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Oxidized casein impairs antioxidant defense system and induces hepatic and renal injury in mice

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ABSTRACT

Scope: Oxidized protein products (OPPs) can be easily found in meat and milk during processing and storage. Evidence supports that accumulation of endogenous OPPs plays a negative role in physiological metabolism. However, the impacts of dietary OPPs and the mechanisms have not been elucidated yet. The present study evaluated whether oral oxidized casein would destruct the antioxidant defense system and cause potential oxidized injury in mice liver and kidney.

Methods and results: We performed oxidized casein (modified respectively by H_2O_2–Cu and HClO) feeding experiments using KM mice (20–22 g). A 10-weeks feeding of oxidized casein as basal protein caused oxidative stress by increasing protein carbonylation (PC), advanced oxidation protein products (AOPPs), dityrosine (Dityr), lipid peroxidation and ROS levels in mice liver, kidney and blood ($P < 0.05$). In mice liver and kidney, the mRNA expression of Nrf2, γ-GCS, HO-1, GPX-3, and GPX-4 up-regulated, the protein level of Nrf2 in nucleus increased. However, activities of anti-oxidant enzymes (CAT, SOD, and GPX) decreased ($P < 0.05$). Moreover, histopathological examination displayed the formation of fibrous septa in mice liver and kidney after oxidized casein feeding.

Conclusion: Oxidized casein impairs antioxidant defense system and induces hepatic and renal fibrosis.

1. Introduction

During food processing and storage, proteins are targets to many radicals and non-radical oxidants (e.g., peroxides, hypochlorous acid, peroxynitrite, and singlet oxygen), including a wide range of environmental agents, such as radiation (e.g., X-ray, UV, or visible light), and metal ions (e.g., Cu^{2+}, Mn^{3+}, and Fe^{3+}). Oxidant generation in food system can also occur as a response to high temperature, extrusion, shearing force, and many by-products of lipid peroxidation, glycation/glycoxidation reactions (Davies, 2005). The most remarkable and measurable changes caused by protein oxidation (Pox) in food have been studied and verified to mainly consist of formation of protein carbonyls (PC), loss of sulfydryl groups, and formation of protein cross-linking (Stadtman and Levine, 2000). These oxidized modification of protein backbone, and/or amino acid residue side chains can further lead to changes in functional properties of protein, including loss of essential amino acids, protein aggregation, and decreased susceptibility to protease (Kim et al., 2010; Utrera and Estévez, 2012; Youling et al., 2010). Studies in meat and/or milk system have proved that oxidized protein products (OPPs), such as methionine sulfoxide, PC, and dityrosine (Dityr), reduce the nutritional value of oxidized proteins (Dalsgaard et al., 2011; Meltretter et al., 2008; Stadtman, 1993).

Proteins in cells are also a major target for oxidants as a result of their abundance in biological systems and their high rate constants for reaction. Many studies have examined potential associations between oxidized protein products (OPPs) and relevant degenerative diseases. Abundant evidence indicated that PC, AOPPs, and Dityr play an essential role in a number of age-related diseases (e.g., Parkinson's Disease, asthma, and senescence) (Davies, 2005; Tong et al., 2012; Voss and Grune, 2006).

Following the above discoveries, it is noteworthy that if oral OPPs from food system can accumulate in cells or cause Pox in vivo, they may moreover have potential associations with some diseases. Epidemiological studies show that spray dried milk powder gave lower growth response (El-Shafei et al., 1988), and caused infants' eczema and dry stool (Beyerlein and Von Kries, 2011). Our recent study showed that oxidized casein caused redox stress in mice after short-term gavage (Wei et al., 2012; Zhuqing Leslie

Abbreviations: AOPPs, advanced oxidation protein products; ARE, antioxidant responsive element; Dityr, dityrosine; ET-1, endothelin-1; H–E, hematoxylin and eosin; MDA, malondialdehyde; OPPs, oxidized protein products; Pox, protein oxidation; PC, protein carbonyl; ROS, reduced oxygen species; TGF-β1, transforming growth factor-β1; Nrf2, nuclear factor erythroid 2-related factor.

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et al., 2013). These previous published investigations have provided some proof that oxidized protein may have potential damage to the body. However, mechanisms of the relationship between dietary Pox and its impact on human nutrition, including the effects on redox state, material and energy metabolism disorder, have been largely unexplored.

In this study, we performed oxidized casein (modified respectively by H_2O_2–Cu and HClO) feeding experiments using KM mice (20–22 g) to evaluate the relationship between oral oxidized casein and oxidative status, and to investigate whether oral oxidized casein will cause potential oxidized injury in vivo.

2. Materials and methods

2.1. Materials

Casein, bovine serum albumin (BSA), 5-amino-2,3,-dihydro-1,4-phthalazinedione (luminol) and horseradish peroxidase (HRP) were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Trizol reagent and Accu Power TM Green Star Qpcr PreMix kit were purchased from Applied Biosystems (Foster City, CA, USA). Dityr and AOPPs ELISA kits were obtained from Xiamen Huijia Bioengineering Institute (Xiamen, China). MDA, CAT, GPX and SOD kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals used in the experiments were of analytical grade.

2.2. Preparation of oxidized casein

Casein was dispersed in 0.05 M phosphate buffer pH 7.4 to get samples with concentration 20 mg/mL.

2.2.1. Casein oxidized by H_2O_2–Cu

The whole casein solution system was mixed in sealed tubes after adding H_2O_2 (5 mM)–$CuSO_4$ (0.05 mM), and was shaken at a property speed of 120 rpm in a thermostatic bath at 25 ± 0.1 °C for 24 h. Add 0.05 mM EDTA to end the reaction. Dialysis was applied to remove remaining hydrogen peroxide (Kurahashi et al., 2001).

2.2.2. Casein oxidized by HClO

HClO with a final concentration 5 mM were added. The whole solution system was shaken at a property speed of 120 rpm in a thermostatic bath at 25 ± 0.1 °C for 24 h. Dialysis was applied to remove the free HClO (Liu et al., 2006; Xiangwu et al., 2006).

2.3. Determination of casein oxidation

2.3.1. Determination of Dityr and AOPPs by ELISA assay kits

Dityr (or AOPPs) were assayed by ELISA kits and performed according to given guidelines. In short, Dityr (or AOPPs) was added to the microtiter plate wells which coated with purified mouse Dityr (or AOPPs) antibody. After 30 min of reaction and washing completely, HRP labeled Dityr (or AOPPs) antibody was added to the mixture to form an antibody–antigen–enzyme–antibody complex. After washing, the TMB substrate solution was added to react with HRP enzyme resulting in a blue color. To stop the reaction, sulfuric acid solution was added and the color changed to yellow which is detected spectrophotometrically at a wavelength of 450 nm. The concentration of Dityr (or AOPPs) in the samples is then determined by comparing the OD of the samples to the standard curve.

2.3.2. Determination of protein carbonyl (PC)

PC content was determined (2,4-dinitrophenylhydrazine, DNPH) using the method of Oliver et al. (1987) with slight modifications. Casein samples were suspended in deionized water to provide a casein solution of the concentration 3.0–3.5 mg/mL. In 2.0 mL capped polyethylene centrifuge tubes, 0.3 mL casein solution was mixed with 1.2 mL 10 mM DNPH in 2 N HCl and incubated at 25 ± 0.1 °C for 1.0 h in dark. A matching aliquot was mixed in 1.2 mL 2 N HCl as an absorbance blank. Then 1.5 mL 40% trichloroacetic acid (TCA) was added to each tube. The tubes were then vortexed, allowed to stand for 20 min, and centrifuged for 15 min at a speed of 12,000g. The supernatant was discarded, and the precipitate was washed three times with 1.0 mL ethyl acetate solution (1:1, v/v) and then was suspended in 1.25 mL 6 M guanidine hydrochloride solution by incubating at 37 °C for 15 min, with vortexing every 5 min. After centrifuge (12,000g, 15 min), the supernatant was collected and detected by the wavelength of 370 nm. The absorbance at 370 nm was corrected for the absorbance in the HCl blank, and the moles of carbonyl derivative per mg protein were calculated by using the extinction coefficient of 22,000 M^{-1} cm^{-1} (Boatright and Hettiarachchy, 1995).

2.4. Animal care and treatments

A total of 30 male KM mice of weight approximately 22 g (4 weeks old) were obtained from Suzhou University (Suzhou, China). Mice were housed under conditions of controlled temperature (26 ± 2 °C) and humidity (60%) with a 12 h light/dark cycle. The experimental protocol was developed according to the institution's guidelines for the care and use of laboratory animals.

After a 3-days acclimatization period, mice were randomly assigned to three groups (10 for each group): a normal diet was given to all animals for 10 days. Then after, the first group was treated with origin casein; second group with H_2O_2–Cu–oxidized casein; and third group with HClO–oxidized casein diet, for 10 weeks.

Mice were deprived of food for 12 h and then were slightly anesthetized and sacrificed by removing the eyeball at the end of the experimental period. Blood was collected from the orbital sinus into centrifugal tubes. Plasma obtained from blood samples after centrifugation (5000 rpm for 10 min at 4 °C) was frozen and stored at −20 °C for further measurement. Tissues, including liver and kidney, were separate on the basis of morphological features by an animal care technician and were rinsed with 0.1 mM pheylmethanesulfonyl fluoride in physiological saline and flash frozen in liquid nitrogen for further biomarker analyzes.

2.5. Determination of antioxidant enzyme activity and tissue oxidation

Levels of ROS were measured in the whole blood by luminol-dependent chemiluminescence assay described by Kobayashi et al. (2001), using MPI-B ultra-weak luminescence analysis system (Xi'an Remex Analysis Instrument Co., Ltd., (Xi'an, China). ROS production was expressed as relative light units (RLUs).

GSH and GSSG levels were determined according to the fluorimetric method of Hissin and Hilf (1976) by using *O*-pthalaldehyde (OPT) as a fluorescent probe. The method takes advantage of the reaction of OPT with GSH at pH 8.0 and with GSSG at pH 12.0, resulting in a highly fluorescent derivative, which is activated at 350 nm with an emission at 420 nm. MDA, CAT, GPX and SOD were assayed using kits as described by the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Total protein contents were determined by the method of Lowry et al. (1951), using BSA as a protein standard.

2.6. Gene expression in liver and kidney

For determining mRNA expression, total RNA was first extracted from frozen tissues with Trizol reagent. The quantity and quality of the RNA were verified by measuring the A_{260}/A_{280} ratio and by gel electrophoresis. Total RNA was reverse-transcribed to cDNA according to the manufacturer's instructions (MultiScribe Reverse Transcriptase; Applied Biosystems). The mRNA expression was quantified using Real-time Polymerase Chain Reaction (RT-PCR). The primer sequences are listed in Table 1.

RT-PCR was carried out using AccuPowerTMGreenStar Qpcr PreMix kit on a MyiQ Single Color Real-Time PCR Detection System (7900 HT Fast, ABI) using the following conditions: 40 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 30 s, and extension at 72 °C for 20 s. Porcine primers were designed using Primer3v.0.4.0 and synthesized by the Co. Generay Biotechnology (Shanghai, China). The relative expression levels of the target genes were calculated as a ratio to the housekeeping gene β-actin.

2.7. Preparation of tissue homogenates and nuclear extracts

Briefly, 100 mg of tissues (liver and kidney cortex) was homogenized in 0.5 mL of buffer A containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.8), 10 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM Phenylmethanesulfonyl fluoride (PMSF), 1 μM pepstatin, and 1 mM p-amino benzamidine using a tissue homogenizer for 20 s. Homogenates were kept on ice for 15 min, and then 125 μL of a 10% Nonidet p40 (NP-40) solution were added and mixed for 15 s and the mixture was centrifuged for 2 min at 12,000 rpm. The supernatant containing cytosolic proteins was collected. The pelleted nuclei were washed once with 200 μL of buffer A plus 25 μL of 10% Nonidet p40, centrifuged, then suspended in 50 μL of buffer B containing 50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% (v/v) glycerol, mixed for 20 min, and centrifuged for 5 min at 12,000 rpm. The supernatant containing nuclear proteins was removed carefully and stored at −80 °C (Kim and Vaziri, 2010). The protein concentrations in tissue homogenates and nuclear extracts were determined by using the Bicinchoninic Acid (BCA, Sigma, USA) protein assay according to the instructions of the manufacturer.

2.8. Western blot analysis

Protein extracts were separated on 10% SDS–PAGE gel using 10 μg protein per sample and then transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (pH7.4) containing 0.1% Tween-20. Subsequently, the blocked membrane was incubated with primary antibody including rabbit Nrf2 antibody (8882, Cell Signaling) and rabbit GAPDH antibody (8884s, Cell Signaling) at a dilution of 1:1000 at 4 °C overnight. Blots were incubated with IRDye