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Inhibitory Effect of Known Antioxidants and of Press Juice from Herring (*Clupea harengus*) Light Muscle on the Generation of Free Radicals in Human Monocytes

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Reactive oxygen species (ROS) can cause oxidative stress, which has been linked to various diseases. It has been suggested that antioxidant-rich foods can reduce such oxidative stress. However, the lack of suitable model systems to screen for in vivo effects of food-derived antioxidants has prevented a clear consensus in this area. In this study, the aim was to use a single-cell model system (human monocyte) to evaluate whether certain pure antioxidants and complex muscle extracts (herring light muscle press juice, PJ) could prevent ROS formation under in vivo like conditions. ROS were excreted from the monocytes upon stimulation with phorbol myristate acetate and were then detected as isoluminol-enhanced chemiluminescence (CL). Adding 2000 units of catalase and 50 units of superoxide dismutase to the monocytes model lowered the CL response by 35 and 86%, respectively. Ascorbate (14.1 mM) lowered the response by 99%, α-tocoperhol (188 μM) by 37%, and Trolox (50 μM) by almost 100%. Crude herring PJ gave a dose-dependent reduction in the CL response. At 10, 100, and 1000 times dilution, the PJ reduced the CL signal by 93, 60.5, and 10.6%. PJ fractionated into low molecular weight (LMW) (<1000 Da) and high molecular weight (>3500 Da) fractions decreased the CL response by 52.9 and 71.4%, respectively, at a 100-fold dilution. Evaluation of the PJ samples in the oxygen radical absorbance capacity test indicated that proteins may be the primary radical scavenging compounds of PJ, whereas the ROS-preventing effect obtained from the LMW fraction may also be attributed to other mechanisms. Thus, this study proved that the monocyte assay can be a useful tool for studying whether food-derived antioxidants can limit ROS production under physiologically relevant conditions. It also showed that herring contains numerous aqueous compounds demonstrating antioxidative effects in the monocyte model system.

KEYWORDS: Antioxidant; monocyte; reactive oxygen species; ROS; herring; *Clupea harengus*; press juice; ORAC

INTRODUCTION

It is generally agreed that endogenously and exogenously produced reactive oxygen species (ROS) can cause oxidative stress, which in turn is associated with various diseases, such as cancer and cardiovascular diseases (*1*, *2*). It has been speculated for a long time that oxidative stress can be reduced by certain antioxidant-rich foods. This has warranted extensive antioxidant research and screening for antioxidant activity in both whole foods and food extracts. However, in recent years, model systems and analyses used to study the function of dietary antioxidants under conditions relevant to humans have been a matter of increasing debate (*3*). Many of the used models were originally developed for ex vivo food research; thus, they do not take into account aspects such as the cellular metabolic complexity or the physiological conditions. Both the physical and chemical conditions surrounding an antioxidant will largely affect its degree of protonation, solubility, and (if proteins) denaturation. Therefore, the oxidation/antioxidation reactions themselves will also be affected (*4*). Thus, to accurately evaluate the potential of antioxidants in the human body, models must be developed that have the chemical, physical, and environ-

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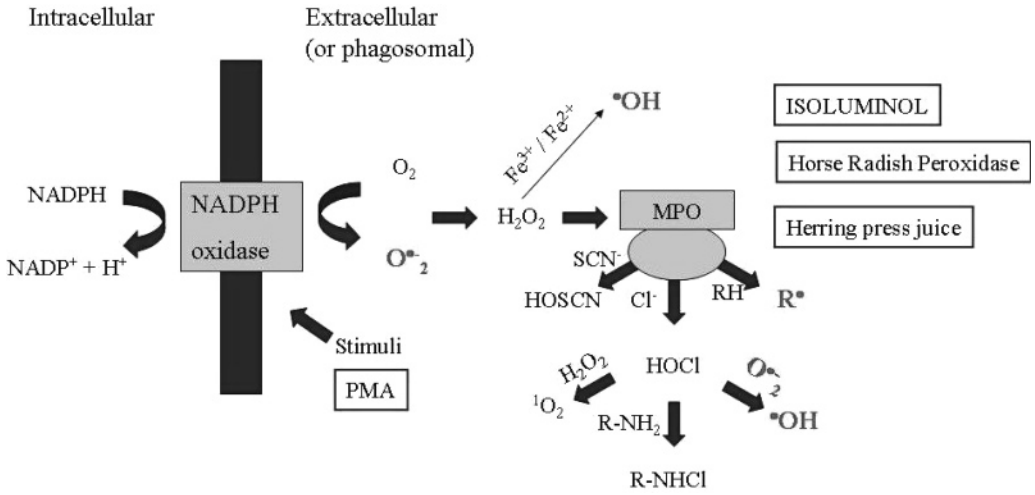

Figure 1. Schematic description of the monocyte model system, describing possible oxidant generating reactions with stimulated monocytes. MPO, myeloperoxidase; PMA, phorbol myristate acetate. Adapted from Hampton et al. (*49*).

mental conditions expected there. The best alternative is clearly human and animal models. However, due to cost, time, and a lack of reliable indirect biomarkers (i.e., relatively stable products) of in vivo oxidation, there is an urgent need for simpler screening models to use in the first step. Here, adequate cell culture methods can play a major role. Cell culture methods have been used extensively in the investigation of anti-inflammatory and inflammatory products as well as studies of oxidative stress. Compared to in vivo experiments, they have the advantages of relative reproducibility and reduced cost. Moreover, cell culture methods can be applied without major ethical concern as compared to higher models.

Here we investigate a cellular model system that has proven to be sensitive to antioxidant activity in some previous studies (*5*−*7*). The model, which is schematically shown in **Figure 1**, is based on ROS production from human monocytes after stimulation of the NADPH oxidase enzyme (*6*, *7*). NADPH oxidase catalyzes the reduction of O_2 to the superoxide anion ($O_2^{\bullet-}$), which has a strong tendency to dismutate, for example, into hydrogen peroxide (H_2O_2). The ROS are detected in terms of isoluminol-enhanced chemiluminescence (CL) (*6*, *7*). Scavenging of ROS, and thus reduction of the CL signal in this system by exogenously added compounds, could then indicate that they have a physiological antioxidative capacity. It was previously shown that ROS production from phorbol myristate acetate (PMA)-stimulated human monocytes and neutrophils could be suppressed by singlet oxygen energy (SOE) (*7*) and by superoxide dismutase (SOD) and catalase (*5*, *6*) as well as by *trans*-resveratrol (*8*). Other than these few antioxidant trials, the monocyte system has mostly been used to follow disease activity, to screen granulocytes for defects in oxidative metabolism, and to study the effects of pharmacological agents on metabolism in granulocytes (*5*).

So far, dietary antioxidants have mostly been a matter of study in plants, with an enormous interest in wines, spices, fruits, and vegetables. However, there are recent results pointing at strong antioxidant activity also in extracts/press juices (PJ) from muscle. This activity has so far only been shown under food-like condition, with most work done in a model system consisting of washed minced cod muscle (*9*). During 10 days of ice storage, PJ from cod and other white fish species fully inhibited the onset of hemoglobin-mediated membrane lipid oxidation in the cod system. Similar antioxidative activities have also been shown in PJ from chicken breast (*10*) and herring light muscle (*11*). Herring PJ has previously also shown protective effects against iron ascorbate stimulated oxidation of isolated fish muscle microsomes (*12*), and emulsified linoleic acid (*13*). It would be of great interest to investigate whether fish muscle PJ could assert antioxidative effects also under physiological conditions relevant for humans. Such knowledge would add to the general debate of dietary antioxidants and health, it would broaden the spectra of possible antioxidant-containing foods, and it would help confirm whether the n-3 fatty acids are the only compounds that have given rise to the numerous positive health effects seen from fish-containing diets (*14*, *15*). It has been speculated that alterations in the eicosanoid metabolism caused by the n-3 fatty acids cannot alone explain the reduced risk for cardiovascular morbidity and mortality, for example. One possible additional pathway through which fish could prevent such disease would be to reduce the release of ROS.

The aims of this study were twofold. First, it was to deepen the existing knowledge on the monocyte-based model as a cellular tool to study exogenously added antioxidants. For this purpose, purified enzymic and nonenzymic antioxidants were tested against the generation of ROS by PMA-stimulated human monocytes. The second aim was to evaluate whether native and fractionated herring light muscle PJ could prevent ROS formation under the physiological conditions of the assay. To confirm whether the response given by the highly complex PJ samples was due to radical scavenging or other mechanisms, the same samples were also analyzed by the oxygen radical absorbance capacity (ORAC) test. To allow for a better understanding of the antioxidative mechanism of herring PJ, a range of compositional analyses were finally to be undertaken.

MATERIALS AND METHODS

Chemicals. Krebs−Ringer phosphate buffer with glucose (KRG), isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione), horseradish peroxidase (HRP), and PMA were purchased from Sigma-Aldrich (Schnelldorf, Germany). Roswell Park Memorial Institute (RPMI) 1640 medium and phosphate-buffered saline (PBS) (with and without Ca^{2+} and Mg^{2+}) were purchased from Göteborgs Termometerfabrik (Göteborg, Sweden). Ficoll Paque Plus was purchased from Amersham Biosciences (Uppsala, Sweden). Ascorbate (purity > 98%) and α-tocopherol (purity > 99%) were purchased from Sigma-Aldrich (Stockholm, Sweden). Beef liver catalase and bovine SOD were purchased from Roche Diagnostics Scandinavia AB (Bromma, Sweden).

Fluorescein (3′,6′-dihydroxyspiro[isobenzo-furan-1[3*H*],9′[9*H*]-xanthen]-3-one) was purchased from Fluka (Sigma Aldrich, Steinheim, Germany), 2,2′-azobis(2-methylpropionamidine)dihydrochloride (AAPH), and Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) were purchased from Sigma Aldrich (Steinheim, Germany).

Fresh herring (*Clupea harengus*) was caught in September 2004 on fishing grounds west of Sweden. It was obtained round from Paul Mattsson AB (Ellös, Sweden) and transported on ice to our laboratory (~1 h). Directly after arrival, the light muscle was manually dissected from the whole fish and ground using an ultrapower grinder (model KS M90, KitchenAid Inc., St. Joseph, MI) with a plate hole diameter of 5 mm.

Preparation and Fractionation of Herring Press Juice. The herring light muscle mince was packed in 200 mL polypropane centrifuge bottles. PJ was obtained by centrifuging the mince at 18368*g* for 2 h at 4 °C. The liquid forming the supernatant was filtered through a Whatman no. 1 filter paper and then frozen at −80 °C.

To obtain the low molecular weight (LMW) fraction, frozen herring light muscle PJ was thawed under cold running water and then subjected to ultrafiltration using a 1000 Da membrane (Millipore, Bedford, MA) and a stirred 50 mL Amicon Ultrafiltration device (model 52, Amicon Corp., Danvers, MA) (*9*).

To obtain the high molecular weight (HMW) fraction, thawed herring light muscle PJ (5 mL) was dialyzed in 3500 Da dialysis tubing (Spectra/Por Membrane, Spectrum, Rancho Dominguez, CA) at 4 °C against (0.5 L) physiological saline buffer (PBS) for 24 h. The buffer was changed three times during this period. During the dialysis operation, the dialysate was diluted 1.24 times, which was corrected for during runs in the monocyte assay.

Compositional Analyses of Herring Press Juice. *Moisture Content, pH, and Conductivity.* The moisture content of press juices was measured using an HA300 moisture balance (Precisa Balance 310M). The pH was recorded with an Orion PerpHect Sure-Flow electrode (Orion Research Inc., Beverly, MA) in conjunction with a pH-meter (model PHM 210; Meter Lab, Radiometer Analy SAS, Villeurbanne Cedex, France). Conductivity was measured with a conductivity meter (CDM 210, Meter Lab, Radiometer Analy SAS).

Proteins and Polypeptides. Total protein measurements were done according to the method of Lowry et al. (*16*) as modified by Markwell et al. (*17*). Quantification of the hemoglobin (Hb) levels in PJ samples was done according to the method of Brown (*18*), as modified by Richards and Hultin (*19*).

The polypeptide pattern of native herring PJ and the HMW fraction was analyzed with SDS-PAGE according to the method of Laemmli (*20*) using a Ready gel cell unit, precast linear minigels (4−20%), buffers, and staining and destaining solutions from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). To confirm the purity of the LMW fraction, the same SDS-PAGE setup was used, but with 10−20% gels.

Lipids. Total lipids were analyzed in the three PJ samples using the method from Lee et al. (*21*) with chloroform and methanol (1:1) as the extraction solvent.

Ascorbic Acid and Uric Acid. Ascorbic acid and uric acid of the three PJ samples were analyzed according to the methods of Margolis et al. (*22*) and Margolis and Davis (*23*) with some modification of the sample preparation step. Here, samples were diluted to required level with 3% metaphosphoric acid (MPA) containing 0.1% dithiothreitol (DTT). Diluted samples were vortexed for 1 min and centrifuged at 12000*g* for 1 min. Five microliters of supernatant was then analyzed by high-performance liquid chromatography (HPLC) (Jasco HPLC unit, AS-2057plus) using an Aquasil C18 column (150 × 4.6 mm, particle size = 3 μm, Thermo Hypersil-Keystone, Chrom Tech AB). The flow rate of the mobile phase was 0.8 mL/min. Eluted components were detected by electrochemical detector (Decade II, Antec, Leyden, The Netherlands), and the area was compared with that of ascorbic acid and uric acid standards. Data were collected and evaluated with Chromeleon chromatographic system (Dionex Corp., Sunnyvale, CA).

Amino Acids. Free amino acids and total amino acids were analyzed with HPLC according to the method of Fontaine and Eudaimon (*24*).

Mineral Content. Minerals (Fe, Cu, Zn) were determined by ion chromatography according to the method of Fredriksson et al. (*25*).

Isolation of Human Monocytes. Buffy coats, blood cell concentrates, prepared from healthy human donors were obtained from Blodcentralen at Sahlgrenska University Hospital (Göteborg, Sweden). The buffy coats were diluted with 1 volume of PBS containing 10 mM EDTA, and a Ficoll−Hypaque density gradient centrifugation (2900 rpm for 20 min) was performed. Monocytes were collected from the interface and were washed three times with PBS containing 10 mM EDTA. The cells were diluted in RPMI 1640 medium, supplemented with nonessential amino acids, sodium pyruvate, and antibiotics and allowed to adhere to cell culture flasks (Nunclon, Nunc, Naperville, IL) (coated with inactivated human serum) for 1 h at 37 °C. Nonadherent cells were removed by rinsing three times with PBS (with Ca^{2+} and Mg^{2+}). By incubation of the remaining monocytes with cold PBS (without Ca^{2+} and Mg^{2+}) containing 5 mM EDTA and 2% fetal calf serum for 20 min in 4 °C, the cells could be detached. Then the cells were washed once with Krebs−Ringer bicarbonate buffer supplemented with glucose (KRG) and finally suspended in KRG to a cell concentration of 5×10^6 cells/mL. They were kept in a bath of melting ice immediately after preparation.

Chemiluminescence. To detect extracellular production of ROS in PMA-stimulated monocytes, the sensitive CL technique was used (*26*). The luminometer used was from Bio-Orbit 1251 with Multiuse Software (Bio-Orbit Oy, Turku, Finland). The concentration of human monocytes in the reaction mixture was 5×10^5 viable cells. The mixture also contained 5.6×10^{-5} M isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione), 4 units of HRP, and the antioxidant solution to be tested (see below). To activate the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, PMA (final concentration = 1×10^{-8} M) was added just before the start of the CL analyses. The reaction volume was adjusted to 1 mL with KRG. The CL was recorded at 37 °C until the maximal peak values were reached, which varied with different antioxidants. The results were expressed in millivolts (mV), produced from 5×10^5 viable cells. To estimate the antioxidative activity of the different samples tested, either the maximum CL intensity (referred to as peak intensity or "shoulder") or the area under the CL curve was calculated and compared to the corresponding values for control samples containing buffer instead of antioxidants.

Samples that were tested for their antioxidative capacity included (i) catalase (final concentration in assay = 2000 units, diluted in PBS), (ii) SOD (final concentration = 50 units, diluted in PBS), (iii) ascorbic acid (final concentration = 2.5 mg/mL, i.e., 14.1 mM, diluted in KRG), (iv) α-tocopherol (final concentration = 1 mg/mL, i.e., 1.88 mM) (stock solutions were prepared in ethanol and then diluted to final concentration in KRG), (v) Trolox (final concentrations = 0.5, 1, 10, and 50 μM diluted in KRG), (vi) native herring light muscle PJ, (vii) ultrafiltered PJ (<1 kDa, LMW fraction), (viii) dialyzed PJ (>3.5 kDa, HMW fraction) (final concentrations of vi−viii were 10, 100, and 1000 times diluted in KRG), and (ix) bovine serum albumin (BSA) (final concentration = 1.84 mg/mL, diluted in KRG).

Oxygen Radical Absorbance Capacity Analyses to Test for General Radical Scavenging Capacity of Press Juice at 37 °C. The ORAC test (*27*) was applied on the PJ samples. Two small modifications were that the fluorescence was recorded every 30 s instead of every minute and the total assay time was extended from 80 to 120 min. The assay was performed on Safire^2, a monochromator-based microplate detection system with the software Magellan, both from Tecan Nordic AB (Mölndal, Sweden). In combination with this equipment, black 96-well untreated FluoroNunc microplates (Nunc, Roskilde, Denmark) were used. The final volume in each well was 200 μL. First, 20 μL of sample, standard, or blank (75 mM phosphate buffer) and 120 μL of 0.117 μM fluorescein solution (final concentration in well = 70 nM) were pipetted into the wells of the microplate. This mixture was preincubated for 15 min at 37 °C. Then 60 μL of 40 mM AAPH solution (final concentration in well = 12 mM) was rapidly added to each well using a multichannel pipet. The plate was immediately placed in the reader (preheated to 37 °C) and the fluorescence measured. The plate was automatically shaken prior to each measurement.

Statistics. All of the antioxidant samples, except Trolox and BSA, were tested in monocytes from six different cell donors ($n = 6$). Each antioxidant sample was then analyzed (*a*) in duplicate on each prep-

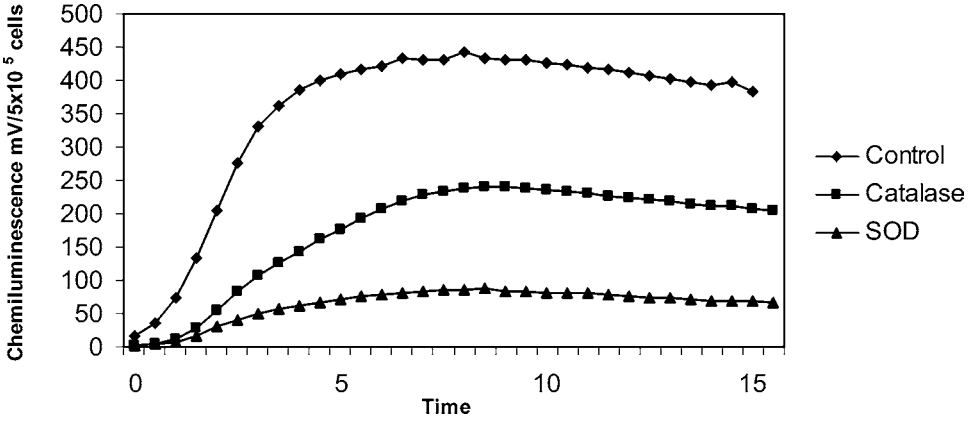

Figure 2. Effect of adding 50 units of SOD and 2000 units of catalase on PMA-induced CL from monocytes compared with control. The assay was performed with 5×10^5 cells, 4 units of HRP, 5.6 μM isoluminol in KRG, and 10 nM PMA. Representative curves are shown from one of the cell donors ($n = 6$ different cell donors with each sample run in duplicate).

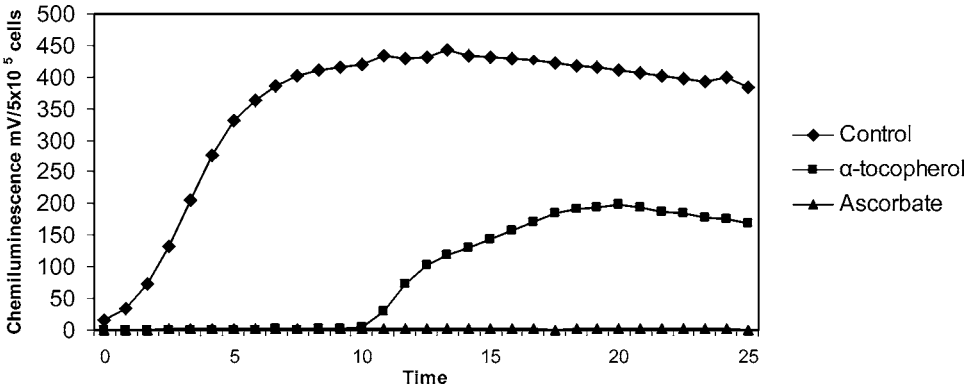

Figure 3. Effect of ascorbate (2.5 mg/mL, i.e., 14.1 mM) and α-tocopherol (0.1 mg/mL, i.e., 188 μM) on PMA-induced CL from monocytes compared with control. The assay was performed with 5×10^5cells, 4 units of HRP, 5.6 μM isoluminol in KRG, and 10 nM PMA. Representative curves are shown from one of the cell donors ($n = 6$ different cell donors, with each sample run in duplicate).

aration of monocytes. For Trolox, four cell donors were used ($n = 4$), and for BSA two cell donors were used ($n = 2$).

ORAC analysis was carried out on eight replicates of each PJ sample (i.e., $n = 8$). Every dilution of a sample was analyzed in at least triplicates (i.e., $a \geq 3$ for each n). pH, conductivity, total proteins, total lipids, and Hb were analyzed on triplicate samples ($n = 3$). All other analyses were made on duplicate samples.

In cases where $n \geq 3$, results are expressed as mean ± standard deviation (SD). Where $n = 2$, mean ± (max−min)/2 value are given.

Differences between the different fractions of herring press juice were tested for significance with one-way ANOVA (statistical analysis was done with SPSS 12.01 for Windows).

RESULTS

Evaluation of the Monocyte Model Using Well-Known Antioxidants. Two enzymic and four nonenzymic antioxidants were added to the monocyte model to test their effect on ROS generated from PMA-stimulated human monocytes. The enzymes were catalase and SOD, and the nonenzymic ones were ascorbate, α-tocopherol, BSA, and Trolox. Catalase, SOD, ascorbate, and α-tocopherol were added at levels found to be efficient in previous work (*6*), whereas BSA was added to match the total protein content of native herring PJ (at the 1/100 dilution). Trolox levels were based on preliminary data on total phenolic content in fish PJ.

Effect of Enzymic Antioxidants on Reactive Oxygen Species. In monocytes incubated with 2000 units of catalase and 50 units of SOD, the maximum CL response was lowered by an average of 35 and 86%, respectively, compared to control cells (207.8 ± 55.9 and 44.8 ± 33 mV/5 × 10^5 cells, respectively, versus 320.7 ± 97.1 mV/5 × 10^5 cells) (mean ± SD, $n = 6$) (**Figure 2**).

Effect of Nonenzymic Antioxidants on Reactive Oxygen Species. In monocytes incubated with 14.1 mM ascorbate, the average inhibition of the maximum CL peak was 99% compared to control cells (3.3 ± 0.5 mV/5 × 10^5 cells versus 320.7 ± 97.1 mV/5 × 10^5 cells) (mean ± SD, $n = 6$) (**Figure 3**).

In monocytes incubated with 188 μM α-tocopherol, a 10 min lag phase was induced, and the average inhibition of the maximum CL response was 37% compared to control cells (202.6 ± 8.3 mV/5 × 10^5 cells versus 320.7 ± 97.1 mV/5 × 10^5 cells (mean ± SD, $n = 6$) (**Figure 3**).

Figure 4 summarizes the average (±SD) maximum CL intensity after testing six different batches of monocytes with buffer (controls), catalase (2000 units), α-tocopherol (0.1 mg/mL, i.e., 188 μM), SOD (50 units), and ascorbate (2.5 mg/mL, i.e., 14.1 mM), respectively.

Trolox was tested at final concentrations of 0.5, 1, 10, and 50 μM (results not shown in graph). Adding 0.5 and 1 μM had little effect on the ROS production and only reduced the total CL area by only 7−10% ($n = 4$). Adding 10 μM Trolox reduced the CL area by 87.3% ($n = 4$), and a 50 μM concentration fully inhibited any ROS production.

BSA at a final concentration of 1.8 mg/mL on average reduced the total CL area by 72% ($n = 2$) (results not shown in graph).

Effect of Whole and Fractionated Herring Light Muscle Press Juice on Reactive Oxygen Species Production from

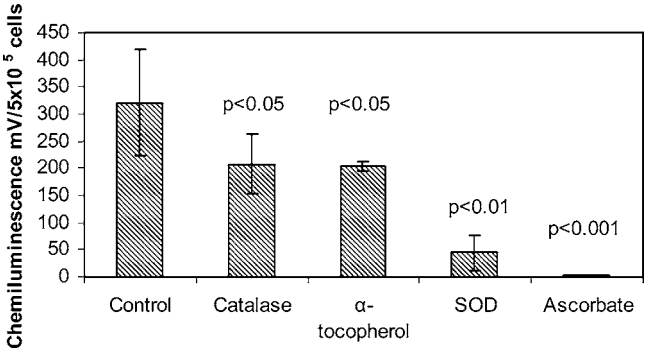

Figure 4. Summary of how catalase (2000 units/mL), α-tocopherol (0.1 mg/mL, i.e., 188 μM), SOD (50 units), and ascorbate (2.5 mg/mL, i.e., 14.1 mM) reduced the maximum CL peak obtained in the monocyte assay. The assay was performed with 5×10^5 cells, 4 units of HRP, 5.6 μM isoluminol in KRG, and 10 nM PMA. The results are the mean ± SD from six experiments, each conducted with monocytes from different donors.

Human Monocytes. Figure 5 shows how the response of the human monocyte ROS system to the herring PJ was dose-dependent when the PJ was tested in different dilutions (from 1/10 to 1/1000). The average reductions in the maximum CL peak obtained were 93, 60.5, and 10.6%, respectively, at the 1/10, 1/100, and 1/1000 dilutions. To take into consideration the somewhat different kinetics obtained, the results were also expressed at percent reduction of the total CL area and then were 94.6, 57.2, and 15.6%, respectively.

Figure 6 shows how the relative inhibitions of the total CL area by herring PJ (at a 1/100 dilution) were strikingly similar among six different cell donors, although the absolute values obtained for each monocyte batch differed (**Figure 5**). In this experiment, the average reduction in CL area given by a 1/100 dilution of herring PJ became 86.7 ± 5.9%, which was a higher area reduction than the one shown in **Figure 5** (57.2%). The PJ batch used in **Figure 6** had a higher protein content than the one shown in **Figure 5** (183.6 vs 94.4 mg/mL, respectively), which could be an underlying reason.

To differentiate between the antioxidative roles of proteins versus LMW substances in herring PJ, it was fractionated into LMW (<1000 Da) and HMW (>3500 Da) fractions. **Figure 7** shows representative curves from one of six cell donors when the LMW and HMW fractions were tested together with native PJ (all at a 1/100 dilution). The native herring PJ, the HMW fraction, and the LMW fraction here decreased the maximum CL peak by 83.3 ± 6.4, 71.4 ± 71,1 and 52.9 ± 10% (mean ± SD, $n = 6$). When expressed as a decrease of the total CL area, the corresponding results were 86.7 ± 5.9, 71.1 ± 10.2, and 55.2 ± 12.0% (mean ± SD, $n = 6$), respectively. The difference between the three groups was significant ($p < 0.001$). It is also shown in **Figure 7** how the protein-free LMW fraction gave kinetics more resembling the control, that is, a sharper and faster CL peak, than the two protein-containing samples.

Peroxyl Radical Scavenging Activity of Native and Fractionated Herring Light Muscle Press Kuice Measured as Oxygen Radical Absorbance Capacity Values. To confirm whether the reduced CL signals given by the three PJ samples were due to radical scavenging, the same samples were also analyzed in the ORAC test. It was found that the native PJ, LMW fraction, and HMW fraction gave rise to ORAC values of 26.7 ± 5.1, 3.1 ± 0.4, and 9.0 ± 1.7 mmol of Trolox equivalents/L of PJ sample (mean ± SD, $n = 8$), respectively. Thus, the LMW and HMW fractions had only 11.5 and 33.8%, respectively, of the activity of native PJ.

Compositional Characteristics of the Herring Light Muscle Press Juice. To allow for speculation on what compounds in the three herring light muscle PJ samples could contribute to the data on ROS production of the monocytes, analyses of dry matter, pH, conductivity, proteins, lipids, polypeptides, amino acids, ascorbic acid, uric acid, and trace minerals were done.

All compositional results, except individual amino acids, are shown in **Table 1**. The pH of all three PJ samples was ~6.7 and the conductivity ~13−16 mS/cm. Lipids were absent in both native PJ and subfractions. Proteins were present at 183.6, 139.4, and 1.39 mg/mL in the native PJ, the HMW fraction, and the LMW fraction, respectively. The lower protein concentration in the HMW fraction compared to native PJ was due to a slight dilution during dialysis. The small response in the Lowry method given by the LMW fraction was due to very small peptides. This was confirmed by the absence of polypeptide bands when the LMW fraction was subjected to SDS-PAGE (data not shown). The SDS-PAGE analyses also illustrated how the polypeptide patterns of the native PJ and HMW fractions were more or less identical, most polypeptides ranging from 7 to 97 kDa (**Figure 8**). The levels of hemoglobin were 11.5 and 13.7 μM in the native PJ and HMW fraction, respectively. Ascorbic acid and uric acid were found at levels of around 20 and 5 μM, respectively, in the native and LMW fractions. There were 2.0 versus 1.3 μg of Fe and 3.0 versus 2.4 μg of Zn per gram of native and HMW PJ samples, respectively. The LMW fraction was deficient in trace metals, indicating that all metals were bound to proteins or polypeptides of >1 kDa. Copper was found in only the native PJ and there at <0.10 μg/g.

Table 2 shows total amino acids of the three PJ samples. In the native PJ and HMW fractions, the amino acids present at the highest levels were aspartic acid, glutamic acid, alanine, leucine, and lysine (4.95−10.55 g/kg). In the LMW fraction, detectable levels (0.1−1.05 g/kg) were found for only glutamic acid, glycine, alanine, valine, histidine, and lysine.

In **Table 3**, the composition of free amino acids is shown. As expected, the HMW fraction was deficient in such acids, whereas the results from the native PJ and LMW fraction reflect each other. The most abundant free amino acids were glycine, alanine, taurine, and histidine; they were present at levels of 0.93−1, 0.4−0.44, 0.43−0.46, and 0.91−0.92 g/kg, respectively.

DISCUSSION

ROS generated by the phagocyte NADPH oxidase are under normoxic conditions important components involved in host defense. However, when excreted in uncontrolled levels, such as during inflammation or postischemic reperfusion, these highly toxic oxidants can cause significant tissue injury. Tight regulation of their generation and inactivation is thus essential.

It is suggested that antioxidant-rich diets may play a major role in preventing disease caused by oxidative injuries. So far, this assumption originates mostly in epidemiological data and in radical scavenging and/or reducing properties observed ex vivo, under food-like conditions (*28*). Solid evidence on the role of dietary antioxidants in vivo or under in vivo like conditions is still scarce. In comparison with food conditions, the latter include among other things higher pH (7.4 instead of ≤7), higher temperature (37 °C instead of ambient, chilled, or frozen storage temperatures), often another ionic strength, and another set of oxidation initiators (mostly enzymic instead of nonenzymic). In this study, it was evaluated whether PMA-stimulated human monocytes (**Figure 1**) could become a model, which is one step closer toward an in vivo situation as compared to classic chemical "test tube" antioxidant methods.

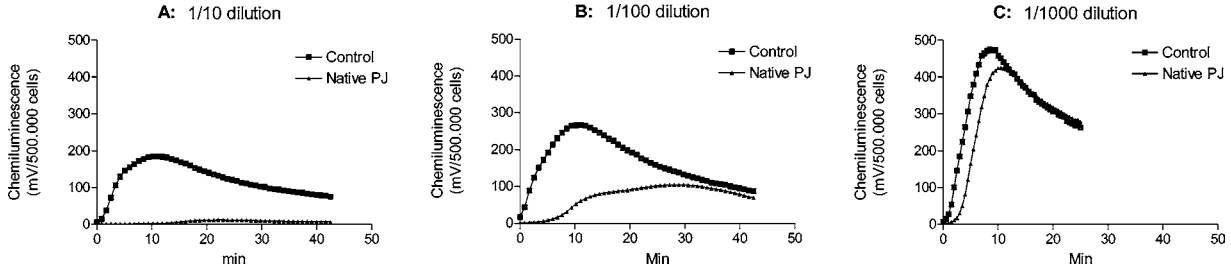

Figure 5. Effect of different dilutions (**A**, 1/10; **B**, 1/100; **C**, 1/1000) of native (unfractionated) herring light muscle PJ on the extracellular ROS production of human monocytes induced by 10 nM PMA. The assay was performed with 5×10^5 cells, 4 units of HRP, 5.6 μM isoluminol in KRG, and 10 nM PMA. Control contains buffer instead of PJ. Representative curves are shown from one of the cell donors ($n = 6$ different cell donors, with each sample run in duplicate).

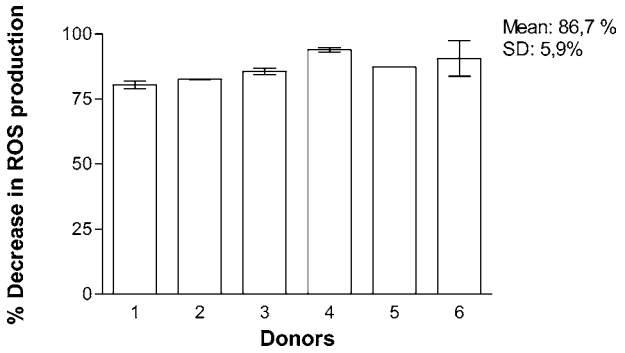

Figure 6. Individual responses from six different cell donors regarding the relative reduction of the total CL area asserted by herring PJ at a 1/100 dilution. The assay was performed with 5×10^5 cells, 4 units of HRP, 5.6 μM isoluminol in KRG, and 10 nM PMA. The bars show mean CL area reduction ± SD when using monocytes from each of six different donors.

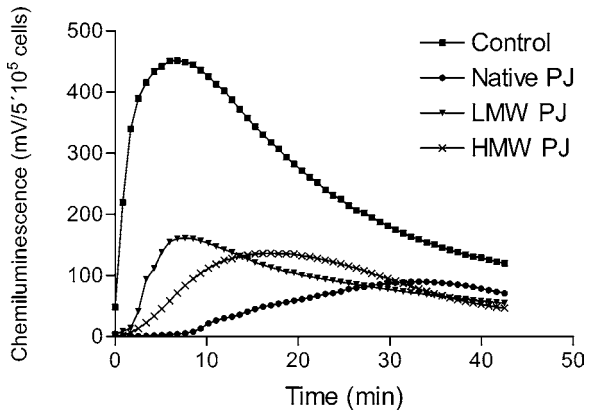

Figure 7. Effect of native herring light muscle PJ as well as LMW (<1000 Da) and HMW (>3500 Da) fractions on the CL from monocytes induced by 10 nM PMA. The assay was performed with 5×10^5 cells, 4 units of HRP, 5.6 μM isoluminol in KRG, and 10 nM PMA. Control contains buffer instead of PJ. Representative curves are shown from one of the cell donors ($n = 6$ different cell donors, with each sample run in duplicate).

When monocytes are stimulated with PMA, the enzyme NADPH oxidase generates ROS such as superoxide anions ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). After reaction with peroxidases, both the monocyte-derived granule enzyme myeloperoxidase (MPO) (*5*) and extra externally added HRP, the ROS can be detected in terms of isoluminol-enhanced CL (*5*−*7*). Isoluminol cannot penetrate cell membranes, ensuring that only extracellularly excreted ROS are detected in the assay (*6*). As shown in **Figure 1**, the toxicity of $O_2^{\bullet -}$ can in vivo be further mediated through the secondary generation of HOCl from H_2O_2 and Cl^- by MPO.

Monocytes are thought to play a major role in the pathogenesis of atherosclerosis (*29*) and, therefore, modulating its activation process is a potential therapeutic target (*30*). The adherence and migration of circulating monocytes into the subendothelial space initiates fatty streak formation. From entering a differentiation program, the monocytes are then converted to macrophages, which accumulate oxidized LDL to form the foam cell. Macrophage oxidation of LDL is thought to be dependent on ($O_2^{\bullet -}$) formed by NADPH oxidase (*31*). Numerous cell-signaling intermediates are involved in the activation of this enzyme, some that are also involved in chemotaxis and phagocytosis (*32*). Activation of various cell signaling intermediates such as phopshatidylinositol-3-kinase (PI3K) is thought to enhance ROS production through a so-called "priming" mechanism. Antioxidants added to the monocyte system can thus both directly scavenge ROS produced according to a biologically highly relevant pathway and also inhibit, e.g., kinases involved in ROS production.

In the absence of any antioxidant, the CL response given by the monocytes used in our experiments gave rise to sigmoid curves with a shoulder peak developing after 5−10 min of recording (**Figures 2**, **3**, **5**, and **7**). After the shoulder, the CL response either remained fairly stable (**Figures 2** and **3**) or declined (**Figures 5** and **7**). The CL intensity at the shoulder varied with different blood donors, from 170 to almost 500 mV/5 $\times$ 10^5 cells, which could possibly be due to individual differences in, for example, NADPH oxidase and/or endogenous MPO activity. There was a tendency toward lower shoulder peak values when the purified monocytes had aged for a few hours, such as during the last experiments of the day. However, as shown by subsequent tests with antioxidants/antioxidant extracts, the *relative* inhibitions obtained using different blood donors were strikingly similar (**Figure 6**). This is an important feature of the model for further use in antioxidant research.

The response from SOD and catalase in the monocyte system was tested to elucidate what was the relative importance of different extracellularly excreted ROS in the PMA-induced CL reaction. Catalase and SOD are macromolecular proteins and, thus, just like isoluminol, react only with extracellularly excreted ROS. SOD catalyzes the dismutation of the superoxide anion radical to hydrogen peroxide, whereas catalase catalyzes the dissociation of hydrogen peroxide to dioxygen and water. Although SOD was tested at a much lower level than catalase (50 units compared to 2000 units), SOD reduced the maximum CL response to a higher degree than catalase (86 vs 35%). This indicates that $O_2^{\bullet -}$ is of much greater importance than H_2O_2 in the isoluminol-amplified CL response. This result is strengthened by results from previous studies in which SOD and catalase were tested at the same concentrations as here (*6*) or at similar concentrations (500 units of SOD and 2000 units of catalase)

Table 1. Compositional Data of Native Herring Press Juice, the Low Molecular Weight (LMW, <1000 Da) Fraction, and the High Molecular Weight (HMW, >3500 Da) Fraction

component	herring whole PJ	herring LMW (<1000 Da)	herring HMW (>3500 Da)
dry matter[a] (%)	11.6 ± 0.03	3.22 ± 0.0	7.76 ± 0.01
pH[b]	6.70 ± 0.032	6.72 ± 0.01	6.73 ± 0.02
conductivity[b] (mS/cm)	13.38 ± 0.02	12.90 ± 0.04	16.05 ± 0.04
total lipids[b] (%)	nd	nd	nd
total protein[b] (mg/mL)	94.4 ± 1.4 to 183.6 ± 4.1	1.39 ± 0.2	139.5 ± 3.74
total hemoglobin[b] (μM)	11.47 ± 1.65	nd	13.72 ± 0.48
ascorbic acid[a] (μM)	21.83 ± 0.33	19.20 ± 0.25	<0.01
uric acid[a] (μM)	6 ± 0.11	5.5 ± 0.15	nd
Fe[a] (μg/g)	2.0 ± 0.11	nd	1.3 ± 0.06
Cu[a] (μg/g)	<0.10	nd	nd
Zn[a] (μg/g)	3.0 ± 0.06	nd	2.4 ± 0.10
total amino acids[a] (g/kg)	96 ± 1.25	3.05 ± 0.20	71.8 ± 1.65

[a] Mean of duplicate samples ± (max–min)/2. [b] Mean of triplicate samples ± standard deviation.

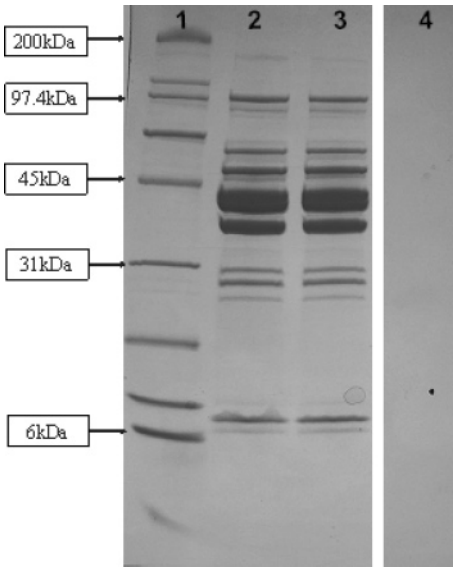

Figure 8. SDS-PAGE of native herring PJ and the HMW and LMW fractions: (lane 1) standards; (lane 2) native herring PJ; (lane 3) HMW fraction; (lane 4) LMW fraction [the gel used for lane 4 was a precast linear minigel (10–20%)]. The gels were precast linear minigels (4–20%), and 20 μg of protein/lane was loaded.

Table 2. Composition of Total Amino Acids in Native Herring Light Muscle Press Juice, the Low Molecular Weight (LMW, <1000 Da) Fraction, and the High Molecular Weight (HMW, >3500 Da) Fraction[a]

	g/kg		
	native herring PJ	LMW (<1000 Da) fraction	HMW (>3500 Da) fraction
cysteine	2.6	<0.1	1.05
methionine	2.85	<0.1	2.05
aspartic acid	10.55	<0.1	8.2
threonine	4.1	<0.1	3.55
serine	3.35	<0.1	3.15
glutamic acid	9.4	0.1	7.05
proline	3.45	<0.1	2.6
glycine	5.8	1.05	3.65
alanine	6.7	0.5	4.95
valine	6.55	0.1	5.15
isoleucine	5.3	<0.1	4.15
leucine	7.8	<0.1	6
tyrosine	3.25	<0.1	2.5
phenylalanine	5.15	<0.1	4.05
histidine	4.55	1	2.6
ornithine	<0.1	<0.1	<0.1
lysine	9.55	0.3	7.3
arginine	5.05	<0.1	3.8
hydroxyproline	<0.1	<0.1	<0.1
sum	96	3.05	71.8

[a] All results are shown as average results from duplicate samples.

(*5*). However, Johansson and Dahlgren (*5*) found that SOD also inhibited the CL response induced by ionomycin, which was not caused by $O_2^{\bullet -}$ production. The specificity for SOD in the CL reaction was therefore questioned.

To evaluate how ROS production in the monocyte assay responded to some common antioxidants, ascorbate, α-tocopherol, Trolox, and BSA were tested. Differently from the enzymic antioxidants, α-tocopherol (188 μM) acted both by inducing a lag phase and by reducing the CL peak intensity (**Figure 3**). The lag-phase induction seen is a typical behavior when a radical chain breaker is added into an oxidizing system (*33*). Reductions in the oxidation *intensity* in lipid-containing systems have previously been ascribed to a lower level of available oxidation catalysts (e.g., Fe, Cu, or Hb) (*34*), for example, through complexing reactions. In the monocyte system, where the ROS are produced enzymically, it is hypothesized that such effects are rather due to disturbance of the cell-signaling intermediates or the NADPH oxidase enzyme itself. α-Tocopherol is normally located in membranes, where it prevents the propagation of free radicals in synergy with ascorbate.

On a molar basis, the aqueous tocopherol analogue Trolox (tested at 0.5−50 μM) inhibited the CL response more efficiently than α-tocopherol (tested at 188 μM), which could be due to its better solubility in the aqueous assay. Ascorbate at 14.1 mM almost fully inhibited the CL signal (**Figure 3**), which can be explained by its well-documented power to scavenge ROS regardless of whether they are formed in the electron transport system or from xanthine oxidase, the myoglobin redox cycle, or leukocytes. The latter is due to the ability of ascorbate to penetrate cellular membranes (*35*, *36*) and, thus, its ability to scavenge the superoxide-, hydroxyl-, and neutrophil-derived hypochlorus radicals (*37*). This property is particularly relevant because ROS can be generated exogenously and endogenously.

Ascorbate and α-tocopherol have previously been shown to reduce oxidative damage in vivo including in animal models of ischemia and reperfusion in rat gastic mucosa (*38*), in rat skeletal muscle (*37*, *39*), and in rabbit kidney (*40*, *41*). Ascorbate and α-tocopherol analogues were most efficient when administered together in the rabbit kidney model, as evidenced by in

Table 3. Composition of Dipeptides and Free Amino Acids in Native Herring Light Muscle Press Juice, the Low Molecular Weight (LMW, <1000 Da) Fraction, and the High Molecular Weight (HMW, >3500 Da) Fraction[a]

	g/kg		
amino acid	native herring PJ	LMW (<1000 Da) fraction	HMW (>3500 Da) fraction
phosphoserine	<0.02	<0.02	<0.02
taurine	0.93	0.995	<0.02
phosphoethanolamine	<0.02	<0.02	<0.02
urea	<0.02	<0.02	<0.02
aspartic acid	<0.02	<0.02	<0.02
threonine	0.07	0.075	<0.02
serine	0.08	0.08	<0.02
aspargine	<0.02	<0.02	<0.02
glutamic acid	0.105	0.105	<0.02
sarcosine	<0.02	<0.02	<0.02
α-aminoadipitic acid	<0.02	<0.02	<0.02
proline	0.035	0.035	<0.02
glycine	0.4	0.435	<0.02
alanine	0.43	0.455	<0.02
citrulline	<0.02	<0.02	<0.02
α-amino-*n*-butyric acid	<0.02	<0.02	<0.02
valine	0.065	0.18	<0.02
cysteine	<0.02	<0.02	<0.02
methonine	<0.02	<0.02	<0.02
cystathionine	<0.02	<0.02	<0.02
isoleucine	0.035	0.025	<0.02
leucine	0.065	0.06	<0.02
tyrosine	0.02	0.02	<0.02
β-alanine	<0.02	<0.02	<0.02
phenylalanine	<0.02	<0.02	<0.02
β-aminoisobutyric acid	<0.02	<0.02	<0.02
L-homocystine	<0.02	<0.02	<0.02
γ-amino-*n*-butyric acid	<0.02	<0.02	<0.02
ethanolamine	<0.02	<0.02	<0.02
ammonia	0.135	0.12	<0.02
γ-hydroxylysine	<0.02	<0.02	<0.02
ornithine	0.02	0.02	<0.02
lysine	0.3	0.29	<0.02
1-methylhistidine	<0.02	<0.02	<0.02
histidine	0.905	0.915	<0.02
3-metylhistidine	<0.02	<0.02	<0.02
anserine	<0.02	<0.02	<0.02
carnosine	<0.02	<0.02	<0.02
arginine	0.02	<0.02	<0.02
hydroxyproline	<0.02	<0.02	<0.02
glutamine	<0.02	<0.02	<0.02

[a] All results are shown as average results from duplicate samples.

vivo MRS (Magnetic Resonance Spectroscopy) demonstrating highly synergistic effects (*40*).

Adding crude herring light muscle PJ at dilutions from 10 to 1000 had a clear dose-dependent inhibitory effect on the CL response (**Figure 5**). At the 1/100 and 1/1000 dilutions, there was both a slight lag-phase induction and a reduction of the CL peak intensity. This could indicate multiple effects from PJ in the assay, which is in line with its complex composition (**Tables 1**−**3**; **Figure 8**). In **Figure 6**, it is shown how proteins and LMW substances in the PJ probably acted by different mechanisms in the monocyte assay. Compared to the protein-containing samples, the LMW compounds gave a more pronounced CL peak, similar to that of the control. To elucidate whether the response given by the HMW fraction was primarily due to antioxidative enzymes present in herring (e.g., SOD, catalase, glutathione peroxidase) or to a general radical scavenging effect from protein-derived sulfhydryl groups, we tested the ROS-preventing properties of a well-known globular protein, BSA. At a BSA concentration similar to the total protein concentration in PJ and HMW samples, the relative inhibitions (percent total area reduction) given by the HMW fraction and BSA were almost identical (72 vs 71.1%). This indicated that the primary action of the herring proteins alone in the assay was nonenzymic. The ability of exposed protein SH groups to reduce free radicals under the formation of S−S bridges has been described previously (*42*). The importance of proteins for the ROS inhibition given by whole PJ was shown when using two different PJ batches with different protein concentrations; the higher the protein, the higher the inhibition (**Figures 5** and **6**). It should be stressed, though, that when the LMW fraction and HMW fractions were combined, such as in the native PJ (**Figures 5** and **6**), the inhibition obtained was stronger than that for any fraction alone. A likely synergy between LMW and HMW compounds in the PJ could be that the herring antioxidant enzymes need LMW cofactors to work properly. However, it must also be stressed that there was a small gap between the LMW and HMW cutoff molecular weights (M_w), 1−3.5 kDa. It is possible that important small antioxidative proteins or peptides could be localized in this M_w range.

That the LMW fraction of herring PJ had a clear inhibitory effect in the assay could be ascribed to numerous compounds. Tentative LMW candidates of fish muscle that previously have been ascribed antioxidative effects ex vivo in muscle-based food model systems include, for example, various polyamines (*43*), trimethylamine oxide (TMAO), histidine-containing dipeptides (e.g., anserine, carnosine), free amino acids (e.g., taurine, histidine), ascorbic acid, uric acid, and glutathione (*42*). Among these, ascorbic acid and uric acid are also regarded as important players in the antioxidative defense system of human blood (*42*). As shown in **Table 3**, anserine and carnosine were not detected in any of the samples, whereas fairly high levels of free taurine and histidine were found. Taurine has been suggested to be a radical scavenger; however, the exact mechanism seems to be unclear (*44*). Niittynen et al. (*45*) suggested that it is particularly important in vivo and then in reaction with hypochloric acid. Histidine has been suggested to be a scavenger, for example, of hydroxyl radicals (*42*). Other free amino acids present in high amounts were glycine and alanine, which, however, not have been specifically mentioned in antioxidant discussions. Ascorbic acid and uric acid were present at 20 and 6 μM, respectively, in the LMW fraction. The antioxidative mechanism of ascorbic acid is discussed above. In a washed cod mince model system, ascorbic acid at 20 μM efficiently inhibited Hb-mediated membrane lipid oxidation during ice storage. In the cod model, the effect was stronger when ascorbic acid was added together with an equimolar level of uric acid (*11*). A suggested antioxidative synergy among these two compounds is the ability of uric acid to chelate Fe^{3+} (*46*), which prevents reductions to the more catalytic Fe^{2+} by ascorbate. In the monocyte assay, where the role of metals is limited, it is, however, probable that the superoxide and radical scavenging properties of uric and ascorbic are dominating (*42*).

It is most likely that the antioxidative effects asserted by herring PJ are due to several mechanisms, connected either to reduction of the ROS production or to scavenging of produced ROS. To separately study the involvement of radical scavenging properties of PJ, we tested these samples in the ORAC assay. This assay works under physiologically relevant conditions in terms of temperature, pH, ionic strength, and radicals involved (peroxyl radicals) (*47*). The antioxidant activity in the monocyte and ORAC assays ranked the three PJ samples in the same order: native PJ > HMW fraction > LMW fraction. However, in the ORAC assay, proteins appeared to have a

more pronounced antioxidant effect than LMW compounds, which was also previously seen by Nifaldi et al. (*48*). Furthermore, the synergy between HMW and LMW compounds was more obvious in ORAC (native PJ was almost 10 times more efficient than the LMW PJ and 3 times more efficient than the HMW PJ). Thus, the ORAC results may indicate that the CL reducing effect given by LMW PJ in the monocyte assay is attributed more, for example, to radical formation than to radical scavenging.

As illustrated in **Table 1**, the herring PJ also contains compounds that ex vivo are regarded as strong pro-oxidants, for example, Fe. As shown by the hemeoglobin data, a large part of the iron is present as heme complexes. Heme can cleave hydroperoxide, for example, into the hydroxyl radical and can also be activated by H_2O_2 and form ferryl complexes with radical properties. Possibly, the different kinetics seen with native PJ and HMW fractions compared to the LMW PJ were caused by slight parallel pro-oxidative activity. However, the net effect from the PJ samples in the monocyte assay was clearly antioxidative.

To conclude, this paper proved that the monocyte assay appears to be a very useful tool for studying whether food-derived antioxidants can limit ROS production under biologically relevant conditions. The ROS-derived CL signal was clearly suppressed both by purified antioxidants and by a more complex food extract, herring light muscle PJ. The latter observation could contribute to explaining the numerous observations that a fish-containing diet prevents cardiovascular disease. It remains to be elucidated whether the primary mechanism behind the reduced CL signal given by antioxidants primarily originates in reduced *formation* of ROS or from *scavenging* of liberated ROS.

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NOTE ADDED AFTER ASAP PUBLICATION.

The original posting of this paper on September 19, 2006 contained typographical errors in the keyword section and in the caption of Figure 8. The corrected version was published ASAP on September 29, 2006.

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