

# Acetaminophen-induced accumulation of 8-oxodeoxyguanosine through reduction of Ogg1 DNA repair enzyme in C6 glioma cells

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Abbreviation: APAP, acetaminophen; DHR-123, dihydrorhodamine-123; NAPQI, N-acetyl-p-benzoquinoneimine; ROS/RNS, reactive oxygen and nitrogen species; 2'-dG, 2'-deoxyguanosine; 8-oxodG, 8-oxo-deoxyguanosine

## Abstract

Large doses of acetaminophen (APAP) could cause oxidative stress and tissue damage through production of reactive oxygen/nitrogen (ROS/RNS) species and quinone metabolites of APAP. Although ROS/RNS are known to modify DNA, the effect of APAP on DNA modifications has not been studied systematically. In this study, we investigate whether large doses of APAP can modify the nuclear DNA in C6 glioma cells used as a model system, because these cells contain cytochrome P450-related enzymes responsible for APAP metabolism and subsequent toxicity (Geng and Strobel, 1995). Our results revealed that APAP produced ROS and significantly elevated the 8-oxo-deoxyguanosine (8-oxodG) levels in the nucleus of C6 glioma cells in a time and concentration dependent manner. APAP significantly reduced the 8-oxodG incision activity in the nucleus by decreasing the activity and content of a DNA repair enzyme, Ogg1. These results indicate that APAP in large doses can increase the 8-oxodG level

partly through significant reduction of Ogg1 DNA repair enzyme.

**Keywords:** acetaminophen; cytochrome P450 CYP2E1; DNA repair enzymes

## Introduction

Acetaminophen (APAP, paracetamol, *N*-acetyl-*p*-aminophenol) is one of the most frequently used analgesics/antipyretics and generally considered safe under therapeutic doses (Thomas, 1993). However, large dosage and long term APAP addictive usage were also reported (Shayiq *et al.*, 1999). When APAP is consumed in large doses, it is known to damage to the liver, kidney and other organs (Thomas, 1993). The APAP-induced toxicity can take place especially when the levels of tissue anti-oxidants such as glutathione are low after fasting or chronic alcohol consumption and/or inadequate nutritional intake or starvation (Seeff *et al.*, 1986; Whitcomb and Block, 1994). APAP was shown to produce reactive oxygen and nitrogen species (ROS/RNS) and reactive metabolites in experimental animals or cultured cells (Adamson and Harman, 1993; Lores Arnaiz *et al.*, 1995) and that cellular DNA can be modified by ROS/RNS, lipid peroxides, or free radical metabolites (Ames, 1989; Wiseman and Halliwell, 1996). Therefore, it is possible that large doses of APAP could modify cellular DNA. However, this question was not systematically evaluated.

In mammalian cells, oxidative DNA damage is mainly repaired *via* the base excision repair pathway (Wood, 1996), although recent studies in yeast showed that nucleotide excision repair pathway (Scott *et al.*, 1999) is also involved in the repair of 8-oxodG in DNA. Base excision repair is initiated by the removal of the damaged base by a glycosylase. The resulting abasic site is further processed by AP lyase, AP endonuclease, DNA polymerase and a DNA ligase (Wood, 1996; Boiteux and Radicella, 2000). This process replaces just one nucleotide, finally filling the gap (Krokan *et al.*, 1997). A gene called *Ogg1* (8-oxoguanine DNA glycosylase) has been recently cloned in various species including human and rodents (Arai *et al.*, 1997; Prieto Alamo *et al.*, 1998; Tani *et al.*, 1998). This gene product, Ogg1 protein was shown

to exhibit DNA glycosylase/AP lyase activity, which removes 8-oxodG from damaged DNA by base excision repair. Furthermore, high levels of 8-oxodG were detected in the genome with significantly elevated spontaneous mutation rates in nonproliferative tissues in homozygous *ogg1*<sup>-/-</sup> null mice (Minowa *et al.*, 2000). These reports strongly suggest that base excision repair of 8-oxodG by Ogg1 protein protects the genome from the ROS-induced oxidative damage in both prokaryotes and eukaryotes. The aim of this current study was to investigate whether large doses of APAP alter the level of 8-oxodG in the nucleus through ROS/RNS production and/or potential modulation of Ogg1 DNA repair enzyme. In the current study, we used C6 glioma cells as a model, because these cells contain catalytically active cytochrome P450 related enzymes such as CYP2E1 and CYP1A2, both of which are important in APAP metabolism and subsequent toxicity (Geng and Strobel, 1993; 1995; Bae *et al.*, 2001).

## Materials and Methods

### Materials

Rabbit anti-mouse Ogg1 antibody was purchased from Alpha Diagnostic Internationals (San Antonio, TX). Horseradish peroxidase conjugated goat anti-rabbit IgG was from Amersham (Buckinghamshire, England). Nuclease P1, bacterial alkaline phosphatase, 8-oxodG, 2'-dG, glutathione (GSH), DMSO and dihydrorhodamine 123 (DHR-123) were from Sigma (St. Louis, MO).

### Cell culture and determination of ROS/RNS production

C6 cells, grown in 96-well microtiter plates (Bae *et al.*, 2001), were incubated for indicated times with or without APAP in the presence of 25 mM DHR-123 in the culture medium as described (Muijsers *et al.*, 2000). After incubation, DHR-123 conversion to rhodamine-123 was measured by fluorometric analysis at excitation/emission wavelengths of 485 and 530 nm, respectively (SpectraFluor Plus, Tecan Inc., Research Triangle Park, NC).

### Determination of GSH concentration

Cells, grown to about 80% confluence in plastic culture dishes (150 mm diameter), were treated with 5 mM APAP for indicated times. Treated cells were harvested by centrifugation at 2,000 *g* for 2 min and resulting cell pellets were sonicated in a cell lysis buffer (Bae *et al.*, 2001). GSH concentration in the whole cell extracts was determined as described

(Dimova *et al.*, 2000) using a fluorometer (Bio-Rad VersaFluor). Excitation was determined at 360 nm while emission was measured at 440 nm.

### Determination of 8-oxodG levels in the nuclear DNA

C6 glioma cells were treated with different concentrations of APAP for indicated times, harvested, and washed with 1×PBS, before their extracts were used for measurement of protein content and enzyme activity. To isolate nuclear DNA, another batch of cell pellets was dissolved in the 1×M-SHE buffer (Croteau *et al.*, 1997) and sonicated for 12 s with Branson Sonifier 250. Nuclear fractions were collected by centrifugation at 500 *g*, suspended in a fresh M-SHE buffer, and re-centrifuged to obtain the final nuclear fractions. Nuclear DNA was then isolated by using a Puregene<sup>®</sup> DNA isolation kit (Gentra Systems, Inc, Minneapolis, MN) following the manufacturer's instruction. Isolated DNA was digested to nucleosides with nuclease P1 and alkaline phosphatase by the method of Lan *et al.* (2000). Levels of 8-oxodG and 2'-dG were determined with HPLC (Waters 625 LC pump, Milford, MA) following the method (Lan *et al.*, 2000) except for the following modifications. An ESA Coulochem II detector (Chelmsford, MA) with potential sets at 850 mV for guard cell, 380 mV for 8-oxodG and 800 mV for 2'-dG was used. The collected data were analyzed using EZChrom chromatography data system (Scientific Software, Inc., San Ramon, CA). The ratios of 8-oxodG to 2'-dG in the DNA sample were determined from the peak heights of the standards.

### Measurement of 8-oxodG incision activity

Nuclear fractions were prepared as described (Croteau *et al.*, 1997). The final extracts were re-suspended in a buffer containing 20 mM Hepes-KOH (pH 7.6), 1 mM EDTA, 2 mM DTT, 300 mM KCl, 5% glycerol and 0.05% Triton X-100. The oligonucleotide containing an 8-oxodG (5'-GAACGACTGTG<sup>oxo</sup>ACTTGACTGCTACTGAT-3') was the same as reported (Croteau *et al.*, 1997) and purchased from the Midland Certified Reagent Company (Midland, TX). The incision activity was determined by the method as described (Souza-Pinto *et al.*, 1999).

### Immunoblot analysis of rat Ogg1 protein in C6 cells

Ogg1 protein level was determined by immunoblot analysis using the rabbit antibody against mouse Ogg1 (1:1,000 dilution) overnight, as described (Bae *et al.*, 2001). The immunoreactive protein was recognized by the secondary antibody (goat anti-rabbit IgG

conjugated with horse radish peroxidase, 1:1,000 dilution) using the Pierce Supersignal chemiluminescent substrate kit (Rockford, Illinois).

### Statistical analysis

The signal density of 8-oxodG incision activity, Ogg1 protein content, or mRNA levels determined by RT-PCR was quantified using NIH image 1.61 (NIH). Statistical analysis was performed by Student's *t* test (Statistica 3.0b) to determine the significance of difference between control and treated groups.  $P < 0.05$  was considered statistically significant. Other materials and methods not specifically described were as same as previously described (Bae *et al.*, 2001).

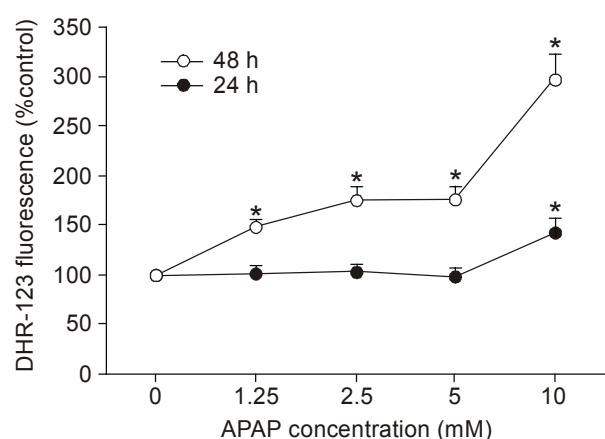
## Results

### Effect of APAP on cell death of C6 glioma cells

Under our current conditions, less than 10% C6 glioma cells died after exposure to 2.5 mM APAP for 24 or 48 h. In addition, less than 12.5% and 25% C6 glioma cells died after treatment with 5 mM APAP for 24 and 48 h, respectively. The cell death rates observed in this study were generally lower than those in our recent study (Bae *et al.*, 2001), possibly due to the fact that more cells were used determine the APAP-induced change in the 8-oxodG level.

### Effect of APAP on ROS/RNS production in C6 glioma cells

Previous studies suggested that APAP produces free

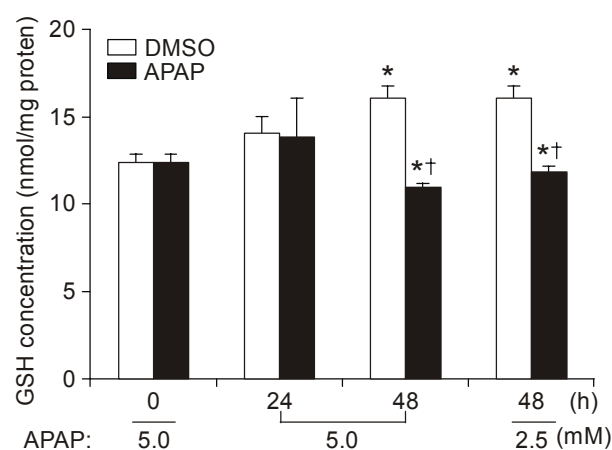


**Figure 1.** Time and APAP-concentration dependent ROS/RNS production. C6 cells grown in microtiter plates were treated with APAP (1.25–10 mM) for 24 h (solid circle) and 48 h (open circle). After APAP treatment for indicated times, conversion of DHR-123 to rhodamine-123 was determined by fluorometric analysis. \*, Significantly different from the untreated control cells.

radical metabolites as well as ROS (Adamson and Harman, 1993; Lores Arnaiz *et al.*, 1995) in different cell systems. Therefore, we investigated whether APAP produces ROS/RNS in C6 glioma cells by using DHR-123 as a sensitive fluorescence probe, since oxidation of DHR-123 to rhodamine-123 can be mediated by peroxynitrite or  $H_2O_2$  (Muijsers *et al.*, 2000) and that peroxynitrite is rapidly formed by a reaction between nitric oxide and superoxide radical. DMSO (vehicle control) did not increase the ROS/RNS level, as compared with untreated control cells. APAP treatment up to 48 h significantly elevated the levels of ROS/RNS in concentration- and time-dependent manners (Figure 1). The rates of ROS/RNS production significantly increased at higher concentrations of APAP and longer exposure time. Approximately 76% and 198% increase in the ROS/RNS level was observed in C6 glioma cells upon treatment with 5 and 10 mM APAP for 48 h, respectively, as compared with the cells treated with DMSO alone. However, cells treated with 5 mM APAP for 24 h did not increase the ROS/RNS level, indicating that a long exposure time is needed for the increased production of peroxides.

### Effect of APAP on GSH concentration in C6 glioma cells

Cellular GSH level was known to change upon exposure to toxic chemicals or stressful conditions (Dimova *et al.*, 2000; Mari and Cederbaum, 2000). Therefore, we determined whether APAP modulates the level of GSH in C6 cells. There was little difference in the GSH level between untreated cells (0 h) and

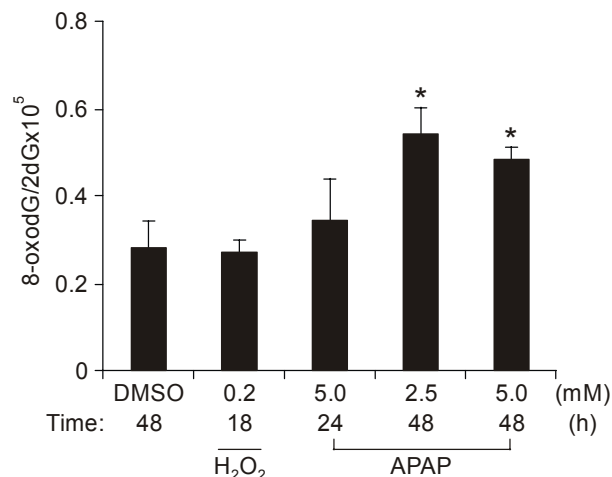


**Figure 2.** Time-dependent changes in the GSH levels by APAP. GSH concentrations in the whole cell extracts were determined after C6 cells were exposed to DMSO (open columns), 2.5 mM, or 5.0 mM APAP (closed columns) for the indicated times. \*, Significantly different from DMSO control (time 0). †, Significantly different from the values of DMSO-treated cells after the same exposure time.

cells treated with 5 mM APAP for 24 h (Figure 2). However, the GSH level after exposure to 5 mM APAP for 48 h was significantly lower than that in the DMSO-exposed cells. GSH concentration was also decreased by exposure to 2.5 mM APAP for 48 h, compared with the DMSO-treated cells.

**Effect of APAP on 8-oxodG levels in nuclear DNA**

The level of 8-oxodG in nuclear DNA was significantly elevated in a concentration- and time-dependent manner after exposure to APAP for up to 48 h (Figure 3). In general, more oxidized DNA was detected after longer exposure to APAP than shorter exposure. For instance, the level of nuclear 8-oxodG was significantly elevated after exposure to 2.5 and 5.0 mM



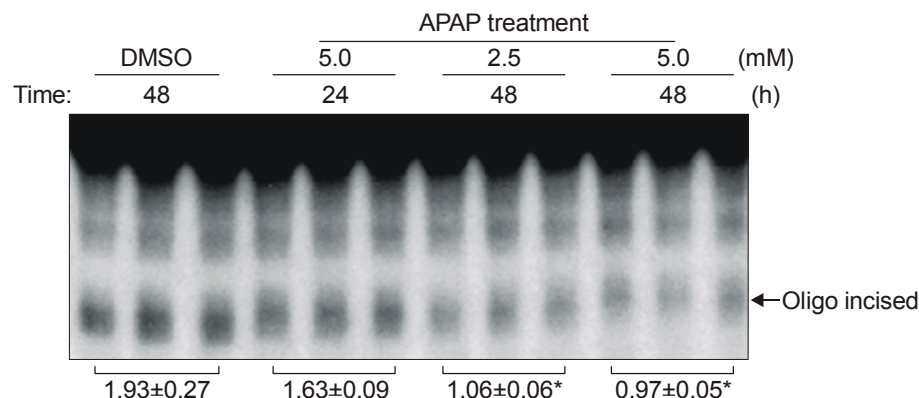
**Figure 3.** APAP-induced changes in the level of nuclear 8-oxodG/2dG. C6 cells were exposed to 0.2 mM H<sub>2</sub>O<sub>2</sub> for 18 h or different concentrations of APAP for 24 or 48 h, as indicated. The ratio of 8-oxodG to 2'-dG in the nucleus was then determined by the method described under Materials and Methods. \*, Significantly different from the DMSO-treated cells.

APAP for 48 h, compared to the DMSO-treated control (Figure 3). In contrast, 5.0 mM APAP for 24 h and 0.2 mM H<sub>2</sub>O<sub>2</sub> for 18 h did not increase the nuclear 8-oxodG level.

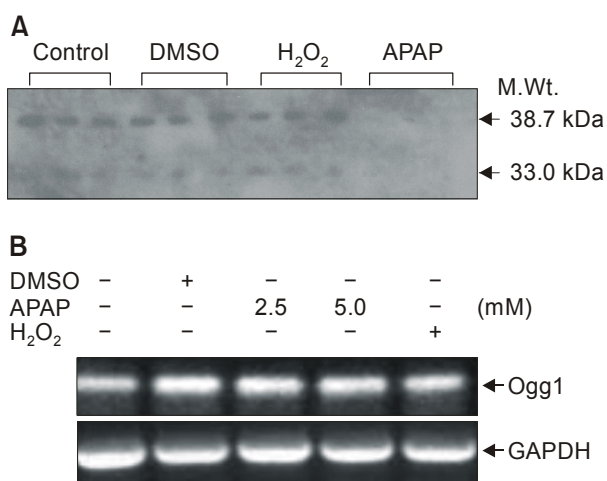
**Effect of APAP on nuclear 8-oxodG incision activity, Ogg1 protein and mRNA levels**

To understand the reason why APAP increased the nuclear 8-oxodG level, we determined the nuclear 8-oxodG incision activity. Densitometric analysis showed that the nuclear 8-oxodG incision activity was significantly decreased by 45% and 50% after treatment with 2.5 and 5 mM APAP for 48 h, respectively, as compared with that of the DMSO-treated cells (Figure 4). Treatment of C6 cells with 5 mM APAP for 24 h slightly (16%) decreased the nuclear 8-oxodG incision activity, whereas DMSO or 0.2 mM H<sub>2</sub>O<sub>2</sub> did not change the activity in the nucleus (data not shown).

Because of the reduction in the 8-oxodG incision activity after APAP treatment, we then measured the level of Ogg1 protein in the nucleus from APAP-treated C6 cells by immunoblot analysis. One major immunoreactive band (apparent M<sub>r</sub> 38.7 kDa), similar to the size of the rat Ogg1 protein (Prieto Alamo *et al.*, 1998) and human Ogg1 protein (Lee *et al.*, 2003), was detected in the nucleus from untreated control, H<sub>2</sub>O<sub>2</sub>-, or DMSO-treated cells (Figure 5A), along with another minor band (M<sub>r</sub> about 33.0 kDa). However, there was little difference in the density of both 38.7 and 33.0 kDa bands among the untreated control, H<sub>2</sub>O<sub>2</sub>-, and DMSO-treated cells (Figure 5A). For instance, treatment with 0.2 mM H<sub>2</sub>O<sub>2</sub> did not reduce the level of nuclear Ogg1 protein. In contrast, treatment with 5 mM APAP for 48 h virtually abolished the 38.7 and 33 kDa proteins (Figure 5A), whereas these proteins were still detected after exposure to 5 mM APAP for 24 h (data not shown). However, the decreased Ogg1 protein did not result from the reduced level of Ogg1 mRNA because neither APAP nor H<sub>2</sub>O<sub>2</sub> significantly changed the levels of Ogg1



**Figure 4.** Effect of APAP on the 8-oxodG incision activity. Activity of 8-oxodG incision was determined for the nuclear extracts prepared from C6 cells exposed to DMSO or different concentrations of APAP for the indicated times. Autoradiographic results and arbitrary densities of the cleaved 10-mer oligo bands representing the 8-oxodG incision activity are presented. \*, Significantly different from the DMSO-treated samples, n = 3 for each treatment.



**Figure 5.** Effect of APAP on the levels of nuclear Ogg1 protein and mRNA. (A) The nuclear extracts were prepared from C6 cells exposed to DMSO, 0.2 mM H<sub>2</sub>O<sub>2</sub> for 18 h, or 5 mM APAP for 48 h. Equal amounts of the nuclear extract (30 mg protein/lane) were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis for Ogg1 protein,  $n = 3$  for each treatment. (B) The levels of Ogg1 and GAPDH mRNA in C6 cells treated differently, as indicated, were determined by RT-PCR. The primers and conditions used for amplification of Ogg1 (503 bp) and GAPDH (304 bp) were described (Tsurudome *et al.*, 1999). PCR-amplified DNA (in 10  $\mu$ l of PCR mixture) was separated on 1% agarose gel and visualized under UV illumination. The correct size of each PCR product was verified with 100 bp-DNA ladder.

transcript determined by RT-PCR analysis (Figure 5B). These results suggest that large doses of APAP can reduce the Ogg1 protein content, mainly by affecting the protein itself without changing its mRNA level.

## Discussion

In this study, we investigated the production of ROS/RNS and subsequent DNA modification in C6 glioma cells by a large dose (5 mM) of APAP, which is the same concentration used by other investigators (Dai and Cederbaum, 1995; Holownia *et al.*, 1997). Our current data clearly showed that APAP significantly increased the levels of ROS/RNS and nuclear 8-oxodG, a marker for oxidative DNA damage. The APAP-induced elevation of nuclear 8-oxodG level is particularly of interest because of the presence of multiple defense mechanisms against oxidative DNA damage in the nucleus (Ames, 1989). The defense system include: nuclear compartmentalization away from the mitochondria where ROS are mainly produced; the presence of histone and polyamines that protect DNA from ROS-induced oxidative damage; and the presence of many different DNA repair en-

zymes in the nucleus. Therefore, the elevation of oxidized DNA observed in C6 cells may be due to the following: increased production of ROS/RNS and reactive metabolites of APAP including *N*-acetyl-*p*-benzoquinoneimine (NAPQI) through cytochrome P450-mediated metabolism; decreased levels of antioxidants including GSH; and substantial reduction in the activity of nuclear Ogg1 protein, one of the major DNA repair enzymes. However, the elevated 8-oxodG level most likely results from an indirect, secondary effect of the significant reduction in Ogg1 DNA repair enzyme activity and content through interaction with reactive metabolites of APAP, because H<sub>2</sub>O<sub>2</sub>, a strong oxidant used as a negative control, neither elevated the level of nuclear 8-oxodG (Figure 3) nor altered the 8-oxodG incision activity or Ogg1 content (Figure 4 and 5). Furthermore, peroxynitrite itself did not elevate the 8-oxodG level (Uppu *et al.*, 1996). Our results are in agreement with the recent report that the inactivation of the *Ogg1* gene lead to accumulation of 8-oxodG in mice (Minowa *et al.*, 2000).

The question whether large doses of APAP causes oxidative DNA damage seems unresolved due to conflicting reports. For instances, Flaks and colleagues (1983 and 1985) reported that APAP can increase the mutation rates through oxidative damage. In contrast, many other reports suggest that APAP is considered safe and non-mutagenic (for a review, see Bergman *et al.*, 1996). These conflicting reports may derive from differences in: experimental models, route and dose of APAP administration, pre-existing conditions of host cells or animals, target tissues examined, etc. However, it is unlikely that low, therapeutic doses of APAP cause oxidative DNA damage, partly based on our current results about the relatively high concentration of APAP and long exposure time needed to observe significant changes in the 8-oxodG level. Furthermore, APAP treatment for 48 h only partially inhibited the nuclear 8-oxodG incision activity. These results, therefore, strongly indicate that another DNA repair enzyme(s) other than Ogg1 must be present in the nucleus and that APAP did not seem to affect the activity of this DNA repair enzyme, because of the 50% reduction in the 8-oxodG incision activity (Figure 4) despite the near-complete depletion of Ogg1 protein (Figure 5A) after APAP exposure.

It would be of interest how the Ogg1 protein was depleted upon exposure to APAP. Our RT-PCR results (Figure 5B) clearly demonstrated that the loss of Ogg1 protein does not result from the reduction in *Ogg1* mRNA content. These results indicate that translation of *Ogg1* mRNA into its protein may be markedly suppressed by APAP. Alternatively, the APAP-induced depletion of Ogg1 protein may result from interaction of Ogg1 protein with the reactive metabolites of APAP such as NAPQI, which is pro-

duced during and after P450-mediated APAP metabolism. The NAPQI-Ogg1 protein adduct may become so different from the unmodified Ogg1 protein that it may be more susceptible to proteolytic degradation than the original protein. In contrast, H<sub>2</sub>O<sub>2</sub>, a strong oxidant which does not produce a protein adduct, did not decrease the Ogg1 content (Figure 5A). Furthermore, the possibility of NAPQI-Ogg1 protein adduct is consistent with the well-established interaction of APAP or NAPQI with various cellular proteins through their cysteinyl-SH moiety (Streeter *et al.*, 1984). For instance, APAP and its metabolites can bind many proteins and thereby decrease their catalytic activities. These include: N-10-formyl-tetrahydrofolate dehydrogenase (Pumford *et al.*, 1997), glutamate dehydrogenase (Halmes *et al.*, 1996), and aldehyde dehydrogenase (Landin *et al.*, 1996). Alternatively, APAP or its reactive metabolites including NAPQI may interact with another amino acid residues of the Ogg1 protein (Arai *et al.*, 1997), producing chemically damaged proteins, which become more susceptible to ubiquitin-dependent and independent proteolytic degradations, as recently reviewed (Goldberg, 2003). However, the production of NAPQI-Ogg1 protein adduct and the mechanism of subsequent degradation remain to be studied.

We observed that APAP diminished the nuclear 39 and 33 kDa proteins recognized by the rabbit anti-mouse Ogg1 antibody, indicating the same epitope between the two immunoreactive proteins. It is possible that the 33 kDa protein represented an alternatively spliced protein of rat *Ogg1* gene, as in the case of the multiple Ogg1 isoforms derived from alternative splicing of the human *Ogg1* mRNAs (Nishioka *et al.*, 1999). The presence of alternatively spliced *Ogg1* gene products in C6 glioma cells needs to be studied in the future.

In summary, APAP in large doses can cause accumulation of 8-oxodG in nuclear DNA of C6 glioma cells partly through significant reduction of the Ogg1 DNA repair enzyme, which results from the increased production of reactive metabolites and oxidative stress accompanied with a reduced glutathione level.

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