Oxidative damage to cellular and isolated DNA by metabolites of a fungicide ortho-phenylphenol

Mariko Murata, Kosuke Moriya, Sumiko Inoue and Shosuke Kawanishi

Department of Hygiene, Mie University School of Medicine, Tsu, Mie 514-8507, Japan

1Department of Public Health, Graduate School of Medicine, Kyoto University, Kyoto 606-8315, Japan

2To whom correspondence should be addressed

ortho-Phenylphenol (OPP) and its sodium salt, which are used as fungicides and antibacterial agents, have been found to cause carcinomas in the urinary tract of rats. To clarify the carcinogenic mechanism of OPP, we compared the DNA damage inducing ability of an OPP metabolite, phenyl-1,4-benzoquinone (PBQ) with that of another metabolite, phenylhydroquinone (PHQ). Pulsed field gel electrophoresis showed that PBQ and PHQ induced DNA strand breakage in cultured human cells, but PBQ did it more efficiently than PHQ. Significant increases in 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) were observed in cells treated with PBQ and PHQ, and the increase of 8-oxodG induced by PBQ was significantly higher than that induced by PHQ. Using 32P-5′-end-labeled DNA fragments obtained from human p53 tumor suppressor gene and c-Ha-ras-1 protooncogene, we showed that PBQ plus NADH, and also PHQ, induced DNA damage frequently at thymine residues, in the presence of Cu(II). The intensity of DNA damage by PBQ was stronger than that by PHQ, showing higher importance of PBQ than other OPP metabolites. Catalase and bathocuproine inhibited Cu(II)-mediated DNA damage by PBQ plus NADH and PHQ, suggesting that H2O2 reacts with Cu(I) to produce active species causing DNA damage. Electron spin resonance and UV-visible spectroscopic studies have demonstrated generation of semiquinone radical and superoxide from the reaction of PBQ with NADH or the Cu(II)-mediated autoxidation of PHQ. The present results suggest that these OPP metabolites cause oxidative DNA damage through H2O2 generation in cells, and the damage may lead to mutation and carcinogenesis. It is concluded that PBQ may play a more important role in the expression of OPP carcinogenicity than other OPP metabolites.

Introduction

ortho-Phenylphenol (OPP) and its sodium salt, sodium ortho-phenylphenate (NaOPP), are used as fungicides and disinfectants (1). Due to their widespread use and potential for human exposure, extensive toxicological testing has been performed on both OPP and NaOPP. Both compounds have been found to cause carcinomas in the urinary bladder and kidney of rats (1–7). However, OPP and NaOPP have not been proved to be mutagenic in bacterial test systems (1,8).

With regard to the mechanism of OPP carcinogenesis, some reports suggested that DNA adducts with OPP metabolites may play a role in the genotoxic effects of OPP (9,10). On the other hand, Inoue et al. (11) revealed that phenylhydroquinone (PHQ), a metabolite of OPP, caused oxidative DNA damage in the presence of Cu(II). There were similar reports (12,13) suggesting that the reactive oxygen species derived from autoxidation of PHQ elicit DNA damage. PHQ was observed to cause oxidative DNA damage in CHO-K1 cells (14,15). Metabolic activation of OPP in rats occurs via a two-step process involving the cytochrome P450-mediated formation of PHQ in the liver and a prostaglandin H synthase-mediated oxidation of PHQ to phenyl-1,4-benzoquinone (PBQ) in the urinary tract (16,17). It has been reported that PBQ injected to rats caused DNA damage in the urinary bladder epithelium, whereas PHQ did not (18). Since PBQ is known to be unable to cause damage to isolated DNA (11,12), the mechanism of PBQ-induced damage to cellular DNA remains to be clarified.

Here we address the question of whether PHQ or PBQ mainly contributes to OPP-metabolite-induced DNA damage. Furthermore, in order to investigate the mechanism of the cellular DNA damage, we compared induction of DNA damage by PBQ with that by PHQ, using HL 60 cells and 32P-5′-end-labeled DNA fragments obtained from human p53 tumor suppressor gene and c-Ha-ras-1 protooncogene. We also measured reactive oxygen species and radicals by UV-visible and electron spin resonance (ESR) spectroscopies.

Materials and methods

Materials

Restriction enzymes (Smal, EcoRI, Apal and SstI) and calf intestine phosphatase and proteinase K were purchased from Boehringer Mannheim GmbH. Restriction enzymes (HindIII, Aval and Xbal) and T4 polynucleotide kinase were purchased from New England Biolabs. A Human p53 Amplimer Panel was from Clontec (CA). The primers designed for the use in the polymerase chain reaction (PCR) process for the amplification of p53 are contained in this product (kit). [γ-32P]ATP (222 TBq/nmol) was from New England Nuclear. CuCl2, ethanol, t-mannitol and sodium formate were from Nakalai Tesque Inc. (Kyoto, Japan). Diethylaminoethylamine-N,N′,N″,N″′,N″″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). Superoxide dismutase (SOD), 3000 U/mg from bovine erythrocytes, catalase (45 000 U/mg from bovine liver), methional, RNase A and bacterial alkaline phosphatase (BAP) were from Sigma. ABI lysis buffer was from Applied Biosystems. Nuclease P1 was from Yasuma Shoyu Co. (Chiba, Japan). Acrylamide, bisacylamide and piperedine were from Wako Chemicals Co. (Osaka, Japan). Phenyl-1,4-hydroquinone (PHQ) was from Tokyo Kasei Co. (Tokyo, Japan). PBQ was purchased from Aldrich. Ethanol solutions of PHQ and PBQ were freshly made up each time.

Detection of cellular DNA damage by pulsed field gel electrophoresis

HL60 cells were grown in RPMI 1640 supplemented with 6% fetal calf serum (FCS) at 37°C under 5% CO2 in a humidified atmosphere. HL60 cells were treated with either PBQ or PHQ at 37°C. The medium contained 0.05% ethanol as the solvent of PBQ and PHQ. Control condition also contained 0.05% ethanol. After the incubation, the medium was removed and the cells

Abbreviations:

8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; DTPA, diethylaminoethylamine-N,N′,N″,N″′,N″″-pentaacetic acid; ESR, electron spin resonance; FCS, fetal calf serum; HPLC-EC-CD, electrochemical detection coupled to high-performance liquid chromatography; NADH, β-nicotinamide adenine dinucleotide; NaOPP, sodium ortho-phenylphenolate; O2−: superoxide; OPP, ortho-phenylphenol; PBQ, phenyl-1,4-benzoquinone; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PHQ, phenylhydroquinone; SOD, superoxide dismutase.

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were washed twice with phosphate-buffered saline (PBS) and resuspended in PBS. The cell suspension was solidified with agarose, followed by treatment with protease K according to the method described previously (19). Electrophoresis was performed in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) by CHEF-Mapper pulsed field electrophoresis system (Bio-Rad) at 200 V and 14°C. Switch time was 60 s for 15 h followed by 90 s switch time for 9 h. The DNA in the gel was visualized in ethidium bromide.

Analysis of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation in HL60 cells treated with OPP metabolites

HL60 cells (1×10⁶ cells/ml) were incubated with either PHQ or PBQ in 5.0 ml of RPMI 1640 supplemented with 6% FCS at 37°C. After the incubation, the medium was removed and the cells were washed twice with PBS. The cells were suspended in 0.05 mg/ml RNase A, 0.5 mg/ml Protease K and 500 µl of ABI lysis buffer and incubated for 60 min at 60°C. After ethanol precipitation, DNA was digested to nucleosides with nuclease P1 and bacterial alkaline phosphatase and analyzed by high-performance liquid chromatography (HPLC–ECD), as described previously (19). The amount of 8-oxodG was measured by a modified method of Takeuchi et al. (20).

Preparation of ³²P-5′-end-labeled DNA fragments

DNA fragments were obtained from the human p53 tumor suppressor gene (21). Two fragments from the p53 gene containing exons were amplified by the PCR method using an Omnigene Temperature Cycling System. The PCR products were digested with Smal I and ligated into Smal-cleaved PUC 18 plasmid, and then transferred to Escherichia coli JM 109. The plasmid PUC 18 was digested with EcoRI and HindIII, and the resulting DNA fragments were fractionated by electrophoresis on 2% agarose gels. The 5′-end-labeled 650 bp fragment (HindIII*13972–EcoRI*14621) was obtained by dephosphorylation with calf intestine phosphatase and repolymerization with [γ-³²P]ATP and T4 polynucleotide kinase (*; ³²P-labeled). The 650 bp fragment was further digested with Apal to obtain singly labeled 433 bp fragment (Apal 14179–EcoRI*14621) and the 211 bp fragment (HindIII*13972–Apal 14182), as described previously (22). DNA fragment was also obtained from human c-Ha-ras-1 protooncogene (23). A DNA fragment was prepared from plasmid pbcNL, which carries a 6.6 kb BamHI chromosomal DNA segment containing c-Ha-ras-1 gene, and a singly labeled 337 bp fragment (PstI 2345–AvaI* 2681) were obtained according to the method described previously (24). Nucleotide numbering starts with the BamHI site (23).

Detection of DNA damage by OPP metabolites in the presence of NADH and Cu(II)

The standard reaction mixture (in a microtube; 1.5 ml; Eppendorf) contained 1 mM NADH and CuCl₂, 3²P-5′-end labeled DNA fragments (50 µl) of sonicated calf thymus DNA (25 µM per base) in 200 µl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 mM DTPA. DTPA was added to remove metal ions, which may be contained in sodium phosphate buffer. After incubation at 37°C for 1 h, the DNA fragments were heated at 90°C in 1 M pipedrline for 20 min where indicated and treated as described previously (25).

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 (26) 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 Ultrascan XL).

Analysis of 8-oxodG formation in calf thymus DNA by OPP metabolites in the presence of NADH and Cu(II)

The amount of 8-oxodG was measured by a modified method of Kasai et al. (27). Native or denatured DNA fragments (25 µM per base) from calf thymus were incubated with PHQ or PBQ in the presence and absence of NADH and CuCl₂ for 1 h at 37°C. For the experiment with denatured DNA, calf thymus DNA was treated at 90°C for 5 min and quickly chilled before incubation. After ethanol precipitation, DNA was digested to the nucleosides with nuclease P1 and calf intestine phosphatase and analyzed by the HPLC–ECD.

Detection of superoxide (O₂⁻) derived from PBQ plus NADH in the presence and absence of Cu(II)

To detect O₂⁻ generation, cytochrome c was added to the reaction mixture, which contained PBQ plus NADH in the presence and absence of Cu(II) in 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 mM DTPA. A maximum absorption at 550 nm due to ferrocytochrome c formed by ferricynochrome c reduction was measured at 37°C with a UV-visible spectrophotometer every 10 min for 1 h. The content of O₂⁻ at a low estimate was calculated by subtracting absorbance with SOD from that without SOD at 550 nm (ε =21.1×10⁵ M⁻¹ cm⁻¹).

Results

Detection of DNA damage in cultured cells treated with OPP metabolites

Figure 1 shows DNA strand breakage in cultured cells treated with OPP metabolites, detected by pulsed field gel electrophoresis. DNA strand breakage to produce 1000–2000 kb fragments was observed in cells treated with 10–20 µM PBQ, but not with PHQ under the condition used. Formation of 50 kb fragments was increased at 10–20 µM PBQ and PHQ. These results reveal that PBQ caused cellular DNA damage more efficiently than PHQ.

Analysis of 8-oxodG formation in HL60 cells induced by OPP metabolites

Figure 2 shows the amount of 8-oxodG in cells induced by OPP metabolites. The formation of 8-oxodG was significantly
Damage of $^{32}$P-labeled DNA fragments by OPP metabolites in the presence of NADH and Cu(II)

Figure 3 shows an autoradiogram of DNA fragments treated with PHQ or PBQ plus NADH in the presence of Cu(II). Oligonucleotides were detected on the autoradiogram as a result of DNA cleavage. In the absence of PHQ and PBQ, DNA damage was not observed in the presence of NADH and Cu(II) (lane 1) under the conditions used. When NADH or Cu(II) was omitted (lanes 2 and 3), no DNA damage was observed. Whereas PHQ-induced DNA damage required Cu(II) alone, PBQ-induced DNA damage required both NADH and Cu(II). The intensity of NADH-dependent DNA damage induced by PBQ was stronger than that of PHQ under the same concentration, although PBQ required 100–200 µM NADH.

Effects of scavengers and bathocuproine on DNA damage by PBQ

Figure 4 shows the effects of scavengers and bathocuproine on DNA damage by PBQ. A Cu(I)-specific chelator, on DNA damage induced by PBQ plus NADH in the presence of Cu(II). Inhibition of DNA damage by catalase (lane 10) and bathocuproine (lane 11) indicates the involvement of H$_2$O$_2$ and Cu(II). Methional inhibited the DNA damage (lane 8), although other typical hydroxyl free radical (OH·) scavengers such as ethanol (lane 5), mannitol (lane 6) and sodium formate (lane 7), and superoxide dismutase(SOD) (lane 9) showed little or no inhibitory effect on DNA damage. When denatured DNA was used, formation of oligonucleotides increased (lane 12).

Increase of oligonucleotides with piperidine treatment (lane 4) suggested that PBQ induced not only strand breakage but also base modification and/or liberation. The similar scavenger effects were observed with DNA damage induced by PHQ plus Cu(II) (data not shown).

Site specificity of DNA cleavage by OPP metabolites

To examine the DNA cleavage site, $^{32}$P-5′-end-labeled DNA fragments treated with PBQ plus NADH or PHQ in the presence of Cu(II), subsequently with piperidine, were electrophoresed. An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensity of DNA cleavage in c-Ha-ras-1 protooncogene and in the human $p53$ gene as shown in Figures 5 and 6. Figure 5 shows site specificity of DNA cleavage in the fragment from c-Ha-ras-1 protooncogene induced by PBQ in the presence of NADH and Cu(II). When native DNA (Figure 5A) was used, DNA cleavage was observed frequently at thymine residues, although there remains a possibility that certain base damage might be over- or under-represented, depending on their sensitivity to piperidine. When denatured DNA (Figure 5B) was used, preferential damage occurred more frequently at guanine sites. Site-specific DNA damage at thymine residues was also observed in the fragment from $p53$ tumor suppressor gene when it was treated with PBQ in the presence of NADH and Cu(II) (Figure 6A). A similar pattern was observed in DNA damage induced by PHQ in the presence of Cu(II) (Figure 6B).

Formation of 8-oxodG in calf thymus DNA by OPP metabolites in the presence of NADH and Cu(II)

Using HPLC–ECD, we measured 8-oxodG content in calf thymus DNA treated with PBQ plus NADH (Figure 7A) and...
Comparison of site specificity of DNA cleavage induced by PBQ in the presence of 200 µM NADH and 20 µM CuCl₂. The 32P-5'-end-labeled 337 bp fragment (PstI 2345–AvaI* 2681) of c-Ha-ras-1 gene, 25 µM/base of sonicated calf thymus DNA, 5 µM PBQ plus 200 µM NADH and 20 µM CuCl₂ in 200 µl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 µM DTPA. Reaction mixtures were incubated at 37°C for 1 h. Native DNA (A) and denatured DNA (B) were used. The DNA fragments were treated with 1 M piperidine for 20 min at 90°C, and then electrophoresed on an 8% polyacrylamide–8 M urea gel. The autoradiogram was obtained by exposing X-ray film to the gel. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL). The horizontal axis shows the nucleotide number of human c-Ha-ras-1 protooncogene starting with the BamHI site (23).

Detection of O₂⁻ derived from PBQ plus NADH in the presence and absence of Cu(II)

Figure 8 shows O₂⁻ produced by PBQ plus NADH in the presence and absence of Cu(II). These results indicate that O₂⁻ was generated by PBQ reduction even in the absence of Cu(II). When Cu(II) was added, the content of O₂⁻ detected by this system was decreased, suggesting rapid reaction between O₂⁻ and Cu(II). Production of O₂⁻ by the autoxidation of PHQ was also detected (date not shown).

Formation of 8-oxodG in calf thymus DNA by PBQ plus NADH and PHQ in the presence of Cu(II).

DNA fragments were incubated with PBQ plus 200 µM NADH or PHQ, and 20 µM CuCl₂ for 1 h at 37°C. After ethanol precipitation, DNA was digested to the nucleosides enzymatically and analyzed by HPLC–ECD as described in the Materials and methods. PBQ and PHQ concentrations are as follows: 0, 5, 10, 15 and 20 µM.
Production of free radicals from OPP metabolites

Figure 9 shows ESR spectra of radicals generated by OPP metabolites. PBQ plus NADH showed the formation of a 1:3:3:1 spectrum radical with hyperfine splitting constant of \( \alpha = 0.23 \) mT, and PHQ also showed the similar signal. The ESR four-line signal was assigned to the PHQ semiquinone radical, as described in our previous study (11). The addition of Cu(II) to the reaction systems resulted in a marked increase in the signal intensity of the semiquinone radical.

Discussion

Pulsed field gel electrophoresis study showed that PBQ and PHQ caused cellular DNA damage, but PBQ did so more efficiently than PHQ. Significant increase of 8-oxodG was observed in cells treated with PBQ and PHQ. The formation of 8-oxodG by PBQ was significantly higher than that by PHQ, at a concentration of 20 \( \mu \)M. These results suggest that PBQ may play a more important role in causing DNA damage than PHQ, although both of these OPP metabolites induced oxidative damage.

To clarify the mechanism of cellular DNA damage induced by OPP metabolites, we investigated damage to \(^{32}\)P-labeled DNA fragments obtained from the human p53 tumor suppressor gene and c-Ha-ras-1 protooncogene. PBQ required both NADH and Cu(II) for DNA damage, whereas PHQ required only Cu(II). Inhibitory effects of catalase and bathocuproine on the DNA damage indicate the involvement of \( \mathrm{H}_2\mathrm{O}_2 \) and Cu(I). Methionol completely inhibited the DNA damage, whereas typical hydroxyl free radical scavengers (ethanol, mannitol, sodium formate) did not. Methionol scavenges not only the hydroxyl radical, but it can also scavenge crypto-OH radicals (28.29). Therefore, it is considered that the species causing DNA damage is an active species other than the hydroxyl free radical. The DNA sequencing experiments revealed that PBQ induced piperidine-labile sites frequently at thymine in the presence of NADH and Cu(II). A similar pattern was observed in the case of PHQ. Several reports showed that the reaction of \( \mathrm{H}_2\mathrm{O}_2 \) with Cu(II) causes DNA damage with a site specificity for thymine residues (30–33). It has been reported that 5-(hydroxymethyl)uracil, one of the major oxidative modifications of thymine is excreted in urine as the result of DNA repair by 5-(hydroxymethyl)uracil-DNA-glycosylase (34). The site modification of DNA bases observed here may contain such oxidized thymine. In any event, the present results support the involvement of reactive oxygen species generated from \( \mathrm{H}_2\mathrm{O}_2 \) and Cu(I), rather than OH·, which causes DNA cleavage at any nucleotides with little site specificity (35,36).

A possible mechanism of DNA damage induced by OPP metabolites in the presence of NADH and Cu(II) is proposed as shown in Figure 10. PBQ is autoxidized to PBQ through the intermediate semiquinone. PBQ is reduced by an endogenous reductant, NADH, to form semiquinone radical. The generation of \( \mathrm{O}_2^- \) takes place during the Cu(II)-mediated autoxidation of PHQ or by the reaction of semiquinone radical with \( \mathrm{O}_2 \). The generation of \( \mathrm{H}_2\mathrm{O}_2 \) by \( \mathrm{O}_2^- \) dismutation and the reduction of Cu(II) to Cu(I) occur concomitantly. \( \mathrm{H}_2\mathrm{O}_2 \) reacts with Cu(I) to form a metal–oxygen complex, such as Cu(I)–\( \mathrm{OOH} \) causing DNA damage. Thus, the NADH-dependent redox cycle of PBQ generates reactive oxygen species, and mediates DNA damage. The production of semiquinone radical and \( \mathrm{O}_2^- \) from PHQ or PBQ plus NADH was confirmed by the data of ESR and UV–visible spectroscopies, respectively. However, the semiquinone radical is not an active species causing DNA damage, because the conversion of Cu(II) into Cu(I) was required for the DNA damage, whereas the semiquinone radical was formed by PBQ plus NADH even in the absence of Cu(II).

The biological importance of NADH as a nuclear reductant has been described (37). The possibility that some chemicals are non-enzymatically reduced by NADH \( \text{in vivo} \) has been shown (38–40). NADH can be a source of endogenous reductant, resulting in oxidative DNA damage. The present study showed that PBQ induced cellular DNA strand break at lower concentration than PHQ, indicating that PBQ has a higher potentiality to cause DNA damage in HL60 cells. This is supported by the report regarding DNA damage of bladder epithelium of rats treated with PBQ, but not with PHQ (18). It is considered that the NADH-dependent redox cycle is important to explain the higher potentiality of PBQ.
concentration of NAD(P)H in certain tissues has been estimated to be as high as 100–200 µM (41), and NAD(P)H possibly plays important roles as a reductant.

Copper occurs in the mammalian cell nucleus, and may contribute to high order chromatin structures (42). Cu(II)/ascorbate/H$_2$O$_2$-mediated DNA damage in aerobic aqueous solutions is believed to be induced in vitro and in vivo (43) through formation of a DNA–Cu(I)–H$_2$O$_2$ complex (32). Copper caused much stronger ascorbate-mediated DNA damage than iron (44). Copper ions exhibit a very high affinity for DNA, and DNA-bound Cu(II) can undergo Cu(II)/Cu(I) redox cycling in a reducing environment, and also O$_2$ reduced to O$_2^{-}$, generating H$_2$O$_2$. Also, the DNA–Cu(I) complex reacts with H$_2$O$_2$, inducing DNA damage through a Fenton-type reaction (45–47). Therefore, the copper-dependent DNA damage by OPP metabolites is of interest in connection with these observations.

Many studies have shown cytotoxicity and genotoxicity of OPP (1–8). Generation of reactive oxygen species from the redox cycle by OPP metabolites has been discussed in relation to OPP carcinogenicity. Although DNA–OPP metabolite adduct formation has been considered as one of the possible mechanisms of OPP carcinogenesis (9,10), a recent study (48) shows the lack of OPP–DNA adduct formation in bladder epithelium of rats exposed to OPP. This report may support the contribution of oxidative DNA damage to the expression of OPP carcinogenicity instead of DNA adduct formation. The present study suggests that OPP metabolites generate reactive oxygen species to induce cellular DNA damage, including 8-oxodG. It has been reported that 8-oxodG formation can cause DNA misreplication resulting in mutation (49,50), leading to carcinogenesis.

OPP is known to cause carcinomas in the urinary bladder and kidney of rats (1–7). Nakao et al. (51) estimated that non-conjugated forms of OPP and PHQ at a dose of 250–300 µM were excreted in the urine of rats receiving 2% OPP in their diet, with which the incidence of tumors was 90%. Interestingly, it is reported that high levels of prostaglandin H synthase are also localized in human bladder and kidney and have been proposed to play a role in the oxidation of PHQ to PBQ exhibiting toxicity to these organs (52). It is noteworthy to find that these OPP metabolites at a low concentration (20 µM) cause oxidative DNA damage in cells, which might lead to mutation and carcinogenesis. In addition, we showed that PBQ caused stronger damage to both cellular and isolated DNA than PHQ. Although there is some difficulty in extrapolating these findings to the whole animal, we concluded that PBQ might play a more important role in OPP carcinogenesis than other OPP metabolites.

References

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