Effects of Dietary Lipid Peroxidation Products on Hormonal Responses in Primary Cultured Hepatocytes of Rats

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Dietary lipid peroxidation products cause endogenous lipid peroxidation with hepatic dysfunction. In this study, we isolated and cultured hepatocytes of rats that were given secondary autodestruction products of linoleic acid (p.o., 400 mg/rat/day for 3 days), and examined the hormonal responses of these hepatocytes. An increase in thiobarbituric acid reactive substances and a depletion of vitamin E persisted in hepatocytes from treated rats for at least 24 h in culture as compared to those from control rats. As markers for hepatic dysfunction, the activities of six enzymes were measured. In each case, there was an initial decrease in the enzyme activity in hepatocytes from the treated rats, and all activities were restored by 48 h in culture. Then, we measured the hormonal responses of these hepatocytes. The responses to insulin or glucagon in hepatocytes from secondary products-treated and control rats were the same. In contrast, the response to dexamethasone was significantly lowered in hepatocytes from secondary products-treated rats as measured by the induction of tryptophan 2,3-dioxygenase and tyrosine aminotransferase. We conclude that primary cultured hepatocytes from the rats treated in vivo with dietary lipid peroxidation products retained symptoms of oxidative stress and had a low response to glucocorticoids.

Key words: lipid peroxidation; glucocorticoid; primary cultured hepatocytes; rats

Polysaturated fatty acids in food products are susceptible to oxidation during the storage, cooking, and frying of foods to form a variety of decomposed products including aldehydes. Some aldehydes, e.g., 4-hydroxyalkenals and related α,β-unsaturated aldehydes, are very reactive with biological molecules such as lipids, proteins, and DNA (reviewed in ref. 1). Recently, the interaction of 4-hydroxy-2-nonenal and protein has been reported in detail.2,3) Therefore, highly reactive aldehydes in secondary autoxidation products of polysaturated fatty acids in food products will impair biological functions. From a nutritional viewpoint, it is worth knowing the effects of secondary autoxidation products on living animals even though these products are a mixture of various compounds. Numerous data have been accumulated on the toxicity and the toxic mechanism of peroxidation products in vivo.4,5,6,7) We have also been studying the hepatic dysfunction caused by secondary products of linoleic acid in the diet.

Secondary products have been shown to cause endogenous lipid peroxidation with subsequent hepatic dysfunction. When secondary products were administered orally to rats, their low-molecular weight products were incorporated into the body, accumulated in the liver, and induced endogenous lipid peroxidation.8) We screened for the compound stimulating endogenous lipid peroxidation in vivo and found it to be 9-oxononoic acid, a major component of the secondary products.9) With regard to hepatic dysfunction, we have almost explained it. The hepatic dysfunction could be ascribed to the reduction in the activities of glucokinase, glucose-6-phosphate dehydrogenase, phosphoglucomutase, aldehyde dehydrogenase (mitochondrial NAD-dependent), and succinate dehydrogenase, and a depletion of coenzyme A.10,11) Previous in vitro study demonstrated that some of these biological changes were caused by the direct attack of aldehydes in the decomposed products on protein molecules and sulfhydryl groups.12) From these results, dietary lipid peroxidation products will affect specific functions of liver such as ureogenesis, gluconeogenesis, and hormonal responses of liver-specific enzymes.

For studies on complex liver functions, primary cultured hepatocytes are useful for in vitro experimental systems.13) Attempts have been made to use isolated and cultured hepatocytes as an intact cell model to study oxidative stress. Almost all of these studies, however, have examined the effects of in vitro treatments of hepatocytes with prooxidants or radical initiators to induce oxidative stress.14,15) Our purpose in this study is to attempt to measure liver functions in primary cultured hepatocytes maintaining in vivo effects of secondary products. We isolated hepatocytes from rats dosed with secondary products and measured lipid peroxidation and enzyme activities as markers for hepatic dysfunction, using these hepatocytes in primary culture. We also investigated the hormonal responses of primary cultured hepatocytes from the secondary products-dosed or the control rats.

Materials and Methods

Materials. Linoleic acid was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). William’s medium E, Leibovitz’s L-15 medium, and newborn calf serum were obtained from Life Technologies Oriental, Inc. (Tokyo, Japan). Collagenase and dexamethasone were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Insulin and glucagon were purchased from Sigma Chemical Co. (St. Louis, MO). [U-14C]Glucose 1-phosphate was obtained from Du Pont (Boston, MA). All other chemicals were commercial products of the highest quality commercially available.

Abbreviations: TBA, thiobarbituric acid; TBARS, TBA reactive substances.

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Preparation of secondary autodioxidation products of linoleic acid. Secondary autodioxidation products of linoleic acid were prepared as described previously.20 Briefly, linoleic acid was oxidized in air at 37°C for 7d. The autooxidized linoleic acid was separated into linoleic acid, its mono-hydroperoxides, and secondary products by silica-gel column and thin-layer chromatography as described previously.20 Peroxides and carbonyl values in the secondary products fraction were about 1400 and 580 meq/kg, respectively. This fraction consisted of 49% polymers, 31% endoperoxides and epoxyhydroperoxides, and 20% low-molecular-weight compounds (molecular weight less than 300) measured by Sephadex LH-20 column (2.5 x 75 cm) chromatography.41 Components in the low-molecular-weight fraction of secondary products were analyzed by gas-chromatography-mass spectrometry as described previously.20 and the fraction consisted of 2.2% (as a percent of secondary products) 9-oxononanoic acid, 1.9% hydroxy alkyls (e.g., 4-hydroxyhexenal), 1.7% hexanal, 1.4% dialdehydes (e.g., malonaldehyde), 1.2% short-chain carboxylic acid, 1.2% 10-formyl-9-decenolic acid, 1.0% nonanedioic acid, 1.0% 8-oxoanctoic acid, 0.4% 2,4-decadienal, 0.3% 12-oxoodecadienoic acid, and other unidentified compounds.

Treatment of animals. Male Wistar rats (ST, SPF, 4 week old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were housed in stainless steel cages, with free access to water and freshly prepared diet as described previously.21 Animals were maintained in a climate-controlled room (temperature 22 ± 3°C, humidity 70%, and 12-h light cycle). Rats were acclimated for 1 week before use in experiments. Rats were orally dosed with 400 mg/kg/day of secondary autioxidation products for 3d. Control animals received the same volume of vehicle (saline solution) alone. Hepatocytes were isolated 64-65h after the first administration.

Isolation of hepatocytes and their culture. Hepatocytes were isolated from secondary-products-dosed and control rats separately by in situ perfusion of the liver by collagenase by the method of Tanaka et al.25 with at least 3 animals per treatment group. The liver was immersed through two layers of stainless mesh (150 µm) and the parenchymal cells were purified by centrifugation (3 times) at 50 x g for 1 min. Viability of isolated hepatocytes was measured immediately by the exclusion of 0.2% trypan blue after the preparation. The isolated cells were suspended in a mixture of 50% William's medium E and 50% Leibovitz's-S-15 medium, with 5% newborn calf serum, 5KU/liter of aprotinin, 10^-8 m insulin, and 10^-6 m dexamethasone. The cells were cultured at a density of 10^7 cells/0.2 ml/cm² on plastic dishes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), which were precoated with collagen type-I, at 37°C under 5% CO2 in air. The medium was replaced at 4, 24, and 48 h after seeding. Viability of hepatocytes during culture was measured by MTT test (21), which is based on the selective ability of mitochondrial dehydrogenases in living cells to reduce the yellow soluble salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to a purple insoluble formazan precipitate. Primary cultured hepatocytes were harvested at 0 (isolated cells), 4, 8, 12, 24, 48, and 72 h. The cells were washed, scraped off, and homogenized in phosphate-buffered saline. Cytosolic and mitochondrial fractions were prepared from the homogenate as described before.10 Protein contents in the cells, homogenate, and the subcellular fractions were measured by the method of Lowry et al.22

Measurement of oxidative stress. Lipid peroxidation in hepatocytes was measured by the thiobarbituric acid (TBA) test as described by Buege and Aust,23 and data were represented as TBA-reactive substances (TBARS). Tocopherol levels in hepatocytes were measured by the method of Taylor et al.24

Enzyme assay. As markers of the toxicity of secondary autodioxidation products,11-15 the following six enzyme activities were analyzed by published methods: glucokinase,25 glucose-6-phosphate dehydrogenase,26 phosphoglucomutase,26 and phosphoglucose-6-phosphate dehydrogenase42 were measured using the cytosolic fraction of hepatocytes. Sucinate dehydrogenase26 and NAD-dependent aldehyde dehydrogenase25 were measured using the mitochondrial fraction.

Effects of hormones on hepatocytes from secondary-products-dosed rats. The effects of insulin on cultured hepatocytes were estimated by the induction of glucokinase, glucose-6-phosphate dehydrogenase, and malic enzyme. After incubation for 4h, hepatocytes were washed with phosphate-buffered saline twice and were treated with 10^-7 m insulin containing medium or hormone-free medium (as a control) for another 20h. The activities of glucokinase,25 glucose-6-phosphate dehydrogenase,26 and malic enzyme26 were measured in the cytosolic fraction.

The effects of glucagon on hepatocytes were assessed by measurement of glycogen phosphorylase activity, ureogenesis, and gluconeogenesis. For glycogen phosphorylase, hepatocytes were cultured with 10^-7 m dexamethasone for 20h, and then were incubated with high-glucose (400 mg/ml) Eagle's minimal essential medium for 30 min. The medium was changed to the mixed culture medium (50% William's medium E and 50% Leibovitz's-S-15 medium) containing 10^-7 m glucagon. After incubation for 5 min, the medium was removed and the cells were frozen immediately by liquid nitrogen. The frozen cells were scraped off and homogenized with 10% glyceral containing 30 mm NaF and 10 mm EDTA. Glycogen phosphorylase activity was stabilized in 0.1M Tris-HCL, pH 7.5. The activity was measured using [U-14C]Glucose-1-phosphate as a substrate as described previously.25 In another experiment, isolated hepatocytes were cultured with hormone-free medium (but with 5% calf serum) for 24h and cultured cells were used for the measurement of ureogenesis and gluconeogenesis in the presence of or absence of 10^-5 m glucagon. For ureogenesis the cells were washed with Earle's buffer twice, and incubated with the same buffer solution containing 5 mm ammonium chloride as a substrate for 2h. Urea concentration in cell-free buffer was measured by the method of Marsh et al.25 For gluconeogenesis the cells were washed with glucose-free Earle's buffer twice and incubated with buffer containing 5 mm fructose or 5 mm pyruvate as a substrate for 30 min. The released glucose was measured with a glucose assay kit (Wako Pure Chemical Ind.).

Response to glucocorticoids of hepatocytes was analyzed by the induction of tryptophan 2,3-dioxigenase and tyrosine aminotransferase. Medium containing various concentrations of dexamethasone (10^-10, 10^-9, 10^-8, 10^-7) was added to 48-cultured hepatocytes and incubated for another 20h. Hepatocytes used for the assay of tryptophan 2,3-dioxigenase were cultured in the presence of 2.5 mm tryptophan, which stabilizes this enzyme.25 For assays of tryptophan 2,3-dioxigenase and tyrosine aminotransferase, the cells were washed with ice-cold phosphate-buffered saline, harvested, and homogenized with 20 mm potassium phosphate buffer (pH 7.0) containing 1 mm EDTA and 10 mm sodium fluoride. The homogenate was used for the assay of tryptophan 2,3-dioxigenase,25 the remainder was centrifuged at 40,000 x g for 20 min, and the resulting supernatant was used for assay of tyrosine aminotransferase.25

Statistical analysis. Data were analyzed using Student's t-test, and the 0.05 level of probability was used as a criterion of significance.

Results

Viability, attachment, yield, and survival of hepatocytes from secondary-products-dosed rats

The isolated hepatocytes from secondary-products-dosed and control rats were identical with respect to their yield (about 3 x 10^6 cells/rat), viability (over 85%), and attachment to plastic culture dishes (83-88%). We have previously observed leakage of aspartate aminotransferase and alanine aminotransferase into the serum of secondary products-dosed rats.81 It is also known that membranes of isolated hepatocytes are injured during the perfusion step. Thus, the leakage of aspartate aminotransferase was measured in both treated and control hepatocytes after 1 h of incubation in fresh medium. The leakage of the enzyme in isolated hepatocytes from treated rats was transiently increased (treated: 262 ± 29 vs. control: 143 ± 13 nmol/min/ml medium) but recovered to a normal level by 4 h in culture (treated: 25.2 ± 5.3 vs. control: 30.1 ± 6.3 nmol/min/ml medium).

To measure the survival of hepatocytes in culture, we measured protein contents in these cells (Fig. 1). No differences were noted in the cells from control and treated animals during 72 h in culture. DNA and RNA contents in hepatocytes also showed no difference during culture in this experiment (data not shown). Viability of cultured hepatocytes, which was assayed by the MTT method, also
Effects of Lipid Peroxidation Products on Hormonal Responses

Fig. 1. Changes in Protein Contents, Cell Viability, and Ureogenesis Activity in Primary Cultured Hepatocytes of Rats Treated with Secondary Products.

Secondary autooxidation products of linoleic acid (400 mg/kg/day for 3 d) were orally administered to rats. Control rats were given the same amount of saline solution. Hepatocytes were isolated by in situ collagenase perfusion method and were cultured in 30% William's medium E and 50% Leibovitz's 15 medium, supplemented with 5% newborn calf serum, 50 U/liter aprotinin, 10^{-8} M insulin and 10^{-7} M dexamethasone. Protein contents in hepatocytes, MTT test as viability of hepatocytes, and ureogenesis were measured as described under Materials and Methods. The data are presented as a percent of control with mean ± S.E. of three animals. Asterisks show significant difference from control hepatocytes at the corresponding time point (p < 0.05, Student's t-test).

Fig. 2. Changes in the Levels of Thiobarbituric Reactive Substances (TBARS) and Tocopherol in Primary Cultured Hepatocytes of Rats Treated with Secondary Products.

Hepatocytes were isolated and cultured as described in Fig. 1 and harvested at indicated time in culture. The level of TBARS and tocopherol in hepatocytes were measured as described under Materials and Methods. Each time point represents the mean ± S.E. of three animals. Asterisks show significant difference from control hepatocytes at the corresponding time point (p < 0.05, Student's t-test).

showed no difference. On the contrary, ureogenesis, a liver specific function, was significantly lowered in hepatocytes from secondary products-dosed rats between 4 through 12 h in culture. These data, therefore, indicated that hepatocytes isolated from secondary products-dosed rats were the same as the cells from control rats with respect to viability and survival in culture, but their ureogenesis was lowered by the in vivo treatment.

Oxidative stress in cultured hepatocytes from secondary products-dosed rats

Since we previously reported that orally administered secondary products induced oxidative stress in the liver of rats,11-14,20 we now investigated whether hepatocytes from secondary products-treated rats had oxidative stress in primary culture. Figure 2 shows the course of changes in the markers for oxidative stress in cultured hepatocytes. The levels of TBARS (Fig. 2A) increased and the contents of tocopherol (Fig. 2B) decreased in hepatocytes from secondary products-dosed rats at 0 (isolated cells) through 24 h in culture.

Next, we also assayed the activities of six marker enzymes for the toxicity of secondary products13,20 and data are presented in Fig. 3. The activity of glutokinase (Fig. 3A) was lower during the 12 h in culture. The activities of phosphoglucomutase (Fig. 3B), glucose-6-phosphate dehydrogenase (Fig. 3C), and phosphogluconate dehydrogenase (Fig. 3D) also decreased in the treated cells from 0 through 24 h in culture. Decreased activities of mitochondrial succinate dehydrogenase (Fig. 3E) and NAD-dependent aldehyde dehydrogenase (Fig. 3F) were observed in treated hepatocytes until 12 and 8 h in culture, respectively. Our data clearly show that endogenous oxidative stress following administration of secondary products remains in hepatocytes.

Effects of insulin, glucagon, and dexamethasone on oxidative-stressed hepatocytes

Hepatocytes from secondary products-dosed rats had oxidative stress (Figs. 2 and 3) and showed decreased

Fig. 3. Changes in the Activities of Marker Enzymes for Hepatic Dysfunction in Primary Cultured Hepatocytes of Rats Treated with Secondary Products.

Hepatocytes were isolated and cultured as described in Fig. 1 and harvested at indicated time in culture. The activities of glutokinase, phosphoglucomutase, glucose-6-phosphate dehydrogenase, and phosphogluconate dehydrogenase in the cytoplasmic fraction, and succinate dehydrogenase and NAD-dependent aldehyde dehydrogenase in the mitochondrial fraction were measured as described under Materials and Methods. Each time point represents the mean ± S.E. of three animals. Asterisks denote a significant difference from control hepatocytes at the corresponding time point (p < 0.05, Student's t-test).
Table I. Effects of Insulin on Hepatocytes from Secondary Product-dosed and Control Rats

<table>
<thead>
<tr>
<th>Treatment with</th>
<th>Administration of</th>
<th>Activity (nmol/min/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Secondary products</td>
<td></td>
</tr>
<tr>
<td>Glucokinase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>8.6 ± 0.4</td>
<td>7.9 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Insulin (100 nm)</td>
<td>12.5 ± 0.6</td>
<td>10.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Induction %</td>
<td>(145)</td>
<td>(137)</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>61.5 ± 2.0</td>
<td>37.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Insulin (100 nm)</td>
<td>73.5 ± 1.4</td>
<td>45.7 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Induction %</td>
<td>(120)</td>
<td>(121)</td>
<td></td>
</tr>
<tr>
<td>Malic enzyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>37.3 ± 1.1</td>
<td>35.0 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Insulin (100 nm)</td>
<td>44.6 ± 1.4</td>
<td>40.9 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Induction %</td>
<td>(120)</td>
<td>(117)</td>
<td></td>
</tr>
</tbody>
</table>

Secondary oxidation products of linoleic acid (400 mg/ rat/day for 3 d) were orally administered to rats. Control rats were given the same amount of saline solution. Hepatocytes were isolated by in situ perfusion method as described under Materials and Methods. After the incubation for 4 h, hepatocytes were treated with or without 100 nm insulin for 20 h. Effects of insulin were estimated by the increased activities of glucokinase, glucose-6-phosphate dehydrogenase, and malic enzyme as described under Materials and Methods.

Values are mean ± S.E. (n = 3).

* Significant difference from saline-dosed group (p < 0.05, Student's t-test).

Table II. Effects of Glucagon on Hepatocytes from Secondary Product-dosed and Control Rats

<table>
<thead>
<tr>
<th>Treatment with</th>
<th>Administration of</th>
<th>Activity (nmol/min/mg protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Secondary products</td>
<td></td>
</tr>
<tr>
<td>Glycogen phosphorolase (nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>52.2 ± 1.6</td>
<td>54.4 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>Glucagon (100 nm)</td>
<td>104.2 ± 5.3</td>
<td>114.8 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>Induction %</td>
<td>(200)</td>
<td>(211)</td>
<td></td>
</tr>
<tr>
<td>Ureogenesis (μg urea/hour/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>3.25 ± 0.30</td>
<td>3.00 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>Glucagon (100 nm)</td>
<td>6.84 ± 0.63</td>
<td>6.11 ± 0.83</td>
<td></td>
</tr>
<tr>
<td>Induction %</td>
<td>(210)</td>
<td>(204)</td>
<td></td>
</tr>
<tr>
<td>Gluconeogenesis (μg glucose/hour/mg protein) from fructose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>11.1 ± 0.7</td>
<td>11.6 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Glucagon (100 nm)</td>
<td>16.1 ± 1.2</td>
<td>16.5 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Induction %</td>
<td>(145)</td>
<td>(142)</td>
<td></td>
</tr>
<tr>
<td>from pyruvate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>3.96 ± 0.41</td>
<td>3.57 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>Glucagon (100 nm)</td>
<td>6.06 ± 0.36</td>
<td>5.33 ± 0.45</td>
<td></td>
</tr>
<tr>
<td>Induction %</td>
<td>(153)</td>
<td>(149)</td>
<td></td>
</tr>
</tbody>
</table>

Hepatocytes were isolated and cultured as described in the legend to Table I. After 24 h of culture, hepatocytes were treated with or without 100 nm glucagon for 20 min. Effects of glucagon were estimated by the increased activities of glycogen phosphorolase, ureogenesis, and gluconeogenesis as described under Materials and Methods.

Values are mean ± S.E. (n = 3).

Values in parentheses expressed as a percent of activities in vehicle-treated hepatocytes.

Fig. 4. Effects of Dexamethasone at Various Concentrations on the Activities of Tryptophan 2,3-Dioxygenase and Tyrosine Aminotransferase in Primary Cultured Hepatocytes of Rats Treated with Secondary Products.

Hepatocytes were isolated as described in Fig. 1 and cultured for 4 h. Hepatocytes were incubated with the indicated concentrations of dexamethasone and without insulin for another 20 h and harvested. The activities of tryptophan 2,3-dioxygenase and tyrosine aminotransferase were measured as described under Materials and Methods. Data were represented as mean ± S.E. of three animals. Asterisks show significant difference from control hepatocytes at the corresponding concentration of dexamethasone (p < 0.05, Student’s t-test).

ureogenesis (Fig. 1), indicating that liver-specific functions were impaired by in vivo treatment with these compounds. Therefore, we attempted to use these stressed hepatocytes for measuring hormone responses. As shown in Table I, effects of insulin on hepatocytes were estimated by the induction of glucokinase, glucose-6-phosphate dehydrogenase, and malic enzyme. As expected, insulin increased enzyme activities of all three enzymes, but the induction percent, (i.e., response to insulin) showed no significant difference between the pretreated hepatocytes and the control cells.

Glucagon’s effect on hepatocytes was assayed by measuring the activities of glycogen phosphorylase, ureogenesis, and gluconeogenesis (Table II). The activities of glycogen phosphorylase and ureogenesis rose about 2-fold, and gluconeogenesis from both fructose and pyruvate increased to nearly 1.5-fold shortly after treatment with glucagon. The response to glucagon showed no difference between the treated and the control cells, as in the case of insulin.

To assess the effects of glucocorticoids, the activities of tryptophan 2,3-dioxygenase and tyrosine aminotransferase were measured in the presence of various concentration of dexamethasone (Fig. 4). These enzyme activities were dose-dependently increased by the treatment with dexamethasone. Induction of tryptophan 2,3-dioxygenase in hepatocytes from rats dosed with secondary products were significantly lower than that of the control cells over the concentration range of 10⁻⁷ to 10⁻⁵ M of dexamethasone (Fig. 4A). The induction curves of tyrosine aminotransferase (Fig. 4B) in control and secondary products-pretreated hepatocytes also showed a different pattern. Maximum induction in control hepatocytes was reached at 10⁻⁴ M of dexamethasone. On the other hand, the activity in the treated hepatocytes was drastically lower and did not reach the maximum activity in the concentration range of dexamethasone tested here. Therefore, there was no difference between hepatocytes from secondary products-
dosed rats and control rats in the responses to insulin and glucagon, but the response to dexamethasone in the hepatocytes from treated animals was clearly deficient.

**Discussion**

In the current report, we have demonstrated that hepatocytes obtained from rats dosed with secondary autoxidation products retained oxidative stress. Many investigators have used isolated and cultured hepatocytes as a tool for studying oxidative stress, since hepatocytes are more functionally active than cell lines and their functions are closer to that of liver. Most of those studies used *in vitro* treatment of hepatocytes with biological oxidants or prooxidative chemicals. We raise a question here whether these *in vitro* treatments reflect the *in vivo* conditions, since exposure to prooxidants is generally negligible. In this study, we have used secondary autoxidation products of linoleic acid as inducers of oxidative stress. The biological action of secondary products is weaker than that of many prooxidative chemicals, but secondary products are the most likely inducers of oxidative stress from food sources. Therefore, isolating hepatocytes from animals treated *in vivo* with secondary products is important for elucidating the mechanism of oxidative stress. Moreover, primary cultured hepatocytes from stressed animals will be useful in filling the gap between *in vivo* and *in vitro* experimental conditions, since it has been shown that freshly isolated cells have impaired functions and that the cells recover their functions during primary culture.1,6,13,14,15)

Our data clearly showed that hepatocytes from secondary products-dosed rats retained the markers of oxidative stress and hepatic dysfunction (Figs. 2 and 3). The time-dependent changes in the markers of oxidative stress showed similar patterns as our previous *in vivo* time-course study.20) In this study, the increase in TBARS and the decrease in tocopherol gradually recovered to control levels by 24 hr cultured hepatocytes from treated rats. In the *in vivo* study,20) the increase in TBARS and the reduction of tocopherol levels were observed between 15 through 72 hr and 15 through 48 hr after the single p.o. dose of secondary products, respectively. These biological changes in cultured hepatocytes occurred earlier than those of the *in vivo* study. This time-lag may be due to the difference in the cellular environment, i.e., differences between the structure of primary cultured hepatocytes and *in vivo* liver cells. Primary cultured hepatocytes form a monolayer allowing for facilitated uptake of nutrients. Additionally turnover of cultured hepatocytes would be faster than that of the intact liver cells.

We previously surveyed several target enzymes of hepatic dysfunction caused by lipid peroxidation products using *in vitro* cell-free conditions,1,15) but our data did not agree with the *in vivo* results.13,20) For example, glyceroldehyde-3-phosphate dehydrogenase was susceptible to peroxidation products under cell free conditions. In addition, Uchida et al.2,3) reported that 4-hydroxynonenal, the most toxic aldehyde in secondary products was capable of attacking the histidine residues of this enzyme. This enzyme, however, did not decrease in the activity after the oral dose with secondary products. In the current study, we observed a recovery in hepatocytes enzyme activities that was similar to the *in vivo* data.20) The recovery of mitochondrial enzymes are faster than that of cytosolic enzymes in both *in vivo* and *in vitro* experiments. Taken together, these data suggest that cultured hepatocytes from secondary products-dosed rats have reproduced the *in vivo* oxidative stress conditions of the liver, even recovering from damage faster than *in vivo*.

With respect to hormonal effects on hepatocytes, actions of insulin and glucagon were similar between secondary products-treated and control rats (see Tables I and II). Both hormones acted on hepatocytes to significantly increase the activities of target enzymes and metabolic systems, but the induction ratios in both hepatocytes from secondary products-dosed and control rats were identical. It is well known that receptors for both insulin and glucagon exist in the plasma membrane and membranes are easily injured under oxidative stress as a result in the action of active oxygen radicals. Secondary products also have the capability to injure membranes, although they are not as potent as prooxidative chemicals such as carbon tetrachloride. Indeed our previous *in vivo* study30) demonstrated that the leakage of aspartate aminotransferase was detected in serum between 12 and 48 hr after the oral dose of secondary products to rats. Contrary to those *in vivo* results, the current results indicated that the leakage of aspartate aminotransferase in cultured hepatocytes slowed by 4 hr in both cultured hepatocytes from treated and control rats. Under our experimental conditions, it was difficult to compare the function of membranes in cultured hepatocytes to that in the liver. Thus, whether actions of insulin and glucagon observed in oxidatively stressed hepatocytes mimic the *in vivo* condition is still unclear.

In contrast, the response to dexamethasone, a glucocorticoid analog, in hepatocytes from secondary products-dosed rats showed differences from control hepatocytes. The induction of tryptophan 2,3-dioxygenase and tyrosine aminotransferase declined in hepatocytes from secondary products-dosed rats as compared to the control hepatocytes (see Fig. 4). Both of these enzymes are liver-specific and are positively regulated at the level of mRNA by glucocorticoids.36,37) It is known that glucocorticoids bind to its cytosolic receptor, and translocate into the nucleus where the activated receptor protein binds to glucocorticoid responsive elements in the promoters of responsive genes to induce transcription. Thus, our data suggest that secondary products, probably highly reactive aldehydes such as 4-hydroxynonenal, would affect the induction systems of both enzymes by glucocorticoids.

In conclusion, this study shows hepatocytes from secondary products-dosed rats retain the markers of oxidative stress, although they gradually recover in culture. We also have found that oxidative stress induced by dietary lipid peroxidation products may impair the functions in the cytosolic proteins, specifically several cytosolic enzymes and the cytosolic glucocorticoids receptor. We have shown that oxidatively-stressed hepatocytes from *in vivo* treated animals are a useful tool to study the mechanism of hepatic dysfunction by dietary lipid peroxidation products. This model will also be useful in studies on the protective effects of natural antioxidants and food components against *in vivo* oxidative stress.
References