EFFECT OF VARIOUS DIURETICS ON LIPID PEROXIDATION IN RAT RENAL CORTICAL MITOCHONDRIA AND IN THE SUPERNATANT

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Abstract—Effect of various diuretics on lipid peroxidation in rat kidney cortical mitochondria and in the supernatant (11,000xg) was studied. Mersalyl (mercurial diuretic) markedly stimulated the lipid peroxidation in mitochondria and in the supernatant at a concentration of 1 mM, and metolazone (sulfonamide derivative, 1 mM) stimulated only that in the supernatant. Mersalyl-induced lipid peroxidation was markedly inhibited by several classical radical scavengers such as sodium diethyldithiocarbamate, 2,6-di-tert-butylphenol and N,N'-diphenyl-p-phenylenediamine, but metolazone-induced lipid peroxidation was not inhibited by these scavengers. The addition of superoxide dismutase to the reaction mixture inhibited the mersalyl-induced lipid peroxidation, but did not inhibit the peroxidation by metolazone or ascorbate. These results suggest that lipid peroxidation by mersalyl is mediated by free radicals or superoxide anion, which were probably produced by mersalyl, and the mercurial diuretics-induced nephrotoxicity may be due to the lipid peroxidation in the kidney.

It has been shown by the results of several lines of research that the formation of lipid peroxides in biological materials can be induced by enzymic (1) or non-enzymic (2) peroxidation in the presence of oxygen. The former is NADPH-induced lipid peroxidation in microsomes, and the latter is accelerated by various free radical initiators such as ascorbic acid, ferrous ion and hemoprotein. On the other hand, it has been reported that chemicals such as phenobarbital (3, 4), carbon tetrachloride (5), and other halogenoalkanes (6, 7) can induce lipid peroxidation in the liver. Biomembranes and subcellular organelles are major sites of damage by lipid peroxidation, because mitochondrial and microsomal membranes contain relatively large amounts of polyunsaturated fatty acids in their phospholipids. If such a reaction takes place in living cells of the kidney by various diuretics, peroxidation would initiate further damage to the structural and functional parts of the cell. Among the various diuretics, there is evidence of renal lesions induced by mercurial diuretics (8), but whether or not other diuretics cause renal disturbances is unknown.

The purpose of the present study was to determine the possible involvement of lipid peroxidation in nephrotoxicity of diuretics and to investigate the possible mechanism by which such lipid peroxidation would occur.

MATERIALS AND METHODS

Male Wistar rats (200–300 g) were...
decapitated, and both kidneys were immediately removed, cooled, and decapsulated in ice-cold saline. The cortex was homogenized in a freshly prepared 0.25M sucrose solution to make a 10% (w/v) homogenate. Mitochondria were prepared according to the method of Hogeboom (9), and the pellet was washed twice with 0.15M KCl-20 mM Tris-HCl buffer (pH 7.4). Finally, the mitochondrial suspension was prepared by gentle homogenization of mitochondrial pellet with the buffer.

Mitochondria and the resultant supernatant (11,000×g) suspended in 5 ml of 0.15 M KCl-20 mM Tris-HCl buffer (pH 7.4) were incubated with various concentrations of drugs at 37°C for 10 min. The reaction was stopped by the addition of 1.5 ml of 40% trichloroacetic acid. Content of lipid peroxides was estimated by the method of Tappel and Zalkin (10) and expressed as thiobarbituric acid (TBA) value (absorbance at 532 nm/mg protein). Protein was determined according to the method of Lowry et al. with bovine serum albumin as standard (11). Metolazine (Zaroxolyn) was dissolved in 99.5% ethanol and added to the reaction mixture before initiation. Final concentration of ethanol in the reaction mixture was less than 6%, which did not affect the lipid peroxidation significantly. Superoxide dismutase (from bovine blood, 2,500 units/mg protein) was purchased from Sigma Chemical Company. Heat inactivation of the enzyme was carried out by boiling the dilute working reagent for 5 min (12). The values presented in this paper are the mean±standard error. Statistical significance was calculated by using student’s paired t-test.

**RESULTS**

Table 1 shows the effect of various diuretics on lipid peroxidation in rat kidney cortical mitochondria and in the supernatant. Ascorbic acid stimulates a non-enzymic lipid peroxidation process in vitro (13, 14). Therefore, we used ascorbic acid at a concentration of 0.1 mM as a reference. When ascorbic acid was added to the incubation mixture, TBA

| Drug                | Mitochondria | Supernatant |
|---------------------|--------------|-------------|
| No addition         | 1.20±0.04    | 2.41±0.06   |
| Ascorbate           | 5.40±0.62*   | 3.11±0.11*  |
| Aminophylline       | 1.03±0.08    | 2.49±0.10   |
| Diamox              | 1.11±0.05    | 2.33±0.10   |
| Bumetanide          | 1.05±0.04    | 2.33±0.16   |
| Mersalyl            | 4.44±0.10*   | 3.72±0.08** |
| Furosemide          | 1.68±0.10    | 2.45±0.08   |
| Ethanol (6%)        | 1.69±0.15    | 2.96±0.12   |
| Metolazine          | 2.12±0.26    | 6.63±0.30*  |
| Hydrochlorothiazide | 1.61±0.22    | 2.66±0.07   |
| Digitoxin           | 1.63±0.06    | 3.67±0.11   |

Mitochondria (8.5 mg protein) or supernatant (9.2 mg protein) were incubated with various concentrations of drugs in a final volume of 5 ml. All values are the mean±S.E. of thiobarbituric acid value (absorbance at 532 nm/mg protein. ×10⁻², n=12). Drug concentration: ascorbate 0.1 mM, others 1 mM. Other conditions are as described in the text. *Compared to the control, significant at P<0.001. **Compared to the control, significant at P<0.01.
values of mitochondria and of the supernatant increased about 4.5 and 1.3-fold, respectively, compared with the control.

Although the various diuretics tested produced no change in the formation of lipid peroxides at the same concentration as ascorbic acid (data not shown), among the compounds tested, mersalyl stimulated the peroxidation in mitochondria and in the supernatant at a concentration of 1 mM. Metolazone (1 mM) promoted the lipid peroxidation only in the supernatant markedly (about 2.7-fold), but it was not capable of stimulating the peroxidation in mitochondria at the concentration used. Metolazone seems to be particularly sensitive to the supernatant rather than to mitochondria. Other diuretics (except mersalyl and metolazone) had no effect on the lipid peroxidation in mitochondria and in the supernatant even at concentrations higher than 1 mM.

Participation of free radicals on the mersalyl or metolazone-induced lipid peroxidation in mitochondria and in the supernatant was examined by using several classical radical scavengers. As shown in Tables 2 and 3 mersalyl-induced lipid peroxidation in mitochondria and in the supernatant was markedly inhibited by scavengers such as sodium diethylthiocarbamate, 2,6-di-tert-butylphenol and N,N'-diphenyl-p-phenyl-enediamine, but was not significantly affected by the addition of 1,2-dimethoxyethane as a scavenger of hydroxyl radicals, or 2,5-dimethylfuran as a scavenger of singlet oxygen (15). The inhibitory effect of scavengers was dependent on their concentrations. However, metolazone-induced lipid peroxidation in the supernatant was not affected by various scavengers at the concentrations used or more (data not shown). These results suggest the possibility of

Table 2. Effect of various scavengers on lipid peroxidation of rat renal cortical mitochondria

| Scavenger                      | Concentration (M) | Mersalyl (1.0 mM) | Ascorbate (0.1 mM) |
|-------------------------------|-------------------|-------------------|-------------------|
| No addition                   | —                 | 2.24±0.18         | 3.46±0.27         |
| Sodium diethyl-dithiocarbamate| 10\(^{-3}\)       | 0.22±0.04\(^*\)  | 0.86±0.02\(**\)  |
|                               | 10\(^{-4}\)       | 0.90±0.02\(**\)  | 0.95±0.02\(**\)  |
|                               | 10\(^{-6}\)       | 1.32±0.09         | 0.90±0.02\(**\)  |
| 2,5-dimethylfuran             | 10\(^{-4}\)       | 2.00±0.02         | 2.28±0.05         |
|                               | 10\(^{-5}\)       | 2.42±0.03         | 2.38±0.02         |
|                               | 10\(^{-6}\)       | 2.46±0.03         | 2.69±0.01         |
| 2,6-di-tert-butylphenol       | 10\(^{-5}\)       | 0.32±0.01\(**\)  | 1.28±0.03\(**\)  |
|                               | 10\(^{-6}\)       | 0.58±0.01         | 1.59±0.02         |
|                               | 10\(^{-7}\)       | 1.02±0.01         | 1.65±0.05         |
| N,N'-diphenyl-p-phenylenediamine| 10\(^{-7}\)   | 0.32±0.03\(**\)  | 0.41±0.01\(^*\)  |
|                               | 10\(^{-8}\)       | 0.51±0.01\(**\)  | 0.61±0.01\(**\)  |
|                               | 10\(^{-9}\)       | 1.29±0.04         | 2.41±0.04         |
| 1,2-dimethoxyethane           | 10\(^{-2}\)       | 1.95±0.02         | 3.32±0.05         |
|                               | 10\(^{-3}\)       | 1.92±0.06         | 3.60±0.05         |
|                               | 10\(^{-4}\)       | 2.06±0.05         | 3.93±0.04         |

Mitochondria (4.3 mg protein) were incubated with various concentrations of scavengers in a final volume of 5 ml. All values are the mean±S.E. of thiobarbituric acid value (×10\(^{-3}\), n=4). For details, see the experimental section. *Compared to the control, significant at P<0.001. **Compared to the control, significant at P<0.01.
contribution of free radicals to the lipid peroxidation by mersalyl, and a lack of participation of free radicals in the formation of lipid peroxides by metolazone.

Superoxide anion is generated in the xanthine-xanthine oxidase system (16) and by NADPH-cytochrome P-450 reductase (17). The superoxide anion is an important intermediate in the formation of singlet oxygen and hydroxy radicals both of which are capable of initiating lipid peroxidation, and it is scavenged catalytically by superoxide

### Table 3. Effect of various scavengers on lipid peroxidation of rat renal cortical supernatant

| Scavenger                        | Concentration (M) | Mersalyl (1.0 mM) | Metolazone (1.0 mM) |
|----------------------------------|-------------------|-------------------|---------------------|
| No addition                      | ---               | 4.38±0.11         | 11.71±0.36          |
| Sodium diethylthiocarbamate      | 10⁻²              | 2.14±0.05*        | 11.58±0.11          |
|                                  | 10⁻⁴              | 3.52±0.01**       | 12.07±0.12          |
|                                  | 10⁻⁶              | 3.84±0.03         | 11.86±1.05          |
| 2,5-dimethylfuran                | 10⁻⁴              | 3.67±0.03         | 9.12±0.03           |
|                                  | 10⁻⁶              | 3.74±0.20         | 10.03±0.14          |
|                                  | 10⁻⁸              | 3.60±0.04         | 10.94±0.24          |
| 2,6-di-tert-butylphenol          | 10⁻⁶              | 1.92±0.01*        | 9.66±0.02           |
|                                  | 10⁻⁸              | 2.52±0.03*        | 9.77±0.11           |
|                                  | 10⁻⁷              | 2.88±0.03*        | 9.79±0.08           |
| N,N’-diphenyl-p-phenylene diamine| 10⁻⁷              | 1.82±0.01*        | 9.25±0.61           |
|                                  | 10⁻⁸              | 2.51±0.31*        | 10.61±0.01          |
|                                  | 10⁻⁹              | 2.50±0.01*        | 10.36±0.01          |
| 1,2-dimethoxyethane             | 10⁻²              | 4.01±0.14         | 11.13±0.32          |
|                                  | 10⁻³              | 4.15±0.07         | 10.75±0.07          |
|                                  | 10⁻⁴              | 4.30±0.13         | 10.94±0.07          |

Supernatant (4.8 mg protein) was incubated with various concentrations of scavengers in a final volume of 5 mL. All values are the mean±S.E. of thiobarbituric acid value (×10⁻², n=4). For details, see the experimental section. *Compared to the control, significant at P<0.001. **Compared to the control, significant at P<0.01.

### Table 4. Effect of superoxide dismutase on lipid peroxidation of mitochondria and the supernatant

| Superoxide dismutase Concentration (µg/ml) | Mitochondria | Supernatant |
|------------------------------------------|--------------|-------------|
|                                          | Mersalyl (1.0 mM) | Ascorbate (0.1 mM) | Mersalyl (1.0 mM) | Metolazone (1.0 mM) |
| 0                                        | 2.25±0.09     | 2.15±0.08   | 6.11±0.14     | 7.61±0.22     |
| 3.14                                      | 2.37±0.30     | 2.10±0.08   | 5.49±0.21     | 7.56±0.19     |
| 31.4                                      | 1.93±0.43     | 1.92±0.01   | 4.07±0.02     | 7.39±0.18     |
| 314.0                                     | 1.14±0.01**   | 1.81±0.01   | 2.86±0.40*    | 8.02±1.30     |

Mitochondria (4.5 mg protein) or the supernatant (8.2 mg protein) were incubated in 0.15 M KCl-0.02 M Tris HCl buffer containing superoxide dismutase at 37°C for 10 min. For other details, see the experimental section. All values are the mean±S.E. of thiobarbituric acid value (×10⁻², n=3). *Compared to the control, significant at P<0.001. **Compared to the control, significant at P<0.01.
Therefore, the effect of superoxide dismutase on diuretics-induced lipid peroxidation in rat renal cortical mitochondria and in the supernatant was examined (Table 4). The addition of superoxide dismutase at a concentration of 314 μg/ml to the reaction mixture inhibited the lipid peroxidation by mersalyl. The inhibition was about 49% in the mitochondria and about 53% in the supernatant. Heat inactivation of the superoxide dismutase almost completely abolished its inhibitory activity.

**DISCUSSION**

As shown in the present experiments, since mersalyl-induced lipid peroxidation was inhibited by diethyldithiocarbamate (Tables 2 and 3), it is considered that free radicals other than superoxide anion might participate in the initiation of the peroxidation. The peroxidation by metolazone in the supernatant was not inhibited by superoxide dismutase at the concentrations used, or even at higher concentrations (data not shown). This result indicates a lack of contribution of superoxide to the enhanced lipid peroxidation in the supernatant.

It appears from the above finding that the lipid peroxidation by metolazone in the supernatant is not induced by free radicals or superoxide. Metolazone-induced lipid peroxidation may require cytochrome P-450 as a peroxidase (18, 19), and P-450 may be involved in the peroxidation. However, the nature of this induction is not clear and no data are available to clarify this point.

Ascorbic acid catalyzed peroxidation in the presence of metal ions is thought to involve the monodehydroascorbate radical (20). Superoxide dismutase had little inhibitory effect on the peroxidation catalyzed by ascorbate, confirming that the superoxide radical was not involved. However, as the peroxidation was inhibited by several classical radical scavengers (except 2,5-dimethylfuran and 1,2-dimethoxyethane), ascorbic acid catalyzed peroxidation might be explained by the involvement of the monodehydroascorbate radical.

Slater and Sawyer reported that p-chloromercuribenzoate (94 μM) increased the endogenous malonaldehyde production in the presence of NADPH as well as the stimulation of malonaldehyde production due to carbon tetrachloride in rat liver microsomes (21). Recently, it has been shown that the toxicity of the alkylmetals is caused not only by the intact molecule, but also by free radicals formed by homolytic fission of the carbon-metal bond, and that methyl mercury toxicity is due to free radical formation and prevented by Selenium or Vitamin E (22). Among diuretics employed in the present study, mersalyl markedly promoted lipid peroxidation in mitochondria and in the supernatant of rat renal cortex. This finding suggests that the peroxidation is mediated by free radicals, or superoxide anion, which probably produced by C-Hg bond splitting in mersalyl. Thus, mersalyl may promote tissue oxidative damage to some extent, by inhibiting the glutathione peroxidase-linked defensive system (23) and possibly by serving as an initiator of radical processes. Therefore, it is considered that one possible cause for mercurial diuretics-induced nephrotoxicity may be due to the lipid peroxidation in the kidney.

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