NADH-mediated DNA damage induced by a new coenzyme, pyrroloquinoline quinone, in the presence of copper(II) ion

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Abstract Pyrroloquinoline quinone (PQQ) plays a role as a vitamin or growth factor. Low concentrations of PQQ induced DNA cleavage sites frequently at thymine and cytosine residues in the presence of NADH and Cu(II). Catalase and bathocuproine inhibited DNA damage, whereas free hydroxyl radical scavengers did not. Electron spin resonance and UV-visible spectrometries showed generation of semiquinone radical and superoxide during the reaction of PQQ with NADH. These results suggest that NADH-dependent PQQ redox cycle generated superoxide and hydrogen peroxide to mediate copper-dependent DNA damage. The present study has proposed a requirement to investigate the potentiality of PQQ carcinogenicity.

Key words: DNA damage; Pyrroloquinoline quinone; NADH; Active oxygen species; Copper

1. Introduction

Pyrroloquinoline quinone (PQQ) is nutritionally important as a vitamin or growth factor in mammals [1]. PQQ was identified as a new cofactor of mammalian amine oxidase [2]. The presence of free PQQ in human organs was demonstrated [3]. PQQ is contained in various foods such as vegetables, fruits and beverages [4]. Therapeutic effects of PQQ on various diseases caused by cellular oxidative damage were reported [5,6], and PQQ has been thought a dietary antioxidant. However, there are several reports that antioxidants may have both anticarcinogenic and carcinogenic effects [7–9]. PQQ-induced carcinogenicity and DNA damage have not been studied. Recently, the possibility that some chemicals are non-enzymatically reduced by NAD(P)H in vivo has been pointed out [10]. We investigated DNA damage induced by PQQ in the presence of NADH using ^{32}P-5′-end-labeled DNA fragments obtained from human *p53* tumor suppressor gene and c-Ha-*ras*-1 proto-oncogene. We also studied generation of radicals and superoxide (O_2^-) by the reaction of PQQ with NADH, with electron spin resonance (ESR) and UV-visible spectrometries.

2. Materials and methods

2.1. Materials

Restriction enzymes (*Ava*I and *Pst*I) and T_4 polynucleotide kinase were purchased from New England Biolabs. A restriction enzyme (*Sty*I) was from Boehringer Mannheim GmbH. [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear. PQQ was from Ube Industries Ltd. (Tokyo, Japan). Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). Superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes) and catalase (45 000 units/mg from bovine liver) were from Sigma Chemical Co. Nitroblue tetrazolium (NBT) was from Nacalai Tesque, Co. (Kyoto, Japan).

2.2. Preparation of ^{32}P-5′-end-labeled DNA fragments

DNA fragment was obtained from human *p53* tumor suppressor gene [11]. A 448-bp DNA fragment (PU5 13042–PD6 13489) containing exons 5 and 6 of *p53* gene was obtained by polymerase chain reaction performed on human genomic DNA using 5′ amplimer (PU5) and 3′ amplimer (PD6) (Clontech Lab). A ^{32}P-5′-end-labeled fragment (PU5*-PD6*) was obtained by phosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase (*, ^{32}P-labeled). Subsequently, the fragment was digested with *Sty*I to obtain a singly labeled 334-bp fragment (*Sty*I 13156–PD6* 13489). DNA fragment was also obtained from human c-Ha-*ras*-1 proto-oncogene [12]. DNA fragment was prepared from plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA segment containing c-Ha-*ras*-1 gene. A singly labeled 337-bp fragment (*Pst*I 2345–*Ava*I* 2681) was obtained according to the method described previously [13]. Nucleotide numbering starts with the *Bam*HI site [12].

2.3. Detection of DNA damage by PQQ in the presence of NADH

The standard reaction mixture in a microtube (1.5 ml, Eppendorf) contained PQQ, 250 μM NADH, 20 μM $CuCl_2$, ^{32}P-5′-end labeled DNA fragments and sonicated calf thymus DNA (50 μM/base) in 200 l of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. DTPA was added to remove metal ions, which may be contained in the sodium phosphate buffer. After incubation at 37°C, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min where indicated, and treated as described previously [13].

The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure [14] using a DNA-sequencing system (LKB 2010 Macrophor). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer (LKB 2222 UltroScan XL).

2.4. Detection of radicals and O_2^- derived from PQQ

ESR spectra were measured to detect radicals derived from PQQ. Reaction mixture containing 500 μM PQQ, 1 mM NADH and 20 μM $CuCl_2$ in 10 mM sodium phosphate buffer (pH 7.8) was incubated at 37°C for indicated durations. Aliquots of the mixture were taken up in a calibrated capillary and spectra were measured at 25°C using a JES-FE-3XG (JEOL, Tokyo, Japan) spectrometer with 100 kHz field modulation. The spectra were recorded with a microwave power of 2 mW and a modulation amplitude of 0.125 G. No spin trapping agent was used.

To detect O_2^- derived from PQQ and NADH, 50 μM NBT was added to the reaction mixture, which contained 20 μM PQQ and 100 μM NADH in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. A maximum absorption at 560 nm due to blue formazan formed by NBT reduction [15] was measured at 37°C with a UV-Vis-NIR recording spectrophotometer (Shimadzu UV-365).

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Abbreviations: PQQ, pyrroloquinoline quinone; PQQ·H, semiquinone radical of PQQ; ESR, electron spin resonance; DTPA, diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid; SOD, superoxide dismutase; NBT, nitroblue tetrazolium

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3. Results

3.1. Damage of ^{32}P-labeled DNA fragments by PQQ in the presence of NADH and Cu(II)

Fig. 1A shows an autoradiogram of DNA fragments treated with PQQ, NADH and Cu(II). Oligonucleotides were detected on the autoradiogram as a result of DNA cleavage. PQQ induced DNA damage in the presence of NADH and Cu(II). DNA damage was not observed in the presence of NADH and Cu(II) without PQQ under the condition used (Fig. 1A, lane 1), although high concentrations of NADH caused DNA damage in the presence of Cu(II) [10]. The intensity of DNA damage increased with PQQ concentration (Fig. 1A) and incubation time (data not shown). It is noteworthy that 2 μM PQQ caused apparent DNA damage in the presence of NADH and Cu(II). Enhancement of DNA damage with piperidine treatment was observed (Fig. 1A), indicating that PQQ induced not only strand breakage but also base modification and/or liberation. No DNA cleavage was observed when either NADH or Cu(II) was not added, or when other metal ions, Fe(II), Fe(III) and Mn(II), were added instead of Cu(II) (data not shown).

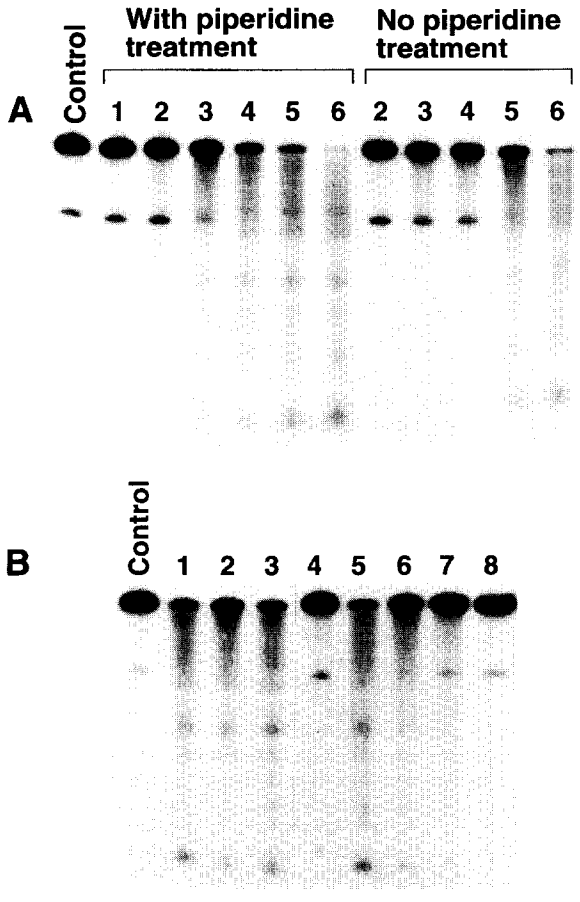

Fig. 1. Autoradiogram of ^{32}P-labeled DNA fragments incubated with PQQ in the presence of NADH and Cu(II). The reaction mixture contained ^{32}P-5′-end-labeled 337-bp DNA fragments, 50 μM/base of sonicated calf thymus DNA, indicated concentrations (A) or 10 μM (B) of PQQ, 250 μM NADH and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated at 37°C for 20 min, and then treated with 1 M piperidine at 90°C for 20 min, unless otherwise noted. The DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and the autoradiogram was obtained by exposing X-ray film to the gel. (A) PQQ concentrations are as follows: lane 1, 0 μM; lane 2, 1 μM; lane 3, 2 μM; lane 4, 5 μM; lane 5, 10 μM; lane 6, 20 μM. (B) Scavenger or bathocuproine was added to the reaction mixture as follows: lane 1, no scavenger; lane 2, 5% (v/v) ethanol; lane 3, 0.1 M mannitol; lane 4, 30 units of catalase; lane 5, 30 units of SOD; lane 6, 10 μM bathocuproine; lane 7, 20 μM bathocuproine; lane 8, 50 μM bathocuproine. Control did not contain PQQ, Cu(II) and NADH.

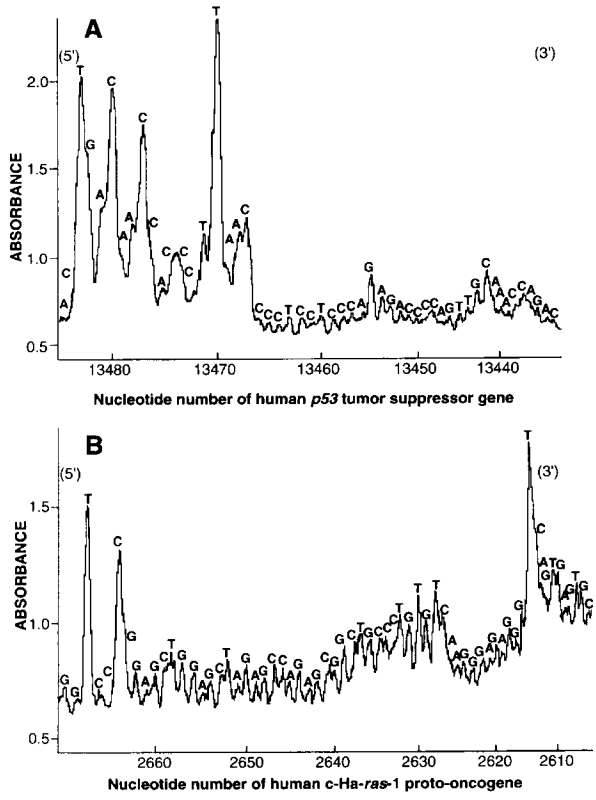

Fig. 2. Site specificity of DNA cleavage induced by PQQ plus NADH and Cu(II). The ^{32}P-5′-end-labeled 334-bp fragment (*Sty*I 13156–PD6* 13489) of *p53* gene (A), or 337-bp fragment (*Pst*I 2345–*Ava*I* 2681) of c-Ha-*ras*-1 gene (B), 50 μM/base of sonicated calf thymus DNA, 10 μM PQQ, 250 μM NADH and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA (*, ^{32}P-labeling). Reaction mixtures were incubated at 37°C for 20 min. After piperidine treatment, the DNA fragments were analyzed as described in the legend to Fig. 1. The relative amounts of oligonucleotide were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL). The horizontal axis shows the nucleotide number of human *p53* tumor suppressor gene (A) [11] or c-Ha-*ras*-1 proto-oncogene starting with the *Bam*HI site (B) [12].

3.2. Effects of scavengers and bathocuproine on DNA damage

Fig. 1B shows the effects of scavengers and bathocuproine, a Cu(I)-specific chelator, on DNA damage induced by PQQ. Inhibition of DNA damage by catalase (lane 4) and bathocuproine (lanes 6–8) suggests the involvement of hydrogen peroxide and Cu(I). However, typical free hydroxyl radical scavengers, ethanol (lane 2) and mannitol (lane 3), and SOD (lane 5) showed little or no inhibitory effect on DNA damage.

3.3. Site specificity of DNA cleavage by PQQ in the presence of NADH and Cu(II)

To examine the DNA cleavage site, ^{32}P-5′-end-labeled DNA fragments treated with PQQ, NADH and Cu(II), sub-

sequently with piperidine, were electrophoresed. An autoradiogram was obtained and scanned with a laser densitometer to measure relative intensity of DNA cleavage as shown in Fig. 2. PQQ induced piperidine-labile sites frequently at thymine and cytosine residues, although there remains a possibility that certain modified bases might be over- or underrepresented, depending on their sensitivity to piperidine.

Because NADH plus Cu(II) is a known DNA cleaving system [10], we performed an experiment to compare the site specificity of DNA cleavage induced by NADH plus Cu(II) in the presence and absence of PQQ. The site specificity of DNA damage induced in the presence of PQQ was similar to that in the absence of PQQ (data not shown). However, in the presence of PQQ, 250 μM NADH induced much stronger DNA damage than 2 mM NADH in the absence of PQQ (data not shown). This suggests that PQQ has a high potentiality to induce DNA damage in the presence of low concentrations of NADH and Cu(II).

3.4. Generation of semiquinone radical and O_2^- during PQQ reduction

Fig. 3 shows ESR spectra of a radical derived from PQQ. Although PQQ alone showed no signal (data not shown), the signals of the radical were observed in the presence of NADH at 10 min and 20 min after starting the reaction (Fig. 3A). The spectrum can be interpreted in terms of hyperfine splitting due to both the two nitrogen atoms and the three hydrogen atoms, and can be reasonably assigned to the semiquinone radical of

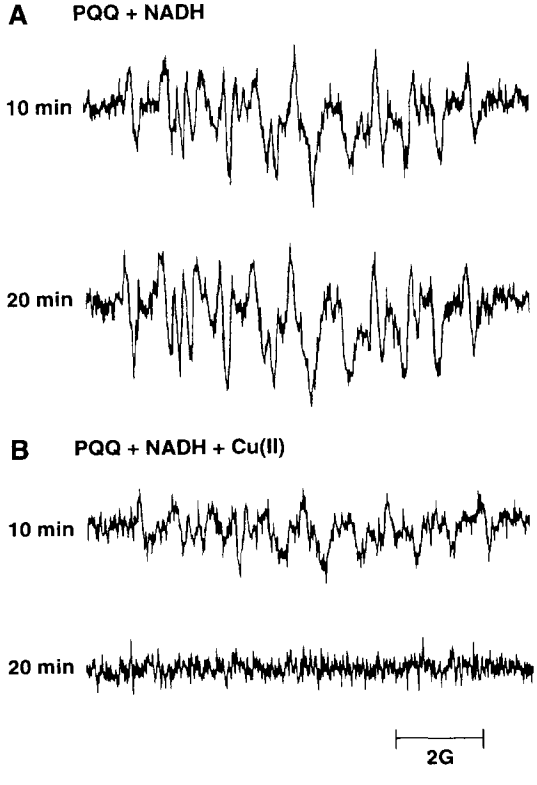

Fig. 3. ESR spectra of radicals derived from PQQ. Reaction mixture containing 500 μM PQQ, 1 mM NADH in the presence (B) and absence (A) of 20 μM $CuCl_2$ in 10 mM sodium phosphate buffer (pH 7.8) was incubated at 37°C. Aliquots of the mixture were taken up in a calibrated capillary and spectra were measured at 25°C. No spin trapping agent was used.

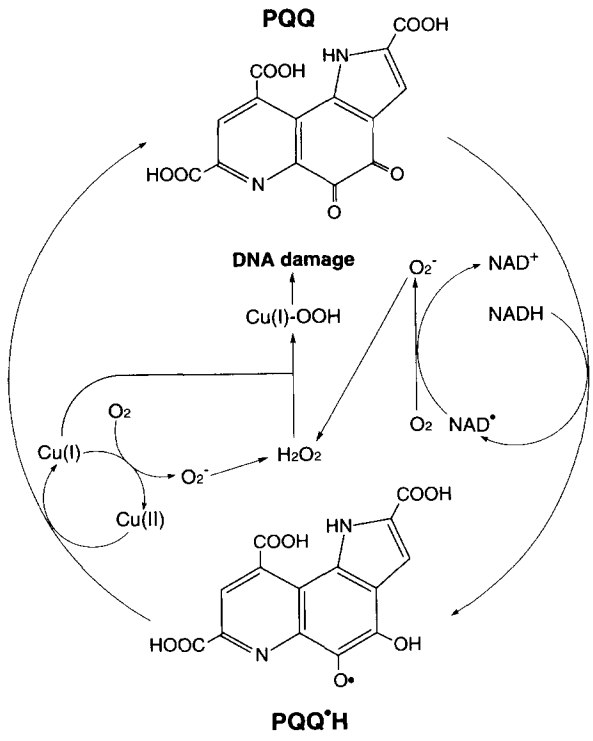

Fig. 4. Possible mechanism of DNA damage induced by PQQ in the presence of NADH and Cu(II).

PQQ (PQQ•H) by reference to the hyperfine coupling constants ($a_1^N = 0.72$, $a_6^N = 0.82$, $a_1^H = 0.99$, $a_3^H = 1.20$ and $a_8^H = 1.99$) [16]. The present ESR data confirmed that PQQ is reduced to PQQ•H by NADH, as reported previously [16]. The addition of Cu(II) decreased the ESR signal, assigned to PQQ•H (Fig. 3B). The decrease of the signal can be explained by assuming that PQQ•H reacts with Cu(II).

PQQ plus NADH induced an increase of absorption at 560 nm due to NBT reduction, even in the absence of Cu(II), and the increase of absorption was inhibited by SOD (data not shown). PQQ alone and PQQ plus Cu(II) showed no changes in the spectra in the absence of NADH (data not shown). These results indicate that NADH is required for generation of O_2^- from PQQ.

4. Discussion

The present study has demonstrated that low concentrations of PQQ have an ability to cause DNA damage in the presence of NADH and Cu(II). Inhibitory effects of catalase and bathocuproine suggested that H_2O_2 and Cu(I) were required for DNA damage. A possible mechanism of DNA damage by PQQ is shown in Fig. 4. Sugioka et al. reported an ESR spectrum of PQQ•H generated by NAD(P)H-mediated reduction of PQQ [16]. It is noteworthy that NADH reduced PQQ to PQQ•H, although NADH is a poor one-electron donor. The production of PQQ•H and O_2^- by the reaction of PQQ with NADH was confirmed by the results of ESR and UV-visible spectrometries, respectively. The generation of O_2^- is supported by reports showing very rapid reaction of NAD radical with O_2 [17]. The decrease in ESR signal intensity by the addition of Cu(II) suggested the reaction of PQQ•H with Cu(II) to produce PQQ and Cu(I).

On the basis of these results, it is concluded that H_2O_2 produced by O_2^- dismutation reacts with Cu(I) to form an active species causing DNA damage.

Typical 'OH scavengers showed little or no inhibitory effect on DNA damage, suggesting that 'OH might not have played an important role. PQQ induced DNA cleavage sites frequently at thymine and cytosine residues. This result supports the involvement of active oxygen species other than 'OH, because 'OH causes DNA cleavage at any nucleotides with little site specificity [18]. Alternatively, it is speculated that Cu(II) bound to DNA in a site-specific manner is reduced to Cu(I), which reacts with H_2O_2 to form a DNA-metal-oxygen complex. This complex may release 'OH to attack an adjacent DNA constituent before being scavenged by 'OH scavengers. Thus, the site-specific DNA damage can be explained both by the formed active oxygen species and by the specific binding manner of Cu(II).

The binding of copper to DNA and/or protein in chromatin has been proposed to serve physiological functions to maintain DNA structure [19]. However, there are several reports about copper-mediated production of active oxygen species and DNA damage [20–22]. A high amount of 8-hydroxydeoxyguanosine, an oxidative product of guanine, was observed in the liver of LEC rats, which develop hepatic cancer associated with abnormal copper metabolism [23]. Therefore, the copper-dependent DNA damage by PQQ is of interest in connection with these observations.

Human organs have been reported to contain PQQ (5.9 ± 3.4 ng/g wet tissue, in spleen) [3]. Because eukaryotic organisms are not thought to synthesize PQQ, PQQ is most likely obtained either from enteric bacteria or from foods, and continuously taken to humans for long periods. Pharmacological effects of PQQ are expected because previous reports have demonstrated its nutritional importance [1] and antioxidative effects [5,6]. However, an excessive intake of PQQ may accelerate the generation of active oxygen species and increase the risk of PQQ toxicity. Therefore, not only nutritional and antioxidative effects, but also toxic effects, especially mediated by active oxygen species, should be considered. To clarify PQQ toxicity and carcinogenicity, further examination is needed.

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References

[1] Killgore, J., Smidt, C., Duich, L., Romero-Chapman, N., Tinker, D., Reiser, K., Melko, M., Hyde, D. and Rucker, R.B. (1989) Science 245, 850–852.
[2] McIntire, W.S. (1994) FASEB J. 8, 513–521.
[3] Kumazawa, T., Seno, H., Urakami, T., Matsumoto, T. and Suzuki, O. (1992) Biochim. Biophys. Acta 1156, 62–66.
[4] Kumazawa, T., Sato, K., Seno, H., Ishii, A. and Suzuki, O. (1995) Biochem. J. 307, 331–333.
[5] Christensen, H.N. (1994) Nutr. Rev. 52, 24–25.
[6] Decker, E.A. (1995) Nutr. Rev. 53, 49–58.
[7] Halliwell, B., Murcia, M.A., Chirico, S. and Aruoma, O.I. (1995) Crit. Rev. Food Sci. Nutr. 35, 7–20.
[8] The Alpha-tocopherol, Beta-carotene Cancer Prevention Study Group (1994) New Engl. J. Med. 330, 1029–1035.
[9] Diplock, A.T., Rice-Evans, C.A. and Burdon, R.H. (1994) Cancer Res. 54, 1952s–1956s.
[10] Oikawa, S. and Kawanishi, S. (1996) Biochemistry, 35, 4584–4590.
[11] Chumakov, P. (1990) EMBL Data Library, accession number X54156.
[12] Capon, D.J., Chen, E.Y., Levinson, A.D., Seeburg, P.H. and Goeddel, D.V. (1983) Nature 302, 33–37.
[13] Kawanishi, S. and Yamamoto, K. (1991) Biochemistry 30, 3069–3075.
[14] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499–560.
[15] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
[16] Sugioka, K., Nakano, M., Naito, I., Tero-Kubota, S. and Ikegami, Y. (1988) Biochim. Biophys. Acta 964, 175–182.
[17] Orii, Y. (1993) Biochemistry 32, 11910–11914.
[18] Celander, D.W. and Cech, T.R. (1990) Biochemistry 29, 1355–1361.
[19] Dijkwel, P.A. and Wenink, P.W. (1986) J. Cell. Sci. 84, 53–67.
[20] Guyton, K.Z. and Kensler, T.W. (1993) Br. Med. Bull. 49, 523–544.
[21] Li, Y. and Trush, M.A. (1994) Cancer Res. 54, 1895s–1898s.
[22] Kawanishi, S., Oikawa, S. and Inoue, S. (1995) In: Genetic Response to Metals (Sarkar, B., Ed.) pp. 131–151, Marcel Dekker, New York.
[23] Yamamoto, F., Kasai, H., Togashi, Y., Takeichi, N., Hori, T. and Nishimura, S. (1993) Jpn. J. Cancer Res. 84, 508–511.