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Development of enzyme-linked immunosorbent assay for 8-iso-prostaglandin $F_{2\alpha}$, a biomarker of oxidative stress *in vivo*, and its application to the quantification in aged rats

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ABSTRACT

8-iso-Prostaglandin $F_{2\alpha}$ (8-iso-PG$F_{2\alpha}$) is one of the isoprostanes that are mainly generated nonenzymatically *in vivo* from arachidonic acid through free radical-induced lipid peroxidation. To assess oxidative stress *in vivo*, we developed a quantitative enzyme-linked immunosorbent assay (ELISA) for 8-iso-PG$F_{2\alpha}$. A sensitive calibration curve allowed the quantification of the amounts from 0.23 pg to 98.4 pg with 4.7 pg of 50% displacement in one assay. The ELISA method was applied to the measurement of the plasma levels of 8-iso-PG$F_{2\alpha}$ in young rats (4–8 weeks of age) and aged rats (106–123 weeks). The average level of esterified form in the plasma from aged rats was 30.6-fold higher than that in the plasma from young rats, reflecting the enhanced status of oxidative stress in aged animals. In addition, the aged rats exhibited higher levels of this F_2-isoprostane esterified to lipids from liver and kidney, suggesting local oxidative injury in specific organs. These results indicate the utility and accuracy of our ELISA for 8-iso-PG$F_{2\alpha}$ as a biomarker *in vivo* to assess systemic oxidative stress in animals or humans as well as oxidative injury at local sites.

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1. Introduction

A series of prostaglandin F_2-like compounds termed F_2-isoprostanes are known to be produced *in vivo* nonenzymatically from arachidonic acid, an essential nutrient, in animals and humans predominantly by a free radical-induced lipid peroxidation [1–3]. A large body of evidence has suggested that the measurement of F_2-isoprostanes is a reliable and useful approach to assess lipid peroxidation and oxidative stress *in vivo* [4]. Of these, the levels of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-PG$F_{2\alpha}$) have been studied more extensively as a biomarker of oxidative marker *in vivo* until now. The majority of arachidonic acid is esterified to the *sn*-2 position of phospholipids in cell membranes of various tissues or blood cells. Earlier studies have shown that F_2-isoprostanes including 8-iso-PG$F_{2\alpha}$ are initially generated *in situ* from arachidonic acid esterified in phospholipids under the oxidative stress and subsequently released as a free form by the action of phospholipases [2]. Thus, 8-iso-PG$F_{2\alpha}$ can be detectable as both free and esterified forms. The free form of 8-iso-PG$F_{2\alpha}$ can be quantified to evaluate total generation of it *in vivo* that reflects the enhanced systemic lipid peroxidation. By contrast, the measurement of 8-iso-PG$F_{2\alpha}$ esterified in phospholipids in tissues would provide the methods for assessing the oxidative injury in specific cells, tissues, or organs. For example, the administration of CCl_4 to animals has been shown to cause the marked elevation of circulating levels of F_2-isoprostanes [1–2]. Alternatively, higher levels of F_2-isoprostanes have been recognized in plasma of smokers due to free radicals in cigarette smoke, whereas the intake of antioxidants such as vitamin C decreased the plasma levels of F_2-isoprostanes as biomarkers of lipid peroxidation [5]. Hence, the development of a sensitive and specific assay for 8-iso-PG$F_{2\alpha}$ should be highly useful as a non-invasive approach by analyzing the levels in plasma and urine to assess the involvement of oxidative stress and lipid peroxidation in human or animal disease from the point of prognostic diagnosis and the evaluation of antioxidants in pharmaceuticals and nutraceuticals.

Until now, most studies have employed gas chromatography–mass spectrometry (GC–MS) to quantify the levels of 8-iso-PG$F_{2\alpha}$ as a more sensitive and specific way [3,6]. However, the mass spectrometric analysis requires high-cost apparatus and an analytical expert to operate the quantitative analysis. Additionally, the GC–MS analysis requires extensive

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sample preparations and derivatization of analytes. Moreover, the use of a deuterium-labeled internal standard required for the quantification with mass spectrometric quantification becomes more expensive for the analysis of multiple samples. As an alternative to GC–MS, liquid chromatography–mass spectrometry (LC–MS) is becoming useful for the analysis and quantification of analytes in clinical laboratories. However, many methods still require extensive sample purification prior to quantitative analysis. More recently, Saenger et al. has reported an improved assay for the measurement of urinary 8-iso-$PGF_{2\alpha}$ by LC–MS without sample pretreatment [7]. Recent advances have been reviewed regarding measurement of oxidative stress parameters using LC–MS [8]. Nevertheless, this mass spectrometric approach is also limited by smaller numbers of analytes assayed from biological sources due to the sequential operation of expensive instrumentation as compared with immunological methods. Therefore, we planed to develop a convenient and sensitive immobilized enzyme-linked immunosorbent assay (ELISA) specific for 8-iso-$PGF_{2\alpha}$. This immunological method is applicable for many samples at the same time without high-cost facilities and an analytical expert. The present study was undertaken to prepare monoclonal antibodies specific for 8-iso-$PGF_{2\alpha}$. Then, using the specific monoclonal antibody, we attempted to develop the immobilized ELISA for 8-iso-$PGF_{2\alpha}$. Finally, we applied our method to the quantification of 8-iso-$PGF_{2\alpha}$ free in plasma and esterified in plasma lipids as well as the form esterified in tissues lipids from liver and kidney in young and old rats.

2. Materials and methods

2.1. Materials

8-iso-$PGF_{2\alpha}$, other related isoprostanes, and $PGF_{2\alpha}$ were obtained from Cayman Chemical (Ann Arbor, MI, USA). Biotin-conjugated rabbit anti-mouse IgG (H + L) antibody was the product of Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Essentially fatty acid-free bovine serum albumin (BSA), bovine γ-globulin, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl, *N*-hydroxysuccinimide, ExtrAvidin-peroxidase conjugate, Dulbecco's modified Eagle's medium with 25 mM HEPES, penicillin G, and streptomycin sulfate were supplied by Sigma (St. Louis, MO, USA). Hypoxanthine, aminopterin, thymidine, NCTC-109 medium, pristane, and *o*-phenylenediamine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum was supplied by Biological Industries (Kibbutz Beit Haemek, Israel). 96-Well and 24-well microplates, and 60-mm and 100-mm plastic Petri dishes for tissue culture were obtained from Iwaki Glass (Tokyo, Japan). For ELISA, 96-well microplates (Type S) were purchased from Sumitomo Bakelite (Tokyo, Japan). All other chemicals used here were of reagent or culture grade. A myeloma cell line, SP-2/*O*-Ag14, was provided by the Japanese Cancer Research Resources Bank (JCRB) (Tokyo, Japan). BALB/c mice and Wistar rats were supplied by Japan SLC (Hamamatsu, Japan).

2.2. Generation of monoclonal antibodies specific for 8-iso-$PGF_{2\alpha}$

For the preparation of the immunogen, 8-iso-$PGF_{2\alpha}$ was chemically linked with fatty acid-free BSA by the *N*-succinimidyl ester method as described earlier [9]. In brief, 8-iso-$PGF_{2\alpha}$ (200 μg) were allowed to react for 3 h at 25 °C with 1.5 mg of *N*-hydroxysuccinimide and 1 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl by dissolving and shaking in 50 μl of 80% dioxane. The *N*-hydroxysuccinimidyl ester of 8-iso-$PGF_{2\alpha}$ was extracted with 2 ml of ethyl acetate after mixing with 2 ml of water and evaporated to dryness. The dried materials were coupled to amino groups of BSA in 200 μl of 50 mM sodium phosphate buffer (pH 7.3) at 4 °C by shaking the tube overnight. The reaction mixture was applied to a NAP-10 cartridge column to prepare the conjugate after removing free 8-iso-$PGF_{2\alpha}$. The resulting conjugate was used for immunizing female 8-week BALB/c mice. After repetitive immunization every 2 weeks, the spleen lymphocytes were prepared and allowed to fuse with SP-2/*O*-Ag14 myeloma cells in the presence of 50% polyethylene 4000. The culture of hybridoma cells and the selection of growing cells in the medium containing hypoxanthine, aminopterin, and thymidine were essentially carried out as reported earlier [10,11]. The diluted antiserum and the culture medium of hybridoma cells were tested for their antibody titers using a conjugate of 8-iso-$PGF_{2\alpha}$ and bovine γ-globulin as the immobilized antigen by solid-phase ELISA. The hybridoma cells secreting the monoclonal antibodies for the target hapten were used for the isolation of cloned cells by the limited dilution method. Several hybridoma clones with a higher titer of the antibody for 8-iso-$PGF_{2\alpha}$ were grown and the resulting cultured cells (1×10^6 cells) were injected into the peritoneal cavity of one mouse after 1 week of administration of 0.5 ml of pristane to prepare monoclonal antibodies in ascites fluid. Additional 10 days later, ascites fluid was collected and then fractionated with ammonium sulfate precipitation. Among cloned hybridoma cells, we eventually used a hybridoma clone called *3B10-10G8-5H10* to produce the monoclonal antibody, which was found to be useful for the generation of standard curve of ELISA due to the higher sensitivity and lower cross-reaction with other isoprostanes and other prostanoids as described below. The fraction precipitating at 50% saturation with ammonium sulfate was redissolved in phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions (PBS (−)) with 20% glycerol to give an original solution of 12.3 mg protein/ml.

2.3. Immobilized ELISA for 8-iso-$PGF_{2\alpha}$

In principle, the immobilized antigen was competitively incubated with monoclonal antibody for 8-iso-$PGF_{2\alpha}$ as a first antibody in the presence of a free authentic standard or a sample to be tested as described previously [12]. Namely, the conjugate of 8-iso-$PGF_{2\alpha}$ and bovine γ-globulin (10 ng protein) in 100 μl of 50 mM sodium phosphate buffer (pH 7.3) was placed in a 96-well microplate for ELISA and incubated at room temperature to immobilize the antigen. After blocking each 96-well microplate with 200 μl of 0.5% gelatin in PBS (−), the resulting immobilized antigen in each 96-well was mixed with 50 μl of a 2×10^6-fold diluted solution of the monoclonal antibody for 8-iso-$PGF_{2\alpha}$ and 50 μl of a standard solution of authentic 8-iso-$PGF_{2\alpha}$ or a sample to be tested. The dilutions of reagents were made using PBS (−) with 0.1% BSA (ELISA buffer). The microplates were incubated at 30 °C for 1 h followed by overnight at 4 °C to allow the formation of the immunocomplex. After washed with PBS (−), the immunocomplex in a 96-well dish was further allowed to react with 100 μl of the ELISA buffer containing biotin-conjugated rabbit–mouse IgG antibody at a dilution of 5×10^4 by incubating at 30 °C for 2 h followed overnight at 4 °C. Following washing as above, the 96-well dish was incubated with 1×10^4-fold diluted solution of the ExtrAvidin-peroxidase conjugate in the ELISA buffer at 25 °C for 30 min. After subsequent washing, the peroxidase reaction was carried out at 30 °C for 20 min with 100 μl of 50 mM sodium phosphate-citrate buffer (pH 5.2) containing 18.5 mM *o*-phenylenediamine and 3.5 mM H_2O_2 as substrates. The peroxidase reaction was stopped by the addition of one-half volume of 2.5 M H_2SO_4. The absorbance at 492 nm was measured for 650 nm as a reference wavelength using a Tecan ELISA microplate reader, a model of Sunrise Thermo (Tecan Japan Co., Kawasaki, Japan).

2.4. Application of immobilized ELISA to the quantification of 8-iso-$PGF_{2\alpha}$ in plasma and tissues from rats

To demonstrate the utility of our developed ELISA for 8-iso-$PGF_{2\alpha}$ in the application to the plasma levels of free and esterified forms with or without increased oxidative stress *in vivo*, we employed rat plasma from young and aged rats as model experimental animals. We also applied our immunological approach to the assessment of the levels esterified in tissue lipids from liver and kidney in young and aged rats, reflecting oxidative injury at local sites. Wistar rats were obtained from Shimizu Laboratory Supplies (Kyoto, Japan) and allowed to the ages of 106–123 weeks. Rats were housed at 23 °C on a 12 h/12 h light/dark cycle and had free access to standard rat chow and tap water. All applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research. Animals were anesthetized with ethyl ether, and blood was drawn from the aorta. The plasma was individually obtained as pooled samples from young (4–8 weeks) and aged rats (106–123 weeks) under the condition that prevent the clotting of blood by treating the needle and silicon tubes with heparin. The plasma samples were applied to the immunological determination of 8-iso-$PGF_{2\alpha}$ free in plasma from young (n = 10) and aged (n = 11) rats as well as the levels esterified in plasma lipids from young (n = 6) and aged (n = 9) rats. In addition, the tissues of liver and kidney were removed from young (8 weeks, n = 6) and aged rats (106 weeks, n = 7) and used for the quantification of 8-iso-$PGF_{2\alpha}$ esterified in tissue lipids after the extraction and subsequent hydrolysis of them as described below. The samples of plasma and tissues were rapidly frozen in liquid nitrogen and stored at −80 °C until use.

For the quantification of the amounts of esterified form of 8-iso-$PGF_{2\alpha}$ in the plasma and tissues from young and aged rats, total lipids were extracted from 1 ml of plasma or 1 g of tissue with 20 ml of ice-cold Folch solution, chloroform/methanol (2:1, v/v), containing 0.005% butylated hydroxytoluene (BHT) as a free radical scavenger and 0.025% triphenylphosphine (TPP) as a reducing reagent, as described earlier [3]. In brief, after mixing with 10 ml of 0.043% $MgCl_2$ for the plasma or 0.9% NaCl for the tissues, the mixture was centrifuged at 800 × g for 10 min to obtain the lower organic phase. The resulting lipid extracts were evaporated to dryness and incubated at 37 °C for 30 min with 4 ml of 7.5% KOH in 50% methanol with BHT and TPP as above to hydrolyze 8-iso-$PGF_{2\alpha}$ esterified in lipids to release the free form. The reaction mixture was acidified to pH 3 with 1 M HCl and followed by the extraction of free 8-iso-$PGF_{2\alpha}$ with 1 ml of ethyl acetate twice. After evaporation of the solvent, the dried materials were reconstituted in 1 ml of fresh ethyl acetate and stored at −30 °C before use. For the ELISA assay of each sample, the extract (500 μl) was evaporated into dryness and redissolved in 500 μl of the ELISA buffer. The resulting aliquots (10–40 μl) using three different volumes were subjected to the assay of ELISA for 8-iso-$PGF_{2\alpha}$ as described above.

2.5. Validation of ELISA for 8-iso-$PGF_{2\alpha}$

For confirming precision of our ELISA, the intraassay coefficients of variation were evaluated for six experiments with n = 3 tubes per assay for samples containing 0.001–10 ng of 8-iso-$PGF_{2\alpha}$. In addition, the interassay coefficients of variation were determined for samples containing 0.001–10 ng of 8-iso-$PGF_{2\alpha}$ five times for the period of 2 months. For the evaluation of the stability of 8-iso-$PGF_{2\alpha}$ during the ELISA assays, the standard at concentrations of 0.2–20 ng/ml was kept at 4 °C for 1 week in the ELISA buffer, after which the samples at different periods of time were used for the quantification of the levels by our ELISA. Our developed immobilized ELISA was applied to the determination of free form of 8-iso-$PGF_{2\alpha}$ in the rat plasma. To evaluate the accuracy of the determined values in the plasma, the samples were fortified with known amounts of authentic 8-iso-$PGF_{2\alpha}$ and then diluted serially by 2-, 4-, and 8-fold with the ELISA buffer. The resulting samples were subjected to the assay of 8-iso-$PGF_{2\alpha}$ by our ELISA.

2.6. Other procedures

High-performance liquid chromatography (HPLC) analysis was performed on a Shimadzu LC-9A liquid chromatograph equipped with a SPD-6AV UV–Vis spectrophotometric detector and a C-R6A Chromatopac recorder (Shimadzu, Kyoto, Japan). The analytes containing 8-iso-$PGF_{2\alpha}$ were individually dissolved in 20 μl of the mobile phase to be used, and applied to a column of reverse-phase L-column ODS (4.6 mm × 250 mm; 5 μm particles; Chemical Evaluation and Research Institute, Sugito, Saitama, Japan). The column was eluted at a flow rate of 1 ml/ml with a mobile phase of acetonitrile/17 mM phosphoric acid (37.2:62.8, v/v). The elution of authentic compound was monitored at 25 °C by detection at 195 nm. For the confirmation of the desired compound in samples, the eluates were collected every 1 min. Each collected fraction (1 ml) was mixed vigorously with 1 ml of ethyl acetate and 1 ml of sodium acetate buffer (pH 3.5), and the upper organic phase was taken after centrifugation and evaporated to dryness. The dried materials were dissolved in 100 μl of ethyl acetate for storage at −30 °C. A portion was removed, dried, redissolved in the ELISA buffer, and used for the determination by ELISA for 8-iso-$PGF_{2\alpha}$. Protein was quantified with fatty acid-free BSA as a standard according to the method of Lowry et al. [13] after precipitating proteins with trichloroacetic acid to remove the interfering substances [14].

3. Results and discussion

3.1. Development of immobilized ELISA for 8-iso-$PGF_{2\alpha}$ using a specific monoclonal antibody

A sensitive standard curve of ELISA for 8-iso-$PGF_{2\alpha}$ was generated using a monoclonal antibody secreted by a hybridoma clone called *3B10-10G8-5H10*. This monoclonal antibody was classified as IgG_1 having isotypes of γ1 heavy chain and κ light chain. The typical standard curve enabled us to quantify the amount from 0.23 pg to 98.4 pg in each 96-well dish, corresponding to those of free 8-iso-$PGF_{2\alpha}$ required to displace 10–90% of the maximal binding of the immobilized antigen to the used monoclonal antibody, respectively (Fig. 1). The amount of free 8-iso-$PGF_{2\alpha}$ to displace 50% of the maximal binding was observed at 4.7 pg in each assay. The intraassay coefficients of variation in our ELISA ranged from 6.5% to 8.2%. In addition, the ELISA method gave interassay coefficients of variation from 12.3% to 18.1%. The standard solutions of 8-iso-$PGF_{2\alpha}$ were stable during the ELISA assays even though those had been kept at 4 °C for 1 week. The cross-reaction of the monoclonal antibody used here was examined using other isoprostanes and the related prostanoids (Fig. 2). The monoclonal antibody exhibited the cross-reaction of less than 0.1% with the related isoprostanes and prostanoids except 9.9% with 8-iso-$PGF_{3\alpha}$ and 0.8% with $PGF_{2\alpha}$ (Table 1).

Here, we were successful to prepare a monoclonal antibody specific for 8-iso-$PGF_{2\alpha}$ as a biomarker of oxidative stress *in vivo*, and to develop a sensitive immobilized ELISA that is more convenient and applicable to many samples from biological sources. Our immobilized ELISA is much easier to conduct the assays than the mass spectrometric assays using expensive instruments like GC–MS and LC–MS although the mass spectrometric assay is highly precise and accurate. The sensitivity of our developed ELISA for 8-iso-$PGF_{2\alpha}$ is considered to be satisfactory for the quantification of normal levels

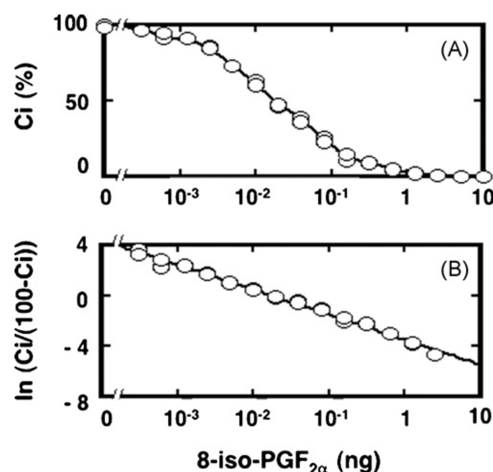

Fig. 1. Standard curve of ELISA for 8-iso-$PGF_{2\alpha}$. (A) Standard curve as represented by the binding percentage of the immobilized antigen (Ci (%)) versus increasing amounts of authentic compound. (B) Linearized standard curve to make a logit transformation of Ci (%) values.

of 8-iso-$PGF_{2\alpha}$ free in plasma since the average levels of our samples were found to be 36.7–45.5 pg/ml in young and old rats, which values were much higher than the detection limit of our sensitive ELISA. A previous study also reported that normal plasma concentrations of F_2-isoprostanes in rats were around 30 pg/ml and basal human plasma contained about 35 pg/ml [3].

Table 1
Cross-reaction of *3B10-10G8-5H10*, a monoclonal antibody for 8-iso-$PGF_{2\alpha}$, with other isoprostanes and $PGF_{2\alpha}$.

Compound	Cross-reaction (%)
8-iso-$PGF_{2\alpha}$	100
8-iso-$PGF_{3\alpha}$	9.9
$PGF_{2\alpha}$	0.8
2,3-Dinor-8-iso-$PGF_{1\alpha}$	0.1
8-iso-PGE_2	<0.1
8-iso-PGE_1	<0.1

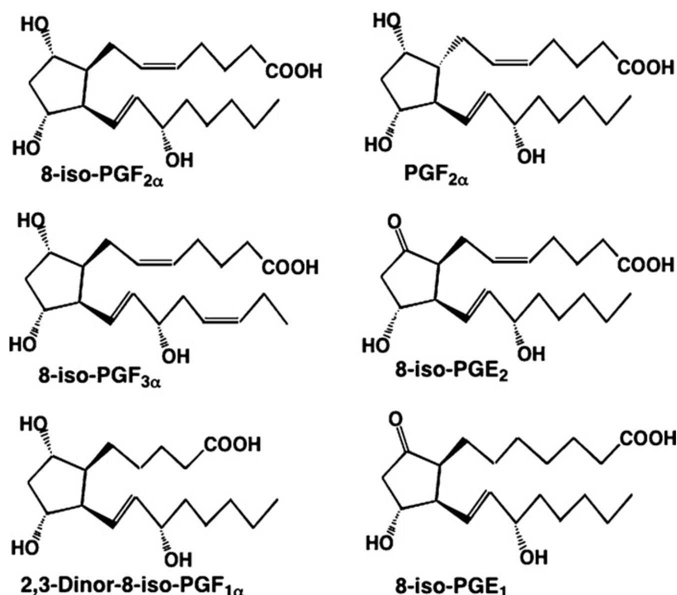

Fig. 2. Chemical structures of isoprostanes and $PGF_{2\alpha}$. These compounds were used for the determination of cross-reaction of monoclonal antibodies for 8-iso-$PGF_{2\alpha}$.

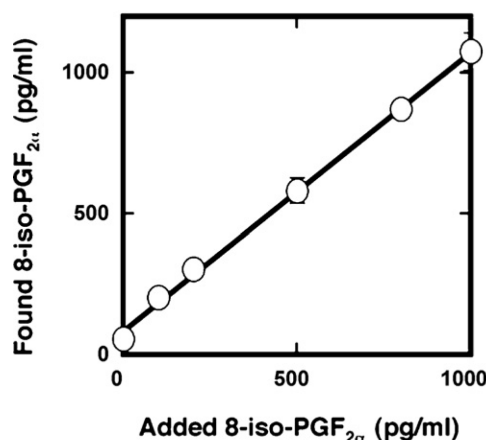

Fig. 3. The accuracy for the determination of 8-iso-$PGF_{2\alpha}$ in the plasma. The plasma was obtained from rats and fortified with increasing amounts of authentic 8-iso-$PGF_{2\alpha}$ in a range from 0 to 1000 pg/ml. The resulting samples were applied to ELISA for 8-iso-$PGF_{2\alpha}$.

3.2. Application of ELISA for 8-iso-$PGF_{2\alpha}$ to the determination of free and esterifed forms of 8-iso-$PGF_{2\alpha}$ in plasma and tissues from rats

Our ELISA for 8-iso-$PGF_{2\alpha}$ was applied to the quantification of free form of 8-iso-$PGF_{2\alpha}$ in rat plasma. The resulting samples were employed for the evaluation of the accuracy in the determination of 8-iso-$PGF_{2\alpha}$ free in the plasma that had been fortified with known amounts of authentic 8-iso-$PGF_{2\alpha}$ (Fig. 3). The analysis gave satisfactory results showing a linear proportionality between the masses found and the amounts added to the sample in concentration rages from 0 pg/ml to 1000 pg/ml with a recovery of 99.0%. The results indicate that our ELISA is reliable enough to apply to the determination of 8-iso-$PGF_{2\alpha}$ in the plasma.

In addition, F_2-isoprostanes such as 8-iso-$PGF_{2\alpha}$ have been shown to be detectable in all types of biological fluids or tissues [3,4]. Therefore, we tried to measure the levels of these compounds esterified in lipids in the plasma or tissues including liver and kidney from rats. The levels esterified in total lipids were determined after alkaline hydrolysis to release free form of 8-iso-$PGF_{2\alpha}$. To determine whether the immunoreactive material detected by our ELISA could coincide with authentic free 8-iso-$PGF_{2\alpha}$, the extracts containing free 8-iso-$PGF_{2\alpha}$ were separated by reverse-phase HPLC, and the resulting eluates were subjected to our ELISA for 8-iso-$PGF_{2\alpha}$. A single immunoreactive peak from the rat plasma was detectable, and the retention time of this peak was consistent with that of the authentic compound. The observations support the reliable measurement of 8-iso-$PGF_{2\alpha}$ released from total lipids (Fig. 4).

3.3. Comparison of 8-iso-$PGF_{2\alpha}$ levels between young and aged rats

We made use of our ELISA to determine the levels of 8-iso-$PGF_{2\alpha}$ free and esterified in total lipids from rat plasma as pooled samples, and compared the values between young and aged rats. The average levels of free form in the plasma from young and aged rats were calculated to be 45.5 pg/ml and 36.7 pg/ml, respectively while those of esterified form were 22.8 pg/ml and 697 pg/ml, respectively (Fig. 5). Therefore, no marked difference was found between young rats and aged rats with respect to the plasma levels of free form of 8-iso-$PGF_{2\alpha}$. On the other hand, the average level of 8-iso-$PGF_{2\alpha}$ esterified in plasma lipids from aged rats was found to be 30.6-fold higher than that in the plasma lipids from young rats although there were considerable variations between the individual aged rats. In addition, we measured the levels of 8-iso-$PGF_{2\alpha}$ esterified in tis-

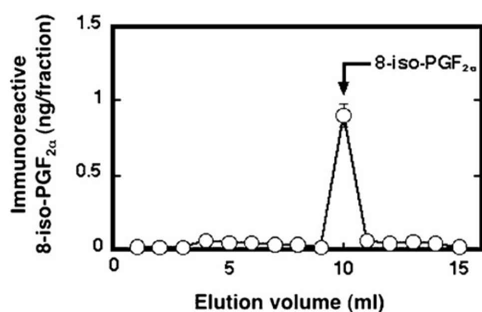

Fig. 4. Separation of hydrolyzed products of esterified lipids in rat plasma by HPLC and detection of immunoreactive 8-iso-$PGF_{2\alpha}$. Total lipids were extracted from rat plasma and subjected to their alkaline hydrolysis. The released products were separated by reverse-phase HPLC, and the resulting eluates were detected by ELISA for 8-iso-$PGF_{2\alpha}$.

sue lipids from liver and kidney in young and aged rats (Fig. 6). The measured levels of 8-iso-$PGF_{2\alpha}$ esterified in lipids from liver were calculated to be 0.521 ng/g tissue and 1.06 ng/g tissue in young and aged rats, whereas those of 8-iso-$PGF_{2\alpha}$ esterified in lipids from kidney were 0.369 ng/g tissue and 0.863 ng/g tissue, respectively. Thus, the aged rats exhibited increased levels of 8-iso-$PGF_{2\alpha}$ esterified in the tissue lipids from both liver and kidney by a mean of approximate 2-fold compared with those measured in young rats.

Free form of 8-iso-$PGF_{2\alpha}$ in plasma can be derived from different sources, indicating total endogenous production of it in the body. On the other hand, esterified forms of 8-iso-$PGF_{2\alpha}$ in tissue lipids should reflect the oxidative injury in specific organs. Earlier, other group measured the levels of 8-iso-$PGF_{2\alpha}$ called F_2-isoprostanes free in plasma and those esterified in plasma lipids by GC–MS in a negative-ion chemical ionization mode [6]. They reported that plasma levels of free F_2-isoprostanes and those esterified in plasma lipids were strikingly increased by means of 20.3- and 29.9-fold in aged rats (22–24 months of age) compared with young rats (3–4 months of age). We also found marked increase in the average levels of 8-iso-$PGF_{2\alpha}$ esterified in plasma lipids from aged rats by a mean of 30.6-fold compared with levels in young rats. In contrast, we did not detect a marked increase in the average levels of 8-iso-$PGF_{2\alpha}$ free in plasma in aged rats. The reason for this discrepancy is unknown, but may be explained by the difference in the experimental conditions or the strains of mice. Our findings point out

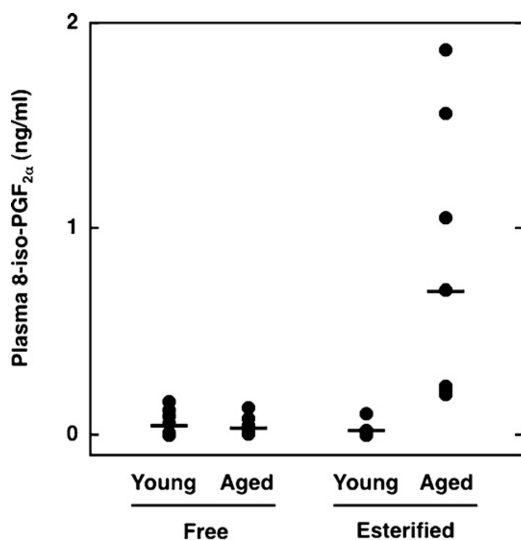

Fig. 5. Levels of 8-iso-$PGF_{2\alpha}$ free and esterified in plasma from young and aged rats. Pooled plasma samples from young (4–8 weeks) and aged rats (106–123 weeks) were analyzed for the quantification of 8-iso-$PGF_{2\alpha}$ free and esterified in plasma lipids following their hydrolysis.

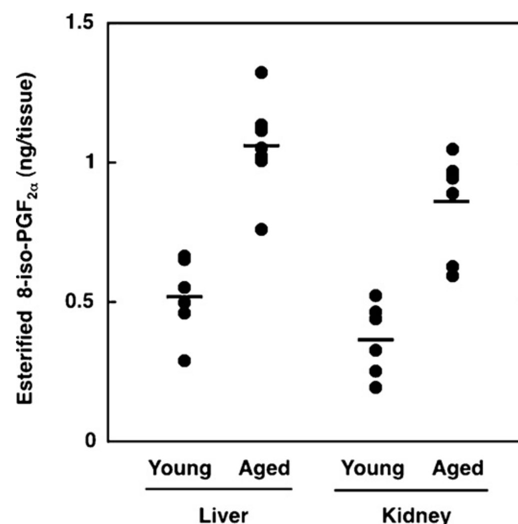

Fig. 6. Levels of 8-iso-$PGF_{2\alpha}$ esterified in tissue lipids from young and aged rats. Total lipids were extracted from pooled tissues of liver and kidney from young (8 weeks) and aged rats (106 weeks) and subjected to their hydrolysis. The released 8-iso-$PGF_{2\alpha}$ was quantified by ELISA.

the importance of assessing the levels of 8-iso-$PGF_{2\alpha}$ esterifed in plasma lipids. Furthermore, the present analysis of the levels of 8-iso-$PGF_{2\alpha}$ esterified in total lipids from liver and kidney clearly revealed the higher levels in the lipids from both tissues in aged rats. These data support the oxidative stress or injury *in vivo* occurring locally in specific organs from old rats. These are closely associated with the free radical theory of aging involving the lipid peroxidation.

3.4. Further implication

To support the usefulness of F_2-isoprostanes as an index for the excess free radical generation in human disease, specific assays for them employing mass spectrometry have previously shown the increased formation of 8-iso-$PGF_{2\alpha}$ in low-density lipoprotein and urine from hypercholesterolemic patients compared with their controls [15]. In addition to these animal disease models associated with oxidative stress *in vivo*, our ELISA would be also useful for the evaluation of the effectiveness of antioxidants from dietary sources to suppress systemic lipid peroxidation in experimental animals or humans. For example, dietary supplementation with aged garlic extract having antioxidant properties has been reported to reduce oxidative stress in humans as assessed by the measurement of plasma and urine concentrations of 8-iso-$PGF_{2\alpha}$ [16]. Taken together, our ELISA is potentially useful for noninvasive approaches using plasma or urine to assess prognostic oxidative stress *in vivo* in animals and humans. Furthermore, this immunological assay for 8-iso-$PGF_{2\alpha}$ can be applicable for the evaluation of antioxidant activity of food factors as well. Although 8-iso-$PGF_{2\alpha}$ from arachidonic acid is best characterized, many other polyunsaturated fatty acids can form different types of isoprostanes. Our immunological approach can be utilized essentially for developing ELISA specific for other related biomarkers to assess lipid peroxidation associated with oxidative stress *in vivo*.

4. Conclusion

We developed an immobilized ELISA for 8-iso-$PGF_{2\alpha}$, an isomer of F_2-isoprostanes that are generated by the free radical-induced peroxidation of arachidonic acid. The quantification of 8-iso-$PGF_{2\alpha}$ has been regarded as a biomarker of oxidative stress *in vivo*. Our ELISA method was demonstrated to be highly sensitive and spe-

cific for 8-iso-$PGF_{2\alpha}$, allowing the determination of a number of samples at the same time. As well as sensitivity and specificity, the utility of our ELISA for the quantification was furthermore validated by precision and accuracy of the assays. When our method was applied to the measurement of 8-iso-$PGF_{2\alpha}$ in plasma from young and aged rats as an experimental animal model, the levels of 8-iso-$PGF_{2\alpha}$ esterified in plasma lipids from aged rats were found to be much higher than those from young rats, indicating the status of oxidative stress in aged animals through the normal aging process. The analysis of levels of 8-iso-$PGF_{2\alpha}$ esterified in tissue lipids also revealed higher levels in liver and kidney from aged rats, supporting local oxidative injury in specific organs. Taken together, our immunological approach without expensive instrumentation would be applicable for assessing enhanced oxidative stress in a number of human or animal disorders or for evaluating the effectiveness of antioxidants from dietary sources *in vivo*.

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