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신피질 세포에서 Organic Hydroperoxide에 의해 유발된 세포사망

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Organic Hydroperoxide-Induced Cell Death in Renal Epithelial Cells

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Abstract

Background : Previous studies have shown that the hydrogen peroxide induces the cell injury through a lipid peroxidation-independent mechanism in renal epithelial cells. This study was therefore undertaken to examine the roles of lipid peroxidation in t-butylhydroperoxide (tBHP)-induced cell death to renal epithelial cells.
Methods : Opossum kidney (OK) cell cultures, a renal tubular epithelial cell line, were used as the renal epithelial cell model. tBHP caused cell death in a dose-dependent manner over the concentration range of 0.05-2 mM.
Results : The cell death was effectively prevented by the hydroxyl radical scavenger (dimethylthiourea), iron chelators (deferoxamine and phenanthroline), and antioxidants (DPPD and BHA), but not by the hydrogen peroxide scavenger catalase. tBHP caused a marked increase in lipid peroxidation, which was completely inhibited by DPPD and deferoxamine. tBHP-induced ATP depletion was prevented by deferoxamine and DPPD. Similar protective effects of antioxidants were observed in cell death induced by cumene hydroperoxide, another organic hydroperoxide. However, inorganic hydroperoxide (H2O2)-induced cell death was not altered by antioxidants. tBHP-induced apoptosis was prevented by DPPD and deferoxamine.
Conclusions : These results indicate that organic hydroperoxide induces cell death through a lipid peroxidation-dependent mechanism, unlike inorganic hydroperoxide, in renal epithelial cells.

Key words : Renal epithelial cell, Organic hydroperoxide, Apoptosis

Introduction

Oxidants have been implicated in the pathogenesis of a number of renal diseases including ischemia/reperfusion injury, aminonucleoside nephrosis, gentamicin nephrotoxicity, and acute nephrotoxic nephritis.[1-3] While the importance of oxidants in the pathogenesis of experimental renal injury has been established, the cellular mechanisms that result in the cell death remain uncertain.

Biological membranes contain a large amount of polyunsaturated fatty acids, which are particularly susceptible to peroxidative attacks by oxidants, resulting in lipid peroxidation.[4]

Therefore, lipid peroxidation has been used as an indirect marker of oxidant-induced cell injury.[5] However, role of lipid peroxidation in the pathogenesis of several studies have suggested that lipid peroxidation from the oxidant-induced cell injury has been shown in various cell types including renal epithelial cells.[5-7]

Recently, we found that an inorganic hydroperoxide H_2O_2 and an organic hydroperoxide *t*-butylhydroperoxide (*t*BHP) induce the cell injury through a different mechanism in renal epithelial cells.[8] The inhibition of inorganic phosphate uptake induced by H_2O_2 is not

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prevented by antioxidants, whereas the inhibition induced by *t*BHP is prevented by antioxidants. These data indicate that the H_2O_2-induced cell injury is associated without lipid peroxidation, while the *t*BHP-induced cell injury is induced by lipid peroxidation.

The present study was therefore carried out to examine the role of lipid preoxidation in cell death induced by *t*BHP in opossum kidney (OK) cells, an established renal proximal tubular cell line. To achieve inhibition of lipid peroxidation, potent phenolic antioxidants N,N'-diphenyl-p-phenylenediamine (DPPD) and butylated hydroxyanisole (BHA) were chosen on the basis of its predominant ability to prevent lipid peroxidation. These compounds have been extensively employed in *in vivo* and *in vitro* studies to specifically inhibit lipid peroxidation.[5, 9-11]

Materials and methods

Chemicals

t-butylhydroperoxide (*t*BHP), cumene hydroperoxide, deferoxamine, phenanthroline, trypsin, N,N'-diphenyl-p-phenylenediamine (DPPD) and butylated hydroxyanisole (BHA), Hoechst 33258, and dimethylthiourea (DMTU) were purchased from Sigma-Aldrich Chemical (St. Louis, MO). All other chemicals were of the highest commercial grade available.

Culture of OK cells

OK cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained by serial passages in 75-cm2 culture flasks (Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, Sigma Chemical Co.) containing 10% fetal bovine serum at 37℃ in 95% air/5% CO_2 incubator. When the cultures reached confluence, subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on 24-well tissue culture plates in DMM/F12 medium containing 10% fetal bovine serum. All experiments started 3-4 days after plating when a confluent monolayer culture was achieved.

Induction of oxidant injury

Cells were treated with *t*BHP in Hanks' balanced salt solution (HBSS) containing (mM) 115 NaCl, 5 KCl, 25 $NaHCO_3$, 2 NaH_2PO_4, 1 $MgSO_4$, 1 $CaCl_2$ and 5 glucose (pH 7.4) at 37℃ for time periods indicated. Following oxidant stress, cell death was measured as described below.

Measurement of cell viability in OK cells

The viability in cultured cells was determined by a trypan blue exclusion assay, since this method was a more sensitive indicator of cell death than LDH release in cultured cells, as demonstrated by Aleo.[12] Following oxidant stress, the cells were harvested using 0.025% trypsin, incubated with 4% trypan blue solution, and were counted using a hemocytometer under light microscopy. Cells failing to exclude the dye were considered nonvialble, and the number of nonviable cells was expressed as a percentage of the total cells.

Measurement of lipid peroxidation

Lipid peroxidation was estimated by measuring the content of malondialdehyde (MDA) in OK cells according to the method of Uchiyama and Mihara.[13] The cells were treated with *t*BHP and then homogenized in ice-cold1. 15% KCL (5% wt/vol). A 0.5 ml aliquat of homogenate was mixed with heated for 45 min on a boiling water bath. After addition of 4 ml of n-butanol the contents were vigorously vortexed and centrifuged at 2,000 g for 20 min. The absorbance of the upper, organic layer was measured at 535 and 520 nm with a diode array spectrophotometer (Hewlett Packard, 8452A), and compared with freshly prepared malondialdehyde tetraethylacetal standards.

Measurement of ATP content

ATP levels were measured on OK cells with a luciferin-luciferase assay. After an exposure to oxidant stress, the cells were solubilized with 500 μl of 0.5 % triton X-100 and acidified with 100 μl of 0.6 M perchloric acid and placed on ice. Cell suspension was then diluted with 10 mM potassium phosphate buffer containing 4 mM $MgSO_4$ (pH 7.4), and 100 μl of 20 mg/ml luciferin-luciferase was added to 10 μl of diluted sample. Light emission was

recorded at 20 sec with a luminometer (MicroLuat LB96P, Berthold, Germany). Protein content was determined on a portion of the cell sample.[14)]

Measurement of apoptotic cell death

Apoptosis was estimated by staining with the fluorescent dye. Cells were grown on 22-mm glass coverslips in 6-well plates. After treatment with reagents as indicated, the cells were washed twice with PBS and fixed cells were washed twice with PBS and stained with 10 μM Hoechst 33258 for 15 min at 37℃. Then cells were washed twice with PBS and examined by confocal microscopy (LSM510, ZEISS, Germany). Apoptotic cells were identified by condensation and fragmentation of nuclei. The number of apoptotic cells was assessed in five randomly selected fields.

Statistical analysis

Data are expressed as mean ± SEM. Comparison between two groups was made using the unpaired t test. Multiple group comparison was done using one-way analysis of variance followed by the Turkey post hoc test. $P<0.05$ were considered statistically significant.

Results

The concentration-dependent effect of *t*BHP on cell death as measured by trypan blue exclusion in OK cells was examined. *t*BHP caused cell death in a dose-dependent manner after exposure of cells to various concentrations of *t*BHP for 120 min (Fig. 1). Concentrations of *t*BHP higher than 0.5 mM did not produce more profound cell death. On the basis of these data, a concentration of 0.5 mM was used in the other studies except for experiments to induce apoptosis.

In order to examine the roles of hydrogen peroxide and hydroxyl radical in *t*BHP cytotoxicity, cells were exposed to *t*BHP in the presence of each scavenger. *t*BHP-induced cell death was not affected by a hydrogen peroxide scavenger catalase but prevented by a hydroxyl radicals scavenger DMTU (Fig. 2A). these results indicate that the formation of hydroxyl radicals is involved in *t*BHP cytotoxicity. The failure of catalase to prevent the cell death suggest that *t*BHP is not a substrate for catalase. Since iron can promote hydroxyl radical production from hydroperoxides via the Fenton/Haber-Weiss reactions, effects of iron chelators were examined. Indeed, the iron chelators deferoxamine and phenanthroline produced a significant protection against *t*BHP-induced cell death (Fig. 2B)

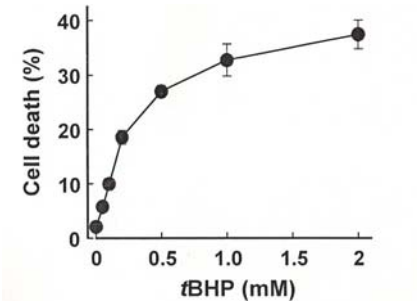

Fig. 1. Concentration-dependent effect of t-butylhydroperoxide (tBHP) in opossum kidney (OK) cells. Cells were treated with various concentrations of tBHP for 120 min at 37℃. Cell death was estimated by a trypan blue exclusion assay. Data are mean ± SEM of five experiments.

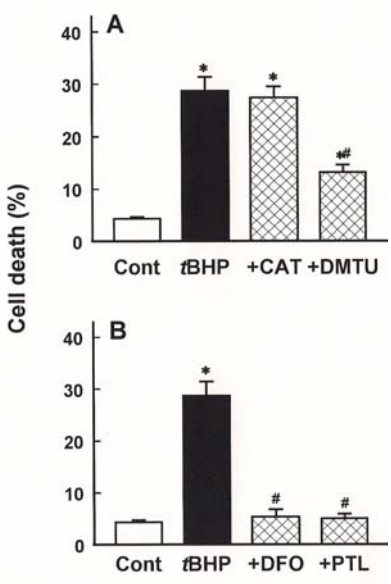

Fig. 2. Effects of radical scavengers (A) and iron chelators (B) on the cell death induced by t-butylhydroperoxide (tBHP) in opossum kidney (OK) cells. Cells were treated with 0.5 mM tBHP for 120 min at 37℃ in the presence or absence of 500 Units/ml catalase (CAT), 30 mM demethylthiourea (DMTU), 2 mM deferoxamine (DFO) or 1 mM phenanthroline (PTL). Cell death was estimated by a traypan blue exclusion assay. Data are mean ± SEM of five experiments.
*$p<0.05$ compared with control (Cont); # $p<0.05$ compared tBHP alone.

The hydroxyl radical has been known to be the most potent oxidant encountered in biological systems, and *t*BHP cytotoxicity maybe attributed to the ability of hydroxyl radical to initiate lipid peroxidation. Therefore, the role of lipid peroxidation in *t*BHP-induced cell death was evaluated. *t*BHP increased lipid peroxidation which was inhibited by the antioxidant DPPD and iron chelator deferoxamine (Fig. 3). To further confirm involvement of lipid peroxidation in *t*BHP cytotoxicity, effects of antioxidants were investigated. The results of Fig 4 indicate that the phenolic antioxidants DPPD and BHA effectively prevented *t*BHP-induced cell death (Fig. 4)

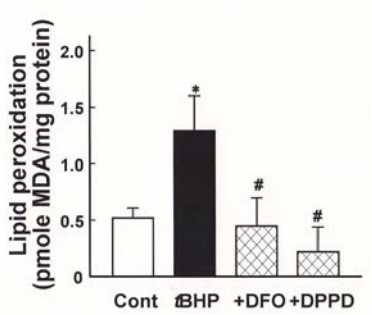

Fig. 3. Effects of antioxidants on lipid peroxidation induced by t-butylhydroperoxide (tBHP) in opossum kidney (OK) cells. Cells were treated with 0.5 mM tBHP for 120 min at 37℃ in the presence or absence of 2 mM deferoxamine (DFO) or 0.02 mM N,N'-diphenyl-p-phenylenediamine (DPPD). Data are mean ± SEM of five experiments.
*p<0.05 compared with control (Cont); # p<0.05 compared tBHP alone.

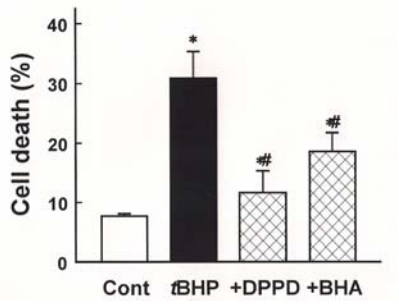

Fig. 4. Effects of phenolic antioxidants on the cell death induced by t-butylhydroperoxide (tBHP) in opossum kidney (OK) cells. Cells were treated with 0.5 mM tBHP for 120 min at 37℃ in the presence or absence of 0.02 mM N,N'-diphenyl-p-phenylenediamine (DPPD) or 0.05 mM butylated hydroxyanisole (BHA). Cell death was estimated by a traypan blue exclusion assay. Data are mean ± SEM of five experiments.
*p<0.05 compared with control (Cont); # p<0.05 compared tBHP alone.

ATP depletion has been demonstrated to be early response of cells to oxidative stress,[15,16] which has been proposed to be involved in the pathogenesis of oxidant-induced cell death.[17] Consistent with these data, *t*BHP induced ATP depletion and its effect was prevented by deferoxamine and DPPD (Fig. 5).

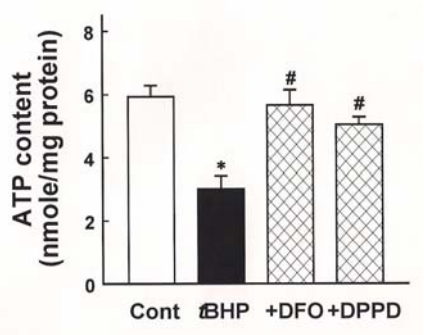

Fig. 5. Effects of antioxidants on ATP depletion induced by t-butylhydroperoxide (tBHP) in opossum kidney (OK) cells. Cells were treated with 0.5 mM tBHP for 120 min at 37℃ in the presence or absence of 2 mM deforoxamine (DFO) or 0.02 mM N,N'-diphenyl-p-phenylenediamine (DPPD). Data are mean ± SEM of five experiments.
*p<0.05 compared with control (Cont); # p<0.05 compared tBHP alone.

Previous studies have shown that H_2O_2-induced cell injury is not affect by antioxidants.[8] To further confirm whether role of lipid peroxidation in the cel death is different between organic hydroperoxide and inorganic hydroperoxide, effects of antioxidants on cell death induced by cumene hydroperoxide and H_2O_2 were examined. Cumene hydroperoxide-induced cell death was prevented by DPPD and BHA. By contrast, H_2O_2-induced cell death was not altered by antioxidants.

In the last series of experiments, role of lipid peroxidation in *t*BHP-induced apoptosis was examined. Apoptosis was induced by exposure of cells to 0.2 mM *t*BHP for 120 min following by the recovery for 18 hr in anormal culture medium. Cells with fragmented chromatin by Hoechst 33258 staining were considered as apoptosis. The nuclei of cells treated with *t*BHP for 2 hr were similar to those of control cells and no apparent apoptotic cell death was observed. On the other hand, when cells were followed by the recovery for 18 hr understandard conditions, they underwent apoptosis, exhibiting

characteristic nuclear fragmentation. Apoptotic cell death induced by *t*BHP was prevented by DPPD and deferoxamine (Fig. 7).

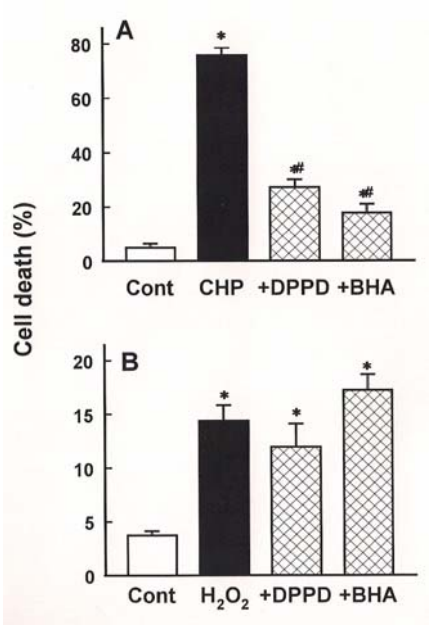

Fig. 6. Effects of phenolic antioxidants on the cell death induced by cumen hydroperoxide (CHP, A) and H2O2 (B) in opossum kidney (OK) cells. Cells were treated with 0.3 mM CHP or 0.5 mM H2O2 for 120 min at 37℃ in the presence or absence of 0.02 mM N,N'-diphenyl-p-phenylenediamine (DPPD) or 0.05 mM butylated hydroxyanisole (BHA). Cell death was estimated by a traypan blue exclusion assay. Data are mean ± SEM of five experiments. * $p<0.05$ compared with respective control (Cont); # $p<0.05$ compared oxidant alone.

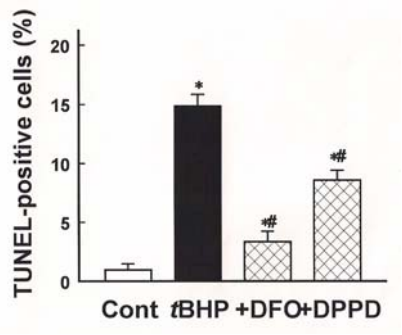

Fig. 7. Quantitation of apoptotic nuclei in opossum kidney (OK) cells treated with tBHP. Cells were treated with 0.2 mM t-butylhydroperoxide (tBHP) for 120 min in the presence or absence of 0.02 mM N,N'-diphenyl-p-phenylenediamine (DPPD) or 2 mM deferoxamine followed by the recovery for 18 hr in normal culture media. Data are mean ± SEM of 5 randomly selected fields in three experiments. * $p<0.05$ compared with control (Cont); # $p<0.05$ compared tBHP alone.

Discussion

Organic hydroperoxides can be converted into more potent radicals by metals such as Fe^{2+}. The alkoxyl radicals (R-O・) formed react with unsaturated fatty acids, thereby initiating lipid peroxidation.[18] In the present study, the importance of alkoxyl radical in the cytotoxicity of *t*BHP was implicated by the fact that the scavenger DMTU prevented *t*BHP-induced cell death (Fig. 2A). thus, it is likely that the alkoxyl radical generated by the Fenton/Haber-Weiss reactions, rather than peroxides themselves, are responsible for the cell death induced by H_2O_2 and *t*BHP.

Requirement of iron in *t*BHP-induced cell death was demonstrated by utilizing deferoxamine and phenanthroline, iron chelators. These compounds prevented cell death, ATP depletion, and lipid peroxidation induced by *t*BHP.

Similar observations have been made in renal cortical slices[11] and isolated proximal tubules.[19] In hepatocytes, however, deferoxamine did not prevent the *t*BHP-induced cell death, although it prevented the H_2O_2- and cumene hydroperoxide-induced cell death,[20] suggesting cell specificity in the *t*BHP cytotoxicity.

Although oxidants may attack a variety of cellular targets, the cell membrane would be of special interest because of its large surface area and the susceptibility of membrane unsaturated fatty acids to oxidant attack. Thus, lipid peroxidation has been considered to be an evidence for the oxidant-induced cell injury. However, the role of lipid peroxidation in the pathogenesis of *t*BHP-induced lethal cell injury has been controversial. It has been reported that lipid peroxidation plays a critical role in *t*BHP-induced renal cell death.[19] Rush[21] reported that lipid peroxidation did not play a role in the acute toxicity of *t*BHP to suspension of isolated rat hepatocytes. In the present study, *t*BHP caused a marked increase in lipid peroxidation, which was completely inhibited by DPPD and deferoxamine (Fig. 3). Although *t*BHP increases lipid peroxidation, this increase can be a result of the cell death or an epiphenonmenon of the action of hazard in question. If lipid peroxidation simply follows the cell death, or is an epiphenonmenon that accompanies the primary action of *t*BHP, then antioxidants

would inhibit the lipid peroxidation without any effect on the cell viability. However, the results of the present study showed that antioxidants prevent lipid peroxidation as well as cell death induced by *t*BHP (Figs. 3 and 4), a result consistent with our previous study in renal cortical slices.[11] Thus, these data suggest that lipid peroxidation may play a critical role in the action of *t*BHP.

Oxidants have been reported to cause ATP depletion by inhibition of the glycolytic and mitochondrial pathways of ADP phosphorylation.[22] However, Importance of ATP depletion in oxidant-induced cell death remains unclear. Several investigators have proposed a close relationship between oxidant-induced ATP depletion and cell viability.[17,22] On the other hand, Andreoli and Mallett[16] reported that antioxidants inhibit lipid peroxidation and cell death induced by H_2O_2 with no effect on ATP depletion or DNA damage in LLC-PK1 cells. However, the presents study showed that ATP depletion is prevented by antioxidants (Fig. 5), supporting the hypothesis that oxidant-induced cell death is associated with ATP depletion.

The present study showed that cell death induced by cumene hydroeproxide, another organic hydroperoxide, was prevented by antioxidants (Fig 6A). However, H_2O_2-induced cell death was not affected by antioxidants (Fig 6B), consistent with the results reported by Min[8] in cultured renal epithelial cells. This may be attributed to the difference in the underlying mechanism of cell injury between organic hydroperoxide and inorganic hydroperoxide. Although the precise mechanism of this discrepancy remains to be defined, a number of studies have shown the remarkable differences between the effects of H_2O_2 and those of *t*BHP in various cell types.[7,20,23,24] The data obtained here with cultured cells are at variance with those observed in renal cortical slices[11] in which both oxidants *t*BHP and H_2O_2 induce cell death through a lipid peroxidation-dependent mechanism.

Cell death is classified as necrosis or apoptosis on the basis of established morphological criteria.[25-27] Necrosis is considered a passive event in which the cell is irreversibly damaged by an environmental insult, leading to cel death. In contrast, apoptosis is an active process in which the cell itself initiates the molecular machinery to trigger cell death in response to either a physiological stimulus or an environmental stress. It has been known that oxidants induce the two modes of cell death, necrosis and apoptosis.[28] Since necrosis and apoptosis have different processes, the role of lipid peroxidation in oxidant-induced cell death could be different between two modes of cell death. However, apoptosis induced by *t*BHP was also prevented by antioxidants (Fig. 7).

Many studies have employed H_2O_2 and *t*BHP as model oxidants in studying using cultured cells. In the present study, organic hydroperoxide cytotoxicity was dependent on lipid peroxidation, unlike H_2O_2 cytotoxicity. Interestingly, H_2O_2-induced cell death was markedly different between cultured cells and renal cortical slices. cell culure is a powerful technique for studying physiological, biochemical, and toxicological processes in vitro. The validity of a cultured model relies on the ability of the cultured cells to accurately reflect the functional properties of the in vivo tissue. Based on the data of the present study and previous studies,[11] the response of cultured cells to H_2O_2 may not reflect the response of cells in the in vivo situation. The underlying mechanisms of *t*BHP in cultured renal epithelial cells are rather similar to those in the in vivo renal epithelial cells.

Conclusion

The *t*BHP cytotoxicity is dependent on iron-mediated free radical formation. Cell death and ATP depletion induced by *t*BHP were prevented by antioxidants. The cell death induced by cumene hydroperoxide but not by H_2O_2 was prevented by antioxidants. These results indicate that organic hydroperoxide causes cell death through a lipid peroxidation-dependent mechanism, which is distinct from that by H_2O_2.

국문초록

배경:신장 세뇨관 상피세포에서 inorganic hydroperoxide인 H_2O_2가 lipid peroxidation-independent mechanism을

통해 세포 상해를 유도한다는 이전 연구 결과가 있었고, 본 연구에서는 신장 세뇨관 상피세포에 organic hydroperoxide인 t-butylhydroperoxide (*t*BHP)에 의해 유도된 세포사망에 있어 lipid peroxidation이 어떤 역할을 하는지 조사하였다.

방법 : 신피질 세포로는 Opossum Kidney (OK) cell이 이용되었다. *t*BHP는 0.05-2mM의 범위에서 농도 의존적으로 세포사망을 유도했다. 이러한 세포사망이 hydroxyl기 제거제인 dimethylthiourea, iron chelator인 deferoxamine과 phenanthroline, 항산화제인 DPPD와 BHA, 그리고 H_2O_2 제거제인 catalase에 의해 어떠한 차이를 나타내는 지를 관찰하였다.

결과 : 세포사망은 dimethylthiourea, deferoxamine과 phenanthroline, DPPD와 BHA에 의해 효과적으로 방지되었으나, catalase에 의해서는 방지되지 않았다. *t*BHP는 lipid peroxidation을 확연하게 증가시켰으며 이러한 효과는 DPPD와 deferoxamine에 의해 완전히 억제되었다. 또 *t*BHP에 의해 유도된 ATP 소모도 deferoxamine과 DPPD에 의해 방지되었다. 항산화제의 이와 같은 방지효과가 다른 organic hydroperoxide인 cumene hydroperoxide에 의해 유도된 세포사망에서도 나타났으나, H_2O_2에 의해 유도된 세포사망은 영향을 받지 않았다. *t*BHP가 apoptosis를 유도하였으며, 이는 DPPD와 deferoxamine에 의해 방지되었다.

결론 : 이상의 결과는 신장 세뇨관 상피세포에서 organic hydroperoxide가 inorganic hydroperoxide와 달리 lipid peroxidation-dependent mechanism을 통해 세포사망을 유발함을 의미한다.

중심단어 : Renal epithelial cell, Organic hydroperoxide, Apoptosis

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