment

Irradiation

Cells were exposed to a single dose of γ-irradiation from a Cesium^{137} source at a dose rate of 2.03 Gy/min at room temperature. To determine the initial DNA damage, cells were placed on ice immediately after treatment. For the kinetic experiments cells were incubated at 37 °C for different periods of time after irradiation.

Hydrogen peroxide

H_2O_2 (Merck) dilutions were prepared in culture medium immediately before use. The H_2O_2 solutions were diluted in the medium of dishes containing cells in culture or thyroid slices.

H_2O_2 generating system

A relatively stable production of H_2O_2 was obtained in the cell culture medium using proline (Sigma–Aldrich) in conjunction with 5 mU/ml D-amino-oxidase (DAO from Sigma–Aldrich).

DL-Buthionine-[S,R]-sulfoximine treatment

Cells were preincubated overnight with 10 mmol/l L-buthionine-sulfoximine (BSO; Sigma–Aldrich) before H_2O_2 or irradiation treatment.

Cell survival test

The cytotoxic potential of the different treatments was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay. This colorimetric method measures the formation of a soluble formazan product that is directly proportional to the number of living cells in culture. After different intervals of time after treatment, cells were incubated with a MTS/phenazine methosulfate solution for 1–3 h in the dark at 37 °C and in a 5% CO_2 atmosphere as described in the Promega (Promega) technical bulletin no. 169. The soluble formazan product has an absorbance maximum at 490–500 nm and was recorded using an ELISA plate reader. Viability was calculated as a percentage of the control. Triton 0.5% v/v was used as a positive control for cell death.

H_2O_2 measurement

H_2O_2 was measured in the cell culture medium by a sensitive fluorimetric assay based on the H_2O_2-dependent oxidation of homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid, HVA; Sigma–Aldrich) to a highly fluorescent dimer (2,2′-dihydroxy-3,3′-dimethoxydiphenyl-5,5′-diacetic acid) by HRP (Benard & Brault 1971).

Iodide organification (protein bound iodide)

Pig thyroid slices were preincubated for 30 min in KRH buffer containing 0.5 g/l BSA supplemented or not with 0.5 mmol/l H_2O_2. The slices were then incubated in fresh medium supplemented with KI (10^{-5} mol/l) and ^{125}I (1 μCi/ml) for 30 min. Methimazole (1 mmol/l), that blocks iodide organification, added during pre-incubation and incubation, was used to estimate the background in the assay. Iodide organification was measured in basal conditions and in slices stimulated by TSH (10 mU/ml) and ionomycin (2 μmol/l). The slices were homogenized in a methimazole solution (1 mmol/l). Proteins were precipitated with 10% v/v trichloroacetic acid and counted. Iodide bound to proteins is expressed as picomoles of iodide organified per 100 mg wet weight tissue/30 min.

Comet assays

The comet assay was adapted from Singh *et al*. (1988) and Olive *et al*. (1990) as previously described (Chico Galdo *et al*. 2006). This assay is based on the separation from supercoiled DNA of DNA loops containing strand breaks (SSBs and DSBs) that become free to migrate out of the nucleus towards the anode during an alkaline electrophoresis in a solution of 0.3 M NaOH, 1 mM EDTA, pH > 13. To detect DSBs more specifically, the comet assay was adapted to neutral electrophoresis in a buffer of 300 mM sodium acetate, 100 mM Tris–HCl, pH 8.3 as previously described (Wojewodzka *et al*. 2002).

DNA images were captured after staining with ethidium bromide (20 μg/ml) with a Zeiss Axioplan 2 imaging microscope with a 40× objective lens. For quantification, the comets were classified into different categories. We subdivided cell DNA damage into five stages (1–5) for the alkaline assay (Collins 2004) and four stages (2–5) for the neutral assay according to the length and the intensity of the comet tail as illustrated in Fig. 1A. Stage 1 (no tail) and stage 2 (halo around the nucleus) corresponded to cells without a significant number of DNA strand breaks. Stages 3–5 corresponded to a gradual increase in DNA damage. We measured the comet score in 200 randomly selected cells per slide. Results were, first, expressed as the percentage of each stage of comets per slide. In a second step, a comet score was calculated, following a modification of Collins' method, as the sum of the percentage of each comet stage n (from 1 to 5) multiplied by $n-1$ (Collins 2004). For this calculation, all the negative comets (stages 1 and 2) were considered as stage 2. The scores were expressed in arbitrary units on a scale from 100 (all the comets are in stage 2) to 400 (all the comets are in stage 5). While the scale of the comet assay was the same in alkaline (reflecting SSBs and DSBs) and neutral condition (more specific for DSBs), the observed score remained semi-quantitative and therefore the values could not be compared to extrapolate the number of SSBs.

Comet immunoassays were performed as previously described (Wojewodzka *et al*. 2002).

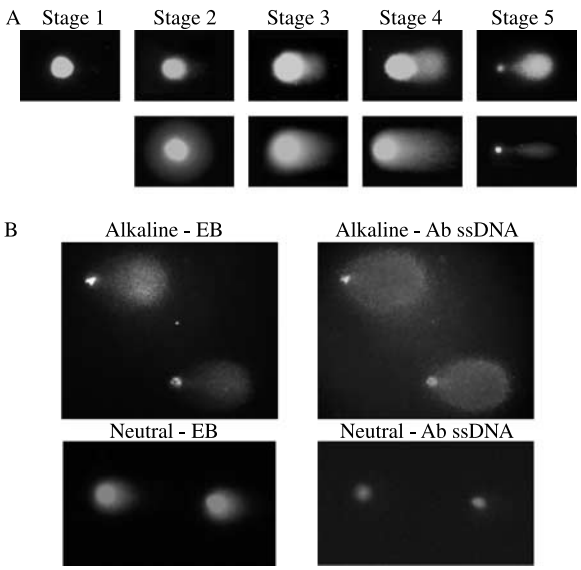


Figure 1 (A) Classification of the comets. Comet categories are defined by the size of the head (nucleus) and the length and intensity of the tail. Five stages are defined in alkaline conditions (upper panel) reflecting global DNA damage (SSBs and DSBs) and four stages in neutral conditions (lower panel) reflecting essentially DSBs. Stage 1, normal nucleus; stage 2, halo around the nucleus; stage 3–5, gradual increase in the length and intensity of the comet tail evolving in parallel with a decrease in the nuclear DNA content. (B) Comet assays performed on PCCl3 cells after incubation with 1 mM H_2O_2. Alkaline assays showed comets in stage 5 after labeling with ethidium bromide and an Ab ssDNA. In neutral assays, the tail of the comet was not labeled by the Ab ssDNA, even though almost all comets were in stage 3 after staining with ethidium bromide. This means that in the neutral condition, the DNA content in the tail results mainly from DSBs. Ab ssDNA, antibody against SSBs; SSBs, single-strand breaks; and DSBs, double-strand breaks.

Western blotting and immunocytochemistry

Two or four µg of histone extracts obtained after lysis in buffer (150 mmol/l NaCl, 1 mmol/l EDTA, 20 mmol/l Tris–HCl (pH 8), 0.5% v/v NP40) were separated by 15% SDS/PAGE and transferred to nitrocellulose. Immune complexes were detected with HRP-coupled anti-rabbit or anti-mouse IgG antibodies according to the ECL method (NEN Life science product) as previously described (De Deken *et al.* 2000). Some nitrocellulose membranes were analyzed by the Odyssey infrared imaging system (LI-COR, Biosciences, Erembodegem, Belgium) using fluorescent secondary antibodies (IRDye 680 Goat Anti-Mouse and IRDye 800 Goat Anti-Rabbit from LI-COR) as previously described (Picariello *et al.* 2006).

For immunocytochemistry, treated cells were washed with cold Tris 0.05 mol/l, NaCl 0.15 mol/l pH 7.4 (TBS) and fixed in 95% v/v ethanol – 5% v/v acetic acid. Cells were treated with 3% v/v horse serum/TBS before incubation with primary antibodies against phosphorylated Ser^{139} of histone H2AX (γH2AX) and total histone H2AX (Upstate Cell Signaling Solutions) (Bioconnect, TE Huissen, The Netherlands) at a 1:500 dilution in blocking buffer for 1 h at room temperature. After washing with TBS, cells were incubated with 1:400 Cy3-conjugated donkey anti-mouse secondary antibody (Jackson Immuno Research, Suffolk, UK) for 1 h at room temperature in the dark. Cells were washed with TBS and counterstained with bis-benzimide. Observations were performed with a Zeiss Axioplan 2 imaging microscope with a 40× objective lens.

Statistical analysis

Non-parametric unpaired tests were performed with the use of GraphPad Prism Software (San Diego, CA, USA).

Results

Assessment of DNA damage after irradiation in a thyroid cell line

We analyzed DNA damage (SSBs and DSBs) by comet assay immediately after γ-irradiation. Irradiation from 1 to 10 Gy of PCCl3 cells induced a dose-dependent increase in the comet score in alkaline condition (SSBs and DSBs) as well as in neutral condition (DSBs). This damage reached a significant score after irradiation of

Table 1 Score of DNA damage measured by comet assay in PCCl3 cells immediately after irradiation (A) and 15 min after addition of various H_2O_2 concentrations (B). DNA damage was evaluated in alkaline conditions (SSBs + DSBs) and in neutral conditions (DSBs). Data are expressed as mean (arbitrary units on a scale from 100 to 400) ± S.E.M.; (*n*), number of measurements. Statistical analyses were made by comparison of control values and values obtained after treatments

	Alkaline	Neutral
(A) Irradiation		
Ctl	120.4 ± 2.1 (32)	113.2 ± 1.9 (26)
1–2 Gy	165.3 ± 15.4 (6)*	201.0 ± 6.1 (6)*
4–5 Gy	202.1 ± 15.8 (10)*	244.5 ± 30.0 (2)*
10 Gy	283.0 ± 6.4 (21)*	253.5 ± 8.6 (24)*
(B) H_2O_2 (mmol/l)		
0	120.1 ± 2.2 (38)	110.5 ± 1.1 (33)
0.01	125.7 ± 5.7 (9)	105.8 ± 1.9 (6)
0.05	128.1 ± 7.2 (10)	132.8 ± 11.2 (4)*
0.1	245.0 ± 11.7 (19)*	215.6 ± 13.4 (14)*
0.2	321.8 ± 45.2 (6)*	241.9 ± 23.2 (6)*
0.5	400.0 ± 0.0 (4)*	225.4 ± 42.3 (4)*
1	391.3 ± 8.1 (15)*	204.5 ± 9.7 (19)*

*$P < 0.001$. SSBs, single-strand breaks; DSBs, double-strand breaks.

Table 2 Cell survival was evaluated by (A) MTS assay in PCCl3 cells and (B) human thyroid in primary culture for 2, 24, and 48 h after addition of various H_2O_2 concentrations and after different doses of irradiation. Triton 0.5% v/v was used as a positive control for cell death. Viability was calculated as a percentage of the control. Data are expressed as mean±S.E.M.; (*n*), number of measurements

	2 h	24 h	48 h
(A) H_2O_2 (mmol/l)			
0	100.0±1.7 (9)	100.0±1.0 (9)	100.0±2.2 (9)
0.1	101.4±1.5 (9)	101.9±0.6 (9)	99.5±2.2 (9)
0.2	100.9±1.1 (9)	100.2±0.8 (9)	95.3±1.3 (9)
0.5	101.1±1.1 (9)	101.0±1.2 (9)	101.9±1.9 (9)
1	77.4±2.3 (9)†	87.4±2.0 (9)†	94.9±1.5 (9)
10	14.2±2.2 (9)†	0.9±0.2 (9)†	0.8±0.2 (9)
Irradiation (Gy)			
1	116.7±1.8 (9)	115.8±1.9 (9)	108.0±1.6 (9)
10	125.1±2.1 (9)	112.7±1.7 (9)	104.2±2.4 (9)
Triton 0.5% v/v	0.8±0.1 (9)†	0.7±0.1 (9)†	0.5±0.2 (9)†
(B) H_2O_2 (mmol/l)			
0	100.0±1.0 (9)	100.0±0.5 (9)	100.0±1.2 (6)
0.1	102.2±1.7 (9)	102.2±1.4 (9)	99.8±1.3 (6)
0.25	100.3±0.9 (9)	99.5±1.5 (9)	101.0±2.7 (6)
0.5	102.9±2.6 (9)	101.6±2.5 (9)	99.2±2.5 (6)
1	104.2±2.4 (9)	100.2±2.8 (9)	101.5±2.1 (6)
10	41.8±8.4 (9)†	53.8±5.2 (9)†	59.3±14.3 (6)*
100	2.0±1.2 (6)†	2.6±1.0 (6)†	1.2±0.6 (6)†
Irradiation (Gy)			
1	106.4±1.0 (9)	101.7±1.2 (9)	102.3±1.7 (6)
5	112.6±3.3 (9)	105.7±3.1 (9)	105.4±2.0 (6)
10	104.9±2.3 (9)	104.5±2.8 (9)	104.9±1.1 (6)
50	106.1±4.3 (9)	101.1±2.5 (9)	104.2±1.7 (6)
Triton 0.5% v/v	1.2±0.3 (9)†	1.2±0.6 (6)†	0.9±0.4 (6)†

*$P<0.05$; †$P<0.001$.

1–2 Gy (Table 1). No significant cell death was observed by the MTS assay after an irradiation of 10 Gy (Table 2). We confirmed the presence of DSBs by analysis of phosphorylation of histone variant H2AX on serine 139 by western blotting. Histone H2AX phosphorylation, that reflects the presence of DSBs, increased in a dose-dependent way from 1 to 10 Gy (Fig. 2A). These results were corroborated by an immunocytochemistry method that detects foci of phosphorylated histone H2AX using the same antibody as in western blotting. A similar relationship was observed between the dose of γ-irradiation and the number of foci (pink spots in the nucleus) per cell as shown in Fig. 2B.

Assessment of DNA damage after exposure to H_2O_2 in thyroid and non-thyroid cell lines

As H_2O_2 is rapidly degraded when added to the cells, we first estimated how long H_2O_2 was present in the incubation medium of the cells in the described experiments. After addition of H_2O_2, the remaining quantities in the medium were measured at different time intervals in comparison with the same concentration of H_2O_2 added to medium without cells. 0.1 mmol/l H_2O_2 added to PCCl3 cells disappeared rapidly; 23 and 1.6% remained after 15 min and 1 h respectively.

DNA damage in PCCl3 cells was quantified by comet assay 15 min after exposure to H_2O_2. In alkaline conditions, no DNA damage was observed up to 0.05 mmol/l H_2O_2 but the scores increased abruptly and significantly above the control values at 0.1 mmol/l H_2O_2 and reached a maximum value (400) between 0.2 and 0.5 mmol/l H_2O_2 (Table 1) corresponding to the upper limit of the assay. The scores obtained in neutral conditions were significantly increased in cells exposed to a concentration of 0.05 mmol/l H_2O_2 compared to non-treated cells (Table 1). These scores reached a plateau at a H_2O_2 concentration of 0.1 mmol/l. We also examined the phosphorylation of histone H2AX after H_2O_2 treatments (Fig. 2C). One hour after the treatment, we observed a concentration-dependent increase of histone H2AX phosphorylation from 0.1 up till 1 mmol/l H_2O_2. Immunocytochemistry confirmed the

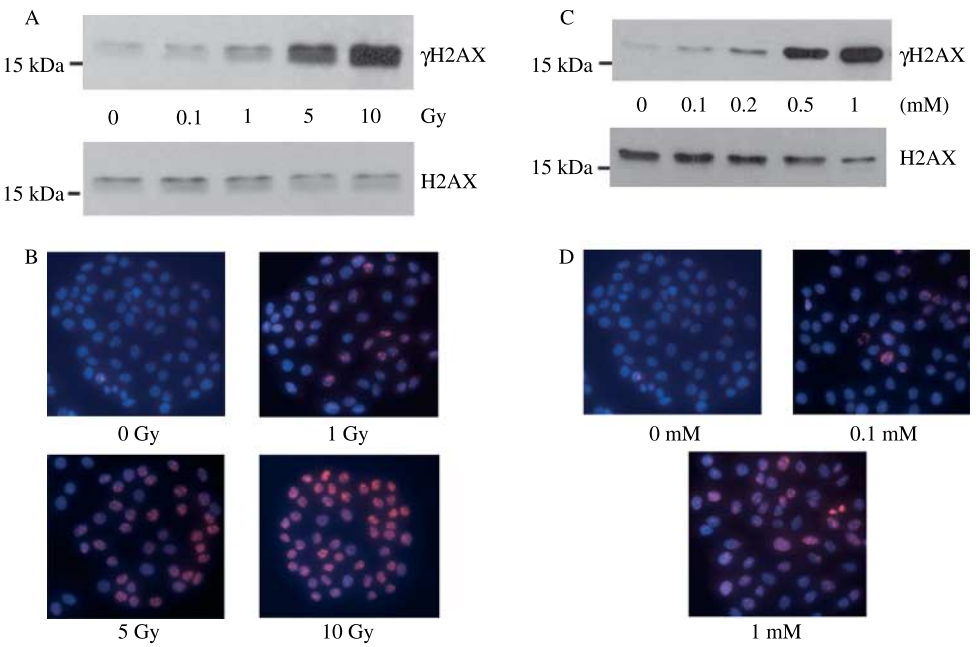


Figure 2 DNA DSBs evaluation using phosphorylation of histone H2AX in PCCl3 cells an hour after irradiation (A and B) or addition of H_2O_2 in the medium (C and D). Phosphorylated (γH2AX) and total histones H2AX were detected with specific antibodies. (A) Western blot showing a dose-dependent effect of irradiation on H2AX phosphorylation. (B) Immunocytochemistry detecting phosphorylated histone H2AX foci (pink spots). (C) Western blot showing dose-dependent effect of H_2O_2 on H2AX phosphorylation. (D) Immunocytochemistry detecting phosphorylated histone H2AX foci (pink spots). DSBs, double-strand breaks.

presence of a rising number of phosphorylated histone H2AX foci, reflecting the presence of DNA DSBs, in cells treated with 0.1 and 1 mmol/l of H_2O_2 (Fig. 2D). H_2O_2 concentrations equal to or below 0.5 mmol/l did not induce significant cell death in contrast to 10 mmol/l H_2O_2 that provoked a massive cell death (Table 2). A viability of 77.4% in comparison with the control cells was observed after 2 h treatment with 1 mmol/l H_2O_2 (Table 2).

Western blotting (γ-H2AX) was also used to evaluate DNA damage in a non-thyroid rat cell line, the F208 fibroblast cell line. We observed the presence of DSBs by the detection of significant levels of phosphorylated H2AX with 0.5–1 mmol/l H_2O_2 (data not shown).

Assessment of DNA damage after exposure to H_2O_2 in pig thyroid slices and in human thyroid primary culture cells

DSBs were detected 1 h after 1 and 10 Gy irradiation in pig thyroid slices by measuring H2AX phosphorylation levels; 3.9±1.0-fold (mean±S.E.M.; $P=0.0063$) and 32.0±5.0-fold (mean±S.E.M.; $P<0.0001$) in comparison with the control for 1 and 10 Gy irradiation respectively. We also evaluated the induction of DSBs after incubation with different concentrations of H_2O_2: 0.1, 0.5, 1, and 10 mmol/l. One hour after treatment, a significant induction of phosphorylated H2AX was observed from 0.5 to 10 mmol/l H_2O_2; 2.3±0.5-fold (mean±S.E.M.; $P=0.01$), 4.9±1.1-fold (mean±S.E.M.; $P=0.0056$) and 33.1±2.9-fold (mean±S.E.M.; $P<0.0001$) in comparison with the control for 0.5, 1 and 10 mmol/l H_2O_2 respectively. Cell survival was indirectly evaluated by the capacity of the pig thyroid slices to organify iodide after treatments with H_2O_2. In the basal condition, 0.5 mmol/l H_2O_2 did not change the capacity of pig thyroid slices to organify iodide (protein bound iodide ($PB^{125}I$) measurements); 97.8±11.2% (mean±S.E.M.) of the control was measured after H_2O_2 treatment. In slices stimulated with 10 mU/ml TSH or 2 μM ionomycin (that stimulate H_2O_2 production), H_2O_2 did not modify extensively the iodide organifying function; 76.5±5.2% of the control and 92.0±11.4% of the control were measured respectively.

In human thyroid in primary culture, the phosphorylation of histone H2AX was increased following a dose-dependent curve between 1 and 10 Gy (data not shown). No significant cell death was observed by the MTS assay 24 and 48 h after an irradiation of 1 to 50 Gy (Table 2). Human thyroid primary culture cells displayed phosphorylation of histone H2AX one hour

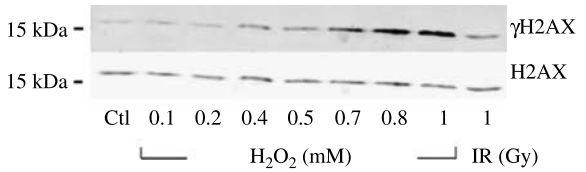


Figure 3 DNA DSBs evaluation by phosphorylation of histone H2AX in human thyrocytes in primary culture an hour after H_2O_2 addition or irradiation. Phosphorylated and total histones H2AX were detected by western blotting with specific antibodies. DSBs, double-strand breaks. This western blot is representative of six independent cell cultures.

after treatment with H_2O_2 from 0.2 to 0.5 mmol/l in a dose-dependent way; 1.9±0.3 fold (mean±S.E.M.; $P=0.0199$) and 8.2±3.5 fold (mean±S.E.M.; $P=0.0161$) in comparison to the control for 0.2 and 0.5 mmol/l H_2O_2 respectively. One representative experiment is shown in Fig. 3. By MTS assay, we measured that H_2O_2 concentrations equal to or below 1 mmol/l did not induce significant cell death 24 and 48 h after treatment in contrast to 100 mmol/l H_2O_2 that provoked a massive cell death already 2 h after treatment (Table 2).

Effect of depletion of glutathione on DNA damage induced by H_2O_2 and irradiation in PCCl3 cells

BSO irreversibly inhibits γ-glutamylcysteine synthetase (γ-GCS) leading to a decrease of glutathione (GSH) concentrations in the cells. GSH is a cofactor for selenium (Se) dependent GPx involved in the detoxification of most cellular H_2O_2. Comet assays in alkaline conditions were performed on PCCl3 cells preincubated overnight or not with 10 mmol/l BSO before H_2O_2 or irradiation treatment (Fig. 4A). BSO by itself did not have any effect on DNA integrity. Preincubation with 10 mmol/l BSO rendered cells more sensitive to H_2O_2 in terms of DNA breaks: 0.05 mmol/l H_2O_2 provoked more DNA damage in BSO preincubated cells than in corresponding control cells (score of 188±6 in BSO treated cells compared with 126±6 in cells not preincubated with BSO; $P<0.0001$). BSO also increased the DNA damage observed at 0.1 mmol/l H_2O_2 ($P<0.0001$). Significant DNA damage was induced after 2 and 4 Gy irradiation but contrary to what we observed after H_2O_2 treatments, BSO did not increase the DNA damage of irradiated cells (Fig. 4A).

H_2O_2 concentrations measured in the incubation medium increased linearly with the dose of irradiation (2–20 Gy) from 1.0 to 10 μmol/l (data not shown). These concentrations of H_2O_2 are largely below those needed to obtain significant DNA damage.

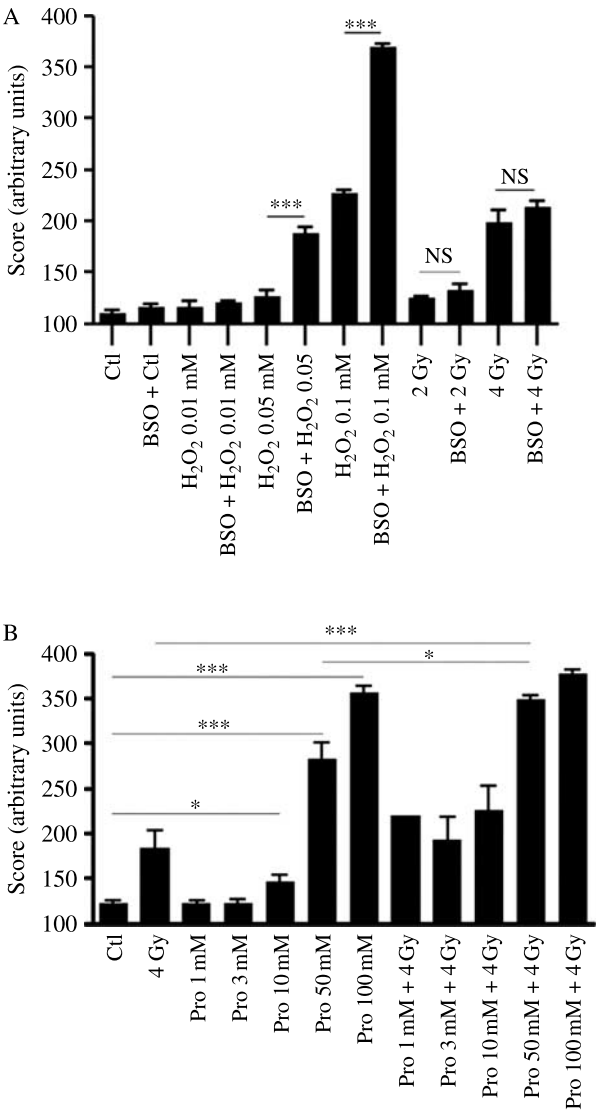
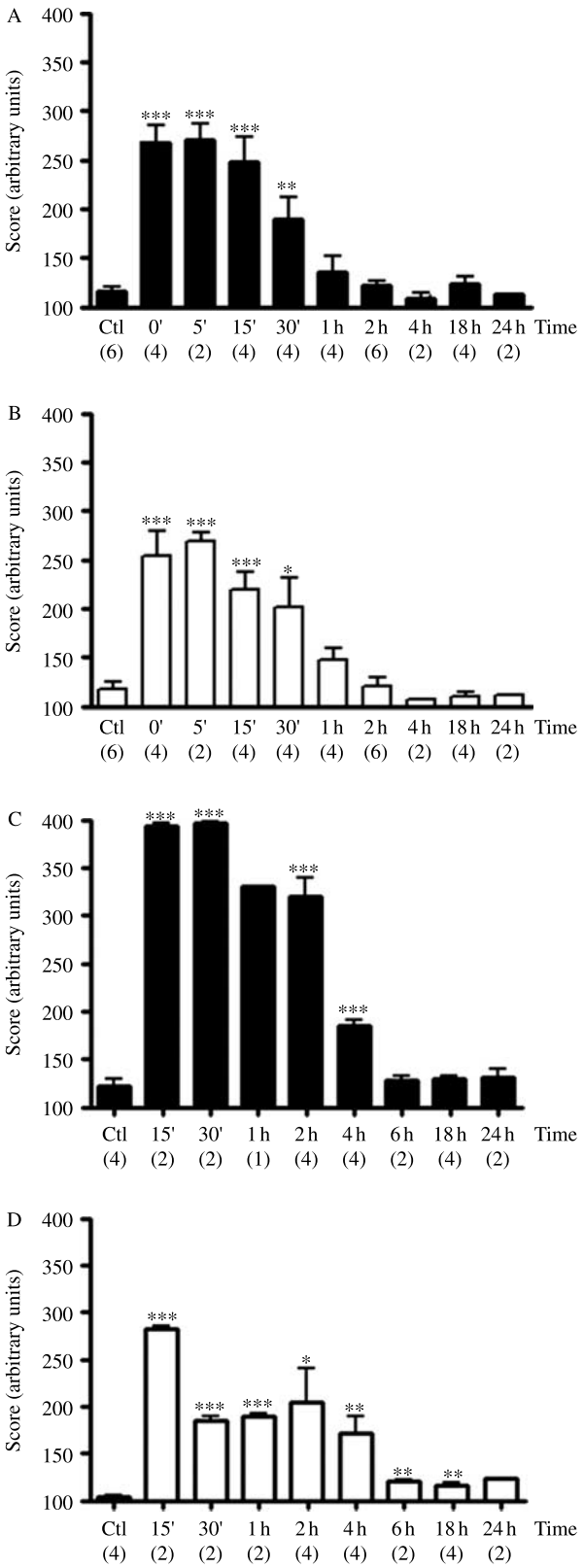


Figure 4 (A) Effect of H_2O_2 and irradiation on comet formation in PCCl3 cells with or without overnight preincubation with 10 mM BSO. Comet assays were performed in alkaline condition. Comparisons were made between scores obtained with and without BSO. Data are expressed as mean±S.E.M. of three experiments in duplicate. NS, non-significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$; BSO, L-buthionine-sulfoximine. (B) Effect of a combined treatment by a H_2O_2-generating system and irradiation on comet formation in PCCl3 cells. The H_2O_2-generating system constituted DAO and various concentrations of proline. Cells were preincubated during 1 h with the H_2O_2-generating system before being irradiated. Induced DNA damage was measured immediately after irradiation by alkaline comet assay. Data are expressed as mean±S.E.M. of three experiments in duplicate. Statistical significance was calculated and illustrated after comparing 1) control score values and values obtained with various concentrations of proline and 2) score values obtained with 50 mM proline, 4 Gy irradiation and combined H_2O_2/irradiation treatment. DAO, D-amino-oxidase; *$P<0.05$; **$P<0.01$; ***$P<0.001$.



DNA damage in PCCl3 cells after a combined treatment with H_2O_2 and irradiation

To evaluate DNA damage induced by a combined H_2O_2 and irradiation treatment, we measured radiation-induced DNA damage on cells preincubated with a H_2O_2-generating system constituted by proline and DAO. This H_2O_2-generating system produced in 1 h from 0.06 to 0.1 mmol/l H_2O_2 with 10 to 100 mmol/l proline (data not shown).

H_2O_2 generated by using 50 and 100 mmol/l proline during 1 h induced DNA damage with a score evaluated in alkaline conditions at 282 ± 18 and 356 ± 7 respectively. When cells preincubated with DAO/proline at 50 mmol/l during 1 h were irradiated with 4 Gy, the damage score recorded immediately after irradiation nearly reached the sum of the score observed after DAO/proline (50 mmol/l) treatment alone and after irradiation alone (Fig. 4B). This additive effect was not observed with 100 mmol/l proline due to the saturation of the assay (Fig. 4B).

Repair of DNA damage in PCCl3 cells

Kinetics of DNA repair after irradiation

DNA damage repair at different time intervals after irradiation was analyzed by the comet assay (Fig. 5A and B). In alkaline conditions (Fig. 5A), cells submitted to 10 Gy showed a high score of DNA damage (268 ± 18) immediately after the treatment, rapidly decreased with time to reach a score of 135 ± 17 after 1 h, close to the basal score (116 ± 4). In the neutral comet assay (Fig. 5B), repair of DNA DSBs was also completed 1 h after a 10 Gy treatment (score of 254 ± 25 immediately after treatment compared with 117 ± 7 and 147 ± 12 for respectively control and 1 h repair).

Kinetics of DNA repair after H_2O_2 treatment

The kinetics of DNA break repair after exposure to H_2O_2 was also studied by the comet assay performed at different time intervals (Fig. 5C and D). Scores of DNA

Figure 5 Kinetics of DNA repair after irradiation (A and B) and H_2O_2 treatment (C and D) in PCCl3 cells. Scores of DNA damage induced by 10 Gy irradiation were evaluated by the comet assay immediately after irradiation and then after various time intervals. Scores of DNA damage induced by 0.2 mM H_2O_2 were evaluated 15 min after the addition of H_2O_2 and then after various time intervals. (A and C) SSBs + DSBs measured in alkaline condition. (B and D) DSBs measured in neutral condition. Data are expressed as mean ± S.E.M.; (*n*), number of measurements for each time interval; *$P<0.05$; **$P<0.01$; ***$P<0.001$; SSBs, single-strand breaks; and DSBs, double-strand breaks.

damage were maximal 15 min after exposure to 0.2 mmol/l H_2O_2. In alkaline conditions, the assay was almost saturated (score around 400). Comet scores decreased by ~50% after 4 h and reached nearly basal values 6 h after treatment (score 128±4; Fig. 5C). The repair of DSBs (comet in neutral conditions) was slower than in irradiated cells; comet scores above control values were still observed 24 h after H_2O_2 treatment (104±2 for the control and 124±1 after 24 h repair; Fig. 5D).

Discussion

The thyroid produces large amounts of H_2O_2 that constitute a potentially mutagenic environment (Bjorkman & Ekholm 1988). The aim of this study was to compare, in a rat thyroid cell line (PCCl3) and in more physiological models (human thyroid primary culture cells and pig thyroid slices), DNA damage induced by a well-known carcinogenic factor (irradiation) with that obtained by a putative carcinogenic agent (H_2O_2).

The physiological levels of H_2O_2 in cells vary from 0.001 μmol/l to a maximum of 0.7 μmol/l (Song *et al.* 2007) but no data are available for the thyrocyte. As the apparent Km of TPO for H_2O_2 is ~300 μmol/l, it has been hypothesized that H_2O_2 reaches especially high concentrations but in a restricted place at the periphery of the thyrocyte, in the postulated thyroxisome (Song *et al.* 2007). In thyroid, H_2O_2 is produced outside the thyrocyte at its apical pole by DUOX and is used by TPO located in the vicinity of DUOX to oxidize iodide. Therefore, our experimental model in which H_2O_2 is added in the incubation medium mimics the *in vivo* extracellular production. The range of H_2O_2 concentrations used in this study is comparable to those probably needed to oxidize iodide. It is difficult to estimate H_2O_2 concentrations achieved in the limited space of the follicular lumen *in vivo* but with the generation of 10 nmol/100 mg per h they could easily reach the micromolar level (Corvilain *et al.* 2000).

In our experiments, extracellular H_2O_2 is rapidly reduced and has nearly disappeared after 15 min. At the concentrations used in this study, H_2O_2 was not lethal as confirmed by viability tests. Apoptosis has not been specifically evaluated in this work but previous studies performed on thyroid cells failed to detect apoptosis for similar doses of irradiation and showed apoptosis only in a very small number of cells when treated with similar concentrations of H_2O_2 (Yang *et al.* 1997, Riou *et al.* 1999). These previous observations, along with the absence of mortality in H_2O_2 or irradiation treated cells exclude that apoptosis may significantly contribute to the measured comet scores.

Comet assays were used to evaluate DNA strand breaks in individual cells. We studied the effects of H_2O_2 and irradiation on generation of SSBs and DSBs. Interestingly, thyroid cancers occur at doses as low as 0.1 Gy with a linear dose–response curve and the related risk increases by 10 times at 1 Gy (Ron *et al.* 1995). In our different thyroid models, we observed a significant number of SSBs and DSBs after an irradiation of 1 Gy or more. No differences in sensitivity to irradiation were observed between PCCl3 cells, pig thyroid slices and human thyroid primary culture cells. High levels of DSBs formation were confirmed by estimation of phosphorylation of histone H2AX by western blotting and by immunocytochemistry. Experiments on non-transformed rat fibroblasts (F208) showed a significant production of DSBs with 1 Gy. These results are in keeping with previous data showing that the formation of ~35 DSBs per gray, per cell, and per cell cycle is a constant (Rogakou *et al.* 1998, Wojewodzka *et al.* 2002, Takahashi *et al.* 2005).

DSBs provoked by H_2O_2 are considered to be rare events: 1 DSBs for ~2000 SSBs (Bradley *et al.* 1979, Takahashi *et al.* 2005). However, Bradley & Kohn (1979) showed that in mouse leukemia L1210 cells, H_2O_2 induced DSBs with a ratio of DSBs to SSBs comparable with that caused by X-rays. Takahashi & Ohnishi (2005) reviewed one study demonstrating formation of DSBsand histone H2AX phosphorylation by immunocytochemistry in normal human fibroblasts exposed to 0.1 mmol/l H_2O_2 for 2 h (Takahashi *et al.* 2005).

DSBs are considered to be more carcinogenic than SSBs. We demonstrated in PCCl3 cells that high but non-lethal concentrations of H_2O_2 provoke a large number of SSBs but also as many DSBs as irradiation. In the presence of large amounts of H_2O_2-induced SSBs, some apparent DSBs could be due to closely spaced SSBs (Bradley *et al.* 1979). Therefore, we confirmed the presence of real DSBs detected by neutral comet assays by highlighting phosphorylated histone H2AX. In this test, PCCl3 cells demonstrated the same apparent damage caused by H_2O_2 (0.05–0.1 mmol/l) as by irradiation. Data obtained on human thyroid primary culture cells and on pig thyroid slices showed a threshold of respectively 0.2 and 0.5 mmol/l H_2O_2 for the appearance of DSBs. We also observed a significant number of DSBs after a 0.5 mmol/l H_2O_2 treatment of a non-thyroid cell line (F208, a non-transformed rat fibroblast cell line) meaning that many

if not all mammalian cells are sensitive to such concentrations of H_2O_2. Thus, our work is clearly demonstrated by two different methods an induction of DSBs by H_2O_2 in a thyroid cell line and, more akin to the *in vivo* situation, in human thyroid primary cultures and pig thyroid slices. The demonstration of H_2O_2-induced DNA damage does not necessarily imply a mutagenic role but it can be extrapolated. A difficulty for this extrapolation is that levels of DNA damage acutely achieved *in vitro* must be compared with lower levels accumulated over years. However, several arguments support such an extrapolation: 1) H_2O_2, as the well accepted mutagen X-ray, induces DNA DSBs, 2) thyroid in which oxidative DNA damage has been demonstrated *in vivo* displays a higher level of mutations than liver (Maier *et al*. 2006), and 3) low levels of Se in serum (i.e. presumably lower activity of Se dependent GPx) constitute a risk factor promoting thyroid cancer development (Duntas 2006).

Comparison of the apparent sensitivity of our different models to extra-cellular H_2O_2 is probably worthless as it may reflect differences in cell membrane permeability and in antioxidant capacities of the cells. When H_2O_2 is applied to the exterior of cultured cells, the intracellular concentrations are estimated to be ~10-fold lower than the extra-cellular concentrations (Song *et al*. 2007). In thyroid, under physiological conditions, a part of the H_2O_2 not used for thyroglobulin iodination may diffuse into the cells where it is degraded by very efficient antioxidant enzymes like Se dependent GPx. BSO decreases intracellular GSH and therefore the activity of Se dependent GPx. In PCCl3 cells exposed to H_2O_2, the presence of BSO decreased the concentration of H_2O_2 needed to observe DNA strand breaks to 0.05 mmol/l without affecting the damage induced by irradiation. BSO alone in absence of externally added H_2O_2 did not increase the level of DNA strand breaks probably because basal H_2O_2 production in PCCl3 cells is not sufficient to induce DNA damage (De Deken *et al*. 2002). Thyroid destruction in myxoedematous endemic cretinism has been related to impaired H_2O_2 degradation in stimulated but Se deficient thyroids (Contempre *et al*. 2004). Therefore, we may extrapolate that *in vivo* the potential DNA damaging effect of H_2O_2 will increase in case of deficient antioxidant defense.

Because radiation increases 8-oxoguanine modifications, it was suggested that both radiation and endogenous oxidative stress could synergistically lead to the initiation of thyroid cancer (Riou *et al*. 1998). We looked therefore for a possible synergic effect of a combined treatment of irradiation and H_2O_2 on DNA damage. The observed effects of irradiation on cells preincubated with a H_2O_2-generating system were additive with no synergy whatever the conditions used. The very low H_2O_2 concentrations measured after irradiation of the culture medium as well as the absence of a potentiating effect of BSO suggest that in PCCl3 cells, H_2O_2 produced through the radiolysis of water is not the main mechanism involved in DNA damage following irradiation.

As it is well known that DNA repair deficiencies are strongly associated with high cancer risk in humans, we compared the kinetics of repair of DNA breaks induced by irradiation and H_2O_2. DNA damage induced by a 10 Gy irradiation and measured by the comet assay in PCCl3 cells was completely repaired after 1 h. The kinetics of repair was clearly made slow for a similar amount of DSBs induced by H_2O_2. This observation is probably related to different parameters: 1) the very high quantity of SSBs produced by H_2O_2 could saturate the repair systems. 2) H_2O_2 may induce DNA damage, but also have direct inhibitory effects on DNA repair. H_2O_2 at 0.1 mmol/l can inactivate the human DNA mismatch repair system (Chang *et al*. 2002) and inhibit the repair of certain types of DNA lesions through redox control of ADP ribosylation and unscheduled DNA synthesis (Pero *et al*. 1990). 3) In any case, the delay in H_2O_2-induced damage repair is not due to the persistence of H_2O_2 in the medium as we demonstrated that in our experimental conditions, H_2O_2 rapidly (15 min) disappeared from the medium.

In conclusion, H_2O_2 produces DNA damage in the thyroid. Concentrations of H_2O_2 that cause significant DNA damage are not lethal for the cells and do not modify cell functioning. These observations reinforce the hypothesis that H_2O_2 is a potential carcinogenic agent in the thyroid. H_2O_2 induces SSBs but also more mutagenic DSBs in amounts comparable with what is obtained with irradiation. The low repair efficiency of DNA DSBs induced by H_2O_2 strengthens the possible role of H_2O_2, generated in the thyroid to oxidize iodide, in thyroid tumorigenesis. Therefore, chronic endogenous exposure of thyroid cells to H_2O_2 could be a key to explain the high frequency of thyroid tumors and thyroid microcarcinoma, particularly in case of antioxidant defense deficiency as demonstrated by the increase of damage observed in the presence of BSO and suggested in epidemiological studies in case of Se deficiency (Duntas 2006, Kaprara & Krassas 2006).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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