A fluorescent homogeneous assay for myeloperoxidase measurement in biological samples. A positive correlation between myeloperoxidase-generated HOCl level and oxidative status in STZ-diabetic rats

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Abstract

Myeloperoxidase (MPO) is a key enzyme derived from leucocytes which is associated with the initiation and progression of many inflammatory diseases. Increased levels of MPO may contribute to cellular dysfunction and tissues injury by producing highly reactive oxidants such as hypochlorous acid (HOCl). Myeloperoxidase-generated HOCl is therefore considered as a relevant biomarker of oxidative stress-related damage and its quantitation is of great importance to the study of disease progression.

In this context, the current study describes a rapid, sensitive and homogeneous fluorescence-based method for detecting the MPO chlorination activity in biological samples. This assay utilizes 7-hydroxy-2-oxo-2H-chromene-8-carbaldehyde oxime as a selective probe for HOCl detection, and is adapted to 96-well microplates to allow high-throughput quantitation of active MPO.

The ability of the method to monitor HOCl release was further investigated in hyperglycemic streptozotocin-treated diabetic rats. The data proved that the present assay has a reliable performance when quantitating the active MPO in the plasma of diabetic animals, a feature of inflammatory disease found concomitant with an elevation of protein carbonyls levels and lipid peroxidation and which was negatively correlated with the ratio of reduced-to-oxidized glutathione.

Keywords

Myeloperoxidase, hypochlorous acid, 7-hydroxy-2-oxo-2H-chromene-8-carbaldehyde oxime, fluorimetric assay

Abbreviations

myeloperoxidase, MPO; protein carbonyls, PC, glutathione, GSH; oxidized glutathione, GSSG; streptozotocin, STZ; 7-hydroxy-2-oxo-2H-chromene-8-carbaldehyde oxime, 7-HCCO.

1. Introduction

Myeloperoxidase (MPO) is a mammalian pro-oxidant enzyme mainly released by activated neutrophils, which catalyzes the conversion of hydrogen peroxide (H_2O_2) and chloride to hypochlorous acid (HOCl) which in turn readily oxidizes key molecules, such as proteins and lipids. It is well established that MPO promotes endothelium dysfunction leading to various vascular damages and elevated blood MPO and HOCl levels could be predictive for increased risk for cardiovascular events [1-4]. Among the numerous pathologies associated with increased cardiovascular risk, high-glucose stimulated H_2O_2 production contributes to MPO-mediated atherosclerosis progression in type 2 diabetes mellitus [5-8]. MPO actions in human health and disease have been widely recognized as specific biomarkers for disease progression, therefore the MPO quantitation in blood and tissues is of great importance in both the experimental and clinical setting.

In clinical practice the most widely used method for MPO detection and quantitation is the enzyme-linked immunosorbent assay (ELISA) method which uses MPO-specific antibodies and which are not relevant for the measurement of the active enzyme. Since then immunological methods have been developed for the measurement of the active content of the enzyme in complex media such as the specific immunological extraction followed by enzymatic detection (SIEFED [9-10]. The immunological methods have been found to be very sensitive and satisfactorily specific but are expensive and time-intensive. In the research field, few alternative methods have been developed for the detection of chlorination or peroxidase activity of the enzyme using chromogenic and fluorogenic substrates [11-15]. However, chlorination and peroxidase activities of the enzyme cannot be measured simultaneously and could be strongly affected by pH, hydrogen peroxide, superoxide, chloride and endogenous inhibitors such as ceruloplasmin, lipoproteins and antioxidants like ascorbic acid [16-19].

There are four distinct peroxidase superfamilies that evolved independently in evolution that can oxidize chloride (e.g. chloroperoxidase) [20]. But MPO is the only human peroxidase that produces HOCl at reasonable rates; note that eosinophil peroxidase is able to oxidize chloride only at low pH values [21]. Thus, for the measurement of myeloperoxidase activity, assays based on measurement of chlorination activity are considered more specific than those based on the detection of peroxidase substrates such as 0-dianisidine hydrochloride or

tetramethylbenzidine [11, 12]. Therefore, investigating chlorination activity appears more attractive in distinguishing myeloperoxidase from other heme proteins with peroxidase activity. For this purpose, several assays have been developed to measure the chlorination activity of myeloperoxidase and most of them are based on the detection of chloramines which are the HOCl oxidation products of proteins or amino acids [13, 15, 22]. However some weaknesses in the reliability, specificity or sensitivity were reported, rendering these assays hardly applicable to complex biological samples in vivo [14].

With the aim to overcome some of these later issues, the present study describes a sensitive homogeneous assay allowing the high-throughput MPO quantitation and rapid processing of multiple samples simultaneously in a short period of time. This method was developed to measure the chlorination activity based on MPO-derived HOCl detection which is an endpoint of the actual oxidative capacity of the enzyme. First, a convenient procedure is reported to synthesize 7-hydroxy-2-oxo-2H-chromene-8-carbaldehyde oxime (7-HCCO, Scheme1) as a highly specific fluorescent probe for HOCl detection since the double bond of the oxime could be selectively cleaved by HOCl leading to a strong increase in fluorescence [23]. Second, the current study reports an accurate method to monitor and quantitate the reaction of HOCl on 7-HCCO, adapted to 96 wells microplate, which is cost-effective and does not require time-consuming steps when compared to ELISA techniques or other methods. The reliability of this method was then investigated by measuring MPO levels in various biologically-relevant milieus such as rat plasma and HL60 cells medium. Finally, the method was successfully applied in vivo (i) to demonstrate MPO-derived HOCl release in streptozotocin (STZ)-treated diabetic rats. (ii) To investigate the relationship between the MPO level and biomarkers of oxidative stress such as protein carbonyls and plasma malondialdehyde or antioxidant defense such as glutathione.

2. Experimental

2.1. Reagents and materials

All chemicals and reagents were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France) and were of the highest grade available. The protein carbonyl assay kit (Carbofax®) based on 7-hydrazino-4-nitrobenzo-2,1,3-oxadiazole (NBDH) was purchased from Yelen (Ensuès-la-Redonne, France; http://www.yelen-analytics.com). Analytical NMR spectra were recorded using a Bruker Avance III Nanobay spectrometer. Chemical shifts (δ) are reported in

parts per million (ppm) relative to internal Me_4Si (^1H and ^{13}C) and coupling constants (*J*) are given in hertz (Hz). Splitting patterns are reported as follows: s = singlet; d = doublet; dd = doublet of doublets; br = broad peak. The screening assay was performed with a microplate spectrofluorimeter TECAN Infinite 200 (TECAN Männedorf, Switzerland). Doubly distilled deionized water filtered through a 0.2-µm Millipore filter was used throughout. Purified MPO (ref MY176) was supplied by Elastin Products Co (Owensville, USA).

2.2. Synthesis of 7-HCCO

The synthesis of probe 7-HCCO was performed according to modifications of a previous procedure [23]. *7-acetylcoumarine* (**1**): 7-hydroxycoumarin (3.09 g, 19.1 mmol) was dissolved in dichloromethane (50 mL). Then acetic anhydride (2 mL, 21.2 mmol) and pyridine (2 drops) were added and the mixture stirred overnight at 38°C. The mixture was then allowed to cool at room temperature and concentrated under vacuum. The residue was dissolved in water (50 mL) and ethyl acetate (100 mL). The aqueous layer was extracted with ethyl acetate (3 × 50 mL). The organic layers were gathered, washed with an aqueous saturated solution of K_2CO_3 (3 × 50 mL), dried over $MgSO_4$, filtered and concentrated to afford **1** as a white powder (3.9 g, quant.). ^1H RMN ($CDCl_3$, 300 MHz) δ 7.69 (d, J = 9.5Hz, 1H, 4-H), 7.49 (d, J = 8.4Hz, 1H, 5-H), 7.11 (d, J = 2 Hz, 1H, 8-H), 7.05 (dd, J = 8.4 and 2.0 Hz, 1H, 6-H), 6.39 (d, J = 9.5Hz, 1H, 3-H), 2.34 (s, 3H, CH_3); ^{13}C RMN ($CDCl_3$, 75 MHz) δ 168.8 (*C*O-CH_3), 160.4 (2-CO), 154.7 (7-C), 153.2 (8a-C), 142.9 (4-C), 128,6 (5-C), 118.4 (6-C), 116.7 (4a-C), 116.1 (3-C), 110.4 (8-C), 21.1 (CH_3).

8-formyl-7-hydroxycoumarine (**2**): Compound **1** (3.9 g, 19.1 mmol) was dissolved in trifluoroacetic acid (26 mL) and the solution was cooled at 0°C. Hexamethylenetetramine (3.8 g, 27.5 mmol) was then added; the resulting solution was allowed to warm at room temperature and then heated under reflux overnight. The mixture was then concentrated under vacuum and the residue was suspended in water (52 mL) and heated at 60°C for 30 min. The mixture was then cooled at 0°C to afford **2** as an orange powder that was filtered and dried (1.35 g, 37 %). ^1H RMN (DMSO-d_6, 300 MHz) δ11.92 (bs, 1H, OH), 10.40 (s, 1H, CHO), 8.01 (d, J = 9.54 Hz, 1H, 4-H), 7.85(d, J = 8.6 Hz, 1H, 3-H), 6.94 (d, J = 8.6 Hz, 1H, 6-H), 6.4 (d, J = 9.5Hz, 1H, 3-H); ^{13}C RMN (DMSO-d_6, 75 MHz) δ 190.8 (CHO), 163.9 (2-C), 159.1 (8a-C), 155.7 (7-C), 144.5 (4-C), 136.3 (5-C), 114.0 (6-C), 112.6 (3-C), 111.2 (4a-C), 109.2 (8-C).

*7-hydroxy-2-oxo-2H-chromene-8-carbaldehyde oxime (**7-HCCO**):*

Hydroxylamine hydrochloride (750 mg, 10.7 mmol) and triethylamine (1.5 mL, 10.7 mmol) were added to a solution of compound **2** (1.35 g, 7.11 mmol) in absolute ethanol (20 mL). The mixture was heated under reflux overnight, and then concentrated under a vacuum. The residue was dissolved in water (40 mL) and ethyl acetate (25 mL). The aqueous layer was extracted with ethyl acetate (4 × 20 mL). The organic layers were gathered, dried over $MgSO_4$, filtered and concentrated to afford 7-HCCO as an orange powder (1.0 g, 69 %). ^{1}H RMN (DMSO-d_6, 300 MHz) δ 8.53 (s, 1H, HC=N), 7.99 (d, J = 9.5 Hz, 1H, 4-H), 7.61 (d, J = 8.6 Hz, 1H, 5-H), 6.93 (d, J = 8.6 Hz, 1H, 6-H), 6.31 (d, J = 9.5 Hz, 1H, 3-H); ^{13}C RMN (DMSO-d_6, 75 MHz) δ 159.5 (CO), 159.5 (8a-C), 152.7 (7-C), 144.6 (4-C), 143.7 (C=N), 130.3 (5-C), 113.2 (6-C), 112.2 (3-C), 111.4 (4a-C), 105.2 (8-C).

2.3. Synthesis of oxidized 7-HCCO

NaOCl (1 mL, 10 % dissolved in water, 16.5 mmol) was added to a solution of compound 7-HCCO (50 mg, 0.244 mmol) dissolved in methanol (6 mL). The mixture was stirred at room temperature for 30 min and concentrated. The residue was purified by column chromatography (SiO_2, gradient AcOEt/MeOH from 100/0, v/v to 90/10, v/v) to afford oxidized-7-HCCO as a yellow powder (20 mg, 44 %). ^{1}H RMN (DMSO-d_6, 300 MHz) δ 7.72 (d, J = 9.2 Hz, 1H, 4-H), 7.37 (d, J = 8.5 Hz, 1H, 5-H), 6.53 (d, J = 8.5 Hz, 1H, 6-H), 5.97 (d, J = 9.2 Hz, 1H, 3-H).

2.4. High-performance liquid chromatography analysis

Chromatographic analyses were performed with a Merck Hitachi System consisting of a LaChrom L-7000 interface module and a L-7480 fluorescence detector controlled by the EZChrome Chromatography manager software. Reverse phase HPLC was carried out at room temperature on a Nucleodur RP-18 column (125 mm × 4.6 mm; 3 μm; Macherey Nagel) using a gradient with a flow rate of 0.8 mL.min^{-1}. Gradient: solvent A (phosphate buffer 10 mM, pH 7.4), solvent B (acetonitrile): 0-15 min, 10-30% B; 16-24 min, 10% B. Derivatives were measured at excitation and emission wavelengths of 350 nm and 470 nm, respectively.

2.5. HOCl production by isolated myeloperoxidase

Myeloperoxidase was mixed at 37°C in 1X PBS pH 6.5 with 7-HCCO probe at 150 µM in 96 wells microplate and H_2O_2 (100 µM) was added to start the reaction. The fluorescence was measured every 3 min at 470 nm, exciting at 350 nm. Fluorescence intensity change (ΔF) was directly proportional to the quantity of active MPO and related to a standard curve to determine the concentration of hypochlorous acid produced. One unit (U) of the enzyme was the activity which formed 1 µmol HOCl per min at 37°C calculated from the initial rate of reaction with peroxide as the substrate.

2.6. HOCl production by HL-60 cells

The characteristics and origin of HL60 cell line used in the present study was purchased from ATCC (Rockville, MD). HL60 (acute promyelocytic leukemia) is a promyelocytic cell line derived by S.J. Collins *et al*. [24]. Peripheral blood leukocytes were obtained by leukopheresis from a 36-year-old Caucasian female with acute promyelocytic leukemia. Cells were grown in DMEM(1X)+GlutaMAX^{TM} containing a stabilized form of L-glutamine, L-alanyl-L-glutamine and supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% carbon dioxide atmosphere. Cells (5×10^6 /mL) were lysed by a freeze/thaw treatment and sonicated on ice for 10 min. Cell lysates were centrifuged for 10 min at 15,000 × g at 4°C. Two hundred µL of supernatant 10 times diluted in 1X PBS pH 6.5 was preincubated 30 min at 37°C with 20 µL PBS 1X or 20 µL of sodium azide (10 mM) in 96 wells microplate. After 10 min of incubation at 37°C, 7-HCCO at 100 µM and H_2O_2 at 100 µM were added and fluorescence was measured every 3 min at 470 nm, exciting at 350 nm.

2.7. Animals and biological samples preparation

A total of twenty-four Sprague-Dawley male rats weighing 120-150 g were purchased from CERJ (Le Genest St Isle, France) and fed *ad libitum* with a standard Teklad 2016 diet (Harlan Laboratories, Gannat, France). Animals were maintained in the local animal house under conventional conditions, including an enriching structural and social environment. Physical and cognitive activity was promoted in a room with a controlled temperature (22 ± 3°C) and a reverse 12h light/dark cycle with food and water available *ad libitum*. All animal care and experimental procedures were performed according to the rules of the Directive 2010/63/EU of the European Parliament. The protocol was approved by the local Animal Research Ethics Committee (2010-2012) and the National Research Committee for the project FEDER-AdiabaOx (2008, N° 13851). The CNRS and Aix-Marseille University have valid

licences for animal housing and experimentation (agreement C13-055-06) delivered by the French Government. Among coauthors M.C. and S.P are graduated for the Certificate in Small Animal Surgery (Aix-Marseille University, Faculté de Pharmacie and Centre de Formation Permanente CNRS DR12, Marseille).

To induce diabetes, streptozotocin (STZ) was administered to twelve overnight fasted rats (STZ-diabetic group) by a single intraperitoneal injection (final dose 60-70 mg/kg in 0.4-0.5 mL). STZ was dissolved beforehand in citrate buffer (1 mM, pH 4.5) and maintained on ice before use. Seventy-two hours after STZ injection, the blood glucose concentration was checked using a commercial glucose auto analyser (AlphaTRAK®, *Abbott* Laboratories). Blood samples (6 µL/sample) were collected from the tail vein under local anaesthesia induced by 0.25% lidocaine application in ointment (B Braun, France). In the study, a blood glucose level higher than 350 $mg.dL^{-1}$ was found in 100% of animals which were considered to be diabetic and used in the experiments. During the 3 weeks following STZ injection blood samples were collected weekly and the blood glucose level was measured in fasting (5-6 h, morning fast) conditions. A second group of twelve untreated rats was used for control (non diabetic control group). All animals were observed daily for clinical signs of suffering, weight loss and moribundity. At the end of the protocol, animals were euthanized by intraperitoneal injection of sodium pentobarbital (120 $mg.kg^{-1}$; Ceva Santé Animale, Libourne, France) before thoratocomy. The blood was withdrawn from the heart cavity and immediately added to serine borate buffer and processed for GSH and GSSG determination (see below) or into heparinized glass tubes for plasma preparation by centrifugation (1,650 × g for 15 min at 3°C) and kept at –80°C for further analysis of MPO, protein carbonyls and MDA-TBA levels.

2.8. Chlorination activity of myeloperoxidase in rat plasma

Plasma samples from control normal rats were diluted ten-fold in 1X PBS pH 6.5. One hundred µL of diluted plasma was placed in 96 wells microplate. Then 7-HCCO at 100 µM and H_2O_2 at 100 µM were added and fluorescence was measured every 3 minutes at 470 nm, exciting at 350 nm during 15 minutes. MPO content was calculated from the relative linear fluorescence intensity and related to a calibration curve constructed in plasma with purified human MPO. The precision of the method was estimated by the coefficients of variation (CV) calculated for several determinations made in triplicate.

2.9. GSH and GSSG quantitation

GSH and GSSG were determined in the blood of diabetic and control rats according to the method previously described by N'Guessan et al. [25] with modifications. Blood was collected and immediately mixed with the equivalent volume of serine borate buffer (pH 8.5) containing: L-serine (100 mM), boric acid (80 mM), sodium tetraborate (20 mM). This buffer was associated with bathophenanthroline disulfonate sodium salt (2 mM), iodoacetic acid (10 mM) and Na-heparin (2020 USP units) to form the collecting solution. The association of blood and collecting solution was plunged rapidly on liquid nitrogen and kept at -80°C. Protein was precipitated by adding 300 µl of 1 M perchloric acid in 0.2 M boric to 150 µl sample. After centrifugation (2000 × g, 10 min at 4°C), 200 µL supernatants were derivatized with 40 µL of iodoacetic acid at 40 mM for at least 10 min at room temperature. The pH was adjusted to 9.0 with 300 µL of NaOH/tetraborate solution at pH 11. Then, dansyl chloride (200 µL at 50 mM) was added and the samples were kept at room temperature overnight in the dark to form *S*-carboxymethyl-*N*-dansyl-GSH and *N*,*N*-9-bis-dansyl-GSSG. Unreacted dansyl chloride was removed by chloroform extraction (500µl). Prior to analysis, the aqueous phase was diluted 5-fold in 100 mM Borate Buffer pH 9. The GSH and GSSG adducts were separated by HPLC using a Merck Hitachi/Lachrom HPLC system: autosampler, pump L-7100, fluorescence detector L-7480 (excitation wavelength, 335 nm; emission wavelength, 515 nm), interface D-7000, degasser L-7612, controlled by the Agilent EZChrom Elite Compact software (Pleasanton, USA). Separation was achieved at room temperature on a 3-aminopropyl column (250 mm × 4.6 mm; 5 µm; Macherey-Nagel, Hoerdt, France) with an isocratic (Solvent A/Solvent B, 90/10, vol/vol) flow rate of 1.2 $mL.min^{-1}$ during 35 min. Solvent A is a 0.2 M sodium acetate buffer (pH 4.6) in methanol (35/65, vol/vol) and solvent B is a methanol/water solution (80/20, vol/vol). Quantification was based on peak area and relative to standards from a calibration curve obtained with commercial GSH and GSSG. Data are expressed in µM and are the means of twelve independent experiments in each group made in duplicate.

2.10. Protein Carbonyls

Protein carbonyls were determined in plasma samples from diabetic and control rats after derivatization with the probe NBDH according to a recently published fluorometric method adapted to 96-microplates (TECAN Infinite 200) [26]. Plasma samples were diluted with 4 vol of PBS 0.1X before analysis. Then 100 µL of diluted samples were placed in 96-well microplates and reacted for 15 min at room temperature with an aliquot (100 µL) of the NBDH assay solution (Carbofax®, Yelen Analytics). Concentration of NBDH-reacted

carbonyls was determined by fluorescence (emission determined at 560 nm using an excitation wavelength at 480 nm) and reduced and oxidized bovine serum albumin was used for calibration. The amount of protein carbonyls was calculated with respect to total protein content obtained using the Pierce BCA protein assay kit (Fisher Scientific, France) and is expressed in nanomoles/mg protein. Data are means of twelve independent experiments in each group made in duplicate.

2.11. Lipid peroxidation measurement

In test tubes aliquots (50 μL) of plasma were added to a mixture of 450 μL of thiobarbituric acid (TBA) solution in orthophosphoric acid (0.3 M, pH 3.5) and 10 μL of butylated hydroxytoluene solution (5 mM) in ethanol. The samples were heated at 95 °C for 30 min, cooled to room temperature, centrifuged (1800 × *g*) for 10 min and the malondialdehyde (MDA)-TBA adduct content of the supernatants were assayed by HPLC. Separation was carried out at room temperature using the HPLC system described above with fluorescence emission determined at 553 nm using an excitation wavelength at 532 nm, and a C18-bonded Macherey-Nagel silica column (250 mm × 4.6 mm, 5 μm). HPLC analyses used solvent A: 20 mM phosphate buffer (pH 6.9) and solvent B: methanol. Samples (20 μL) underwent an isocratic elution (A:B, 50:50) at a flow rate of 1 mL/min for 10 min, with peak area quantification. MDA-TBA levels were calculated against a standard curve of MDA tetrabutylammonium and are the means of twelve independent experiments in each group made in duplicate. Data are expressed in nanomoles/mg protein.

2.12. Statistical analysis

Data are presented as mean ± SD or SEM for the indicated number of independent experiments performed in duplicate. Evaluation of statistical significance was conducted using an unpaired Student's *t* test or Mann-Whitney when appropriate (GraphPad Prism Software). Differences were considered significant when $P < 0.05$.

3. Results

3.1. Synthesis and characterization of probe 7-HCCO

Synthesis of probes 7-HCCO and oxidized 7-HCCO is reported in Scheme 1. Acetylation of 7-hydroxycoumarine, which afforded compound **1** in a quantitative yield. The formulation of compound **1** was performed through a Duff reaction inspired from the synthesis described by Lee *et al*. [27] to give compound **2** in a moderate yield. Reaction of aldehyde with hydroxylamine gave the probe 7-HCCO with a 26 % overall yield. To verify the dehydration of the probe into the nitrile, reaction of 7-HCCO with sodium hypochlorite was performed. The dehydration was proved by the disappearance of the signal attributed to the oxime function (δ 8.53 ppm in 7-HCCO) a result which positively correlates to the previously reported spectra [23].

Chemosensor 7-HCCO is not fluorescent but its oxidation by HOCl gives highly fluorescent derivative. Other reactive oxygen and nitrogen species such as OH, H_2O_2, $^1O_2$, NO_2^-, NO_3^-, NO, $ONOO^-$ only induced negligible fluorescence enhancement [23]. The probe 7-HCCO is then highly selective and sensitive towards HOCl. Fig. 1 shows the absorbance and fluorescence spectra of 7-HCCO after reacting with HOCl in PBS 1X pH 6.5. The absorption spectrum of derivative shows a maximum at 350 nm and the emission spectrum presents a maximum at 470 nm. Fig. 2 shows an HPLC analysis of 7-HCCO and 7-HCCOox. HOCl and 7-HCCO alone did not present a fluorescent signal contrary to the derivative mixture prepared with 7-HCCO and HOCl identified as oxidized 7-HCCOox. The fluorescence emission recorded with 7-HCCO (100 µM in 1X PBS pH 6.5) is proportional to HOCl from 0 to 150 µM and showed a linear regression with good correlation coefficient (F(a.u.) = 10.485c, R^2=0.9992). Under these conditions, the limit of detection for HOCL is estimated to be 8 µM.

3.2. Chlorination activity of purified MPO

It is known that the optimum pH of the MPO-catalyzed chlorination reaction varies with chloride and H_2O_2 concentrations. Under usual experimental conditions the activity of the MPO-H_2O_2-halide system has an optimum pH varying from 4 to 7.4 depending on the $[H_2O_2]/[Cl^-]$ ratio. The H_2O_2 concentration dependences of the human purified MPO activity in 1X PBS pH 6.5 at 37°C are shown in Fig. 3. The MPO activity has a maximum at 100 µM and decreases dramatically at 1 mM. The results of MPO inactivation by hydrogen peroxide are in agreement with previous data [28, 29]. Myeloperoxidase inactivation by hydrogen peroxide was not prevented by chloride-free medium (data not shown). The chlorination specific activity of the native human myeloperoxidase measured by this method was 180 units/mg of protein. Purified human MPO produced HOCl in a concentration-dependent manner. Fluorescence intensity change (ΔF) was recorded in 1X PBS pH 6.5 with 7-HCCO at

100µM is proportional for a range of MPO content between 0 to 120 µg/l. The following relationship was obtained: y = 6.959x ; R^2=0.9978, were y is in µmol/min and x is µg/l). Under the conditions of the assay, concentrations as low as 5 µg/L myeloperoxidase could be reliably detected.

3.3. MPO chlorination activity in HL-60 cells

To study whether the 7-HCCO probe can detect HOCl generated by MPO in biological samples, HL-60 cells were first used for the experiments. Fig. 4 shows the kinetic curves for 7-HCCO oxidation in HL-60 cell lysate. A fluorescence increase is clearly recorded without the heme enzyme inhibitor sodium azide. The inhibition of MPO activity by azide was greater than that observed in the absence of H_2O_2, suggesting that azide action goes beyond simple inhibition of MPO activity.

3.4. MPO activity in rat plasma

Having established that the probe 7-HCCO can detect MPO activity in isolated cells, the probe was then used to provide direct evidence of HOCl formed by MPO in rat plasma. Samples were diluted 10 fold in order to reduce the effects of native MPO inhibitors such as ceruloplasmin or ascorbic acid [30]. At a higher dilution, the limit of the sensitivity is reached by samples with a low content of active MPO. The precision of the method was estimated by repeatedly measuring the same assay with five different concentrations of active MPO. These MPO concentrations were added to the same sample and ranged from 8 to 125 µg/L. Using the current assay, a calibration curve was built (15 µM of 7-HCCO and 100 µM H_2O_2) with twenty different rat plasma samples that were supplemented with incremental purified human MPO concentrations (standard curve $\Delta F = 0.7633c$, R^2=0.9981). Intra-assay CV ranged from 4% to 9.8% and the inter-assay CV ranged from 6.9% to 13.9%. The MPO concentrations were chosen to reach common ranges found in other studies or commercial kits [29-31]. In each sample MPO content was calculated from the calibration curve.

3.5. Comparative measurements of MPO to multiple oxidative stress status biomarkers in diabetic rats

With the aim to test the ability of the method in a relevant physiopathological model known to trigger oxidative stress and inflammation, MPO levels were measured in plasma samples from rats allocated to control or STZ-diabetic groups (n=12/group). It was checked

beforehand that all rats of the STZ-diabetic group have developed hyperglycemia within 3-5 days after STZ injection, as indicated by increased blood glucose concentrations (231 ± 8 $mg.dL^{-1}$ in STZ-treated rats versus 79 ± 2 $mg.dL^{-1}$ in controls, P<.0.01). The content of protein carbonyls, glutathione and oxidized glutathione and the malondialdehyde levels was determined for each animal 3 weeks after STZ injection, and the results are reported in Fig. 5. It was shown that the values of MPO level (± SEM) in diabetic rat plasma samples (433 ± 19 µg/L) were significantly higher (P<0.001) than the values obtained in control animals (106 ± 6 µg/L). The values of GSH blood content in the STZ group (87 ± 11 $\mu mol.L^{-1}$) were found to be significantly lower than that of the controls (264 ± 19 $\mu mol.L^{-1}$). In addition, a significant increase (by 65%) in the GSSG content was observed in the blood of diabetic rats when compared to controls (26 ± 5 $\mu mol.L^{-1}$). As expected, these changes were accompanied by a significant 80% decrease of the ratio GSH/GSSG in the STZ group when compared to normal rats (Fig.5). This drop in GSH/GSSG was found concomitant with a dramatically increased blood glucose level (498 ± 15 $mg.dL^{-1}$) when compared to controls. As described in Fig.5, a significant increase in both levels of protein carbonyls (P<0.005) and MPO values (ranging 330-557 µg/L) was determined in diabetic rats and a positive correlation was obtained (R^2 = 0.8906; Fig. 6A). As a consequence, an inverse correlation (R^2 = 0.8863) between GSH/GSSG and MPO could be drawn (Fig. 6B), which supports the hypothesis that the MPO-derived HOCl may contribute to oxidative stress in the STZ-induced model of experimental diabetes. In addition, Figure 5 shows that the lipid peroxidation degree (MDA-TBA levels) in the plasma of the diabetic group (7.61 ± 0.34 nmol/mg protein) was significantly higher than that of the controls (4.26 ± 0.31 nmol/mg protein; P<0.001).

4. Discussion

The MPO level could be a representative marker of neutrophil activation [32] and it is well established that myeloperoxidase-derived HOCl plays a crucial role in several pathologies and diseases. Therefore, the determination of the enzyme concentration is essential for the understanding of its role as one of the major components of the inflammatory process. The ELISA assay is considered to be robust and sensitive, but it is time intensive, costly and allows the evaluation of the total content of the enzyme and not the active one. Despite other methods based on the quantitation of MPO enzymatic activity that have been proposed, no consensus has been found regarding their ability to specifically measure MPO activity in

biological samples. Most of them are based on the properties of chemical probes such as *o*-dianisidine, guaiacol, luminol, tetramethylbenzidine (TMB) or acetyl-dihydroxyphenoxazine (ADPH) for measuring the peroxidase activity. The ADPH and luminol assays were found to be sensitive enough but not specific to MPO activity since the activity of other peroxidases, including eosinophil and hemoproteins such as hemoglobin and myoglobin present in the tissues, can interfere with the results. In contrast the probes 3'-(p-aminophenyl) fluorescein (APF) and 5-thio-2-nitrobenzoic acid (TNB) have the ability to detect the chlorination activity of MPO but were found to lack sensitivity or required long incubation steps. Another assay of MPO activity has been developed based on the detection of 3-chlorotyrosine, the HOCl oxidation product of protein tyrosyl residues, that can be measured with chromatography/mass spectrometry [33, 34]. Although 3-chlorotyrosine is specifically produced under MPO stimulation, it can be quickly degraded in an inflammatory environment or markedly reduced by thiocyanate ions [35]. The bromide-dependent chemiluminescence has also been shown to be specific towards MPO but this technique can only be used for the quantification of polymorphonuclear leukocytes in selected tissues with an appropriate control [36, 37]. All together, these assays are dependent on the tissue microenvironment and cannot be specific towards MPO without the use of an antibody-capture step. Some authors found a good compromise to measure in complex media the activity of MPO after its capture by specific antibodies by using for example the SIEFED technique [9, 10].

In the present study, a homogeneous assay that uses a new fluorimetric method in order to measure MPO in plasma and other biological samples was proposed as an alternative to ELISA and other assays. This method allows the direct determination of chlorination activity of myeloperoxidase based on a synthetic probe designed to selectively detect HOCl, and has been used to measure active MPO level for the first time in STZ-diabetic rats. This assay has many practical advantages over other methods. The most obvious is that it saves time as it requires only 10 minutes of incubation in order to observe a linear increase in fluorescence intensity. Also, it is homogeneous and requires no labor-intensive sample preparation; biological samples were only diluted in order to reduce the interactions between MPO and other biomolecules. Since it uses a fluorogenic and selective probe for HOCl detection, the technique disclose the MPO activity and allows an easy measurement of the active MPO level with a high specificity and sensitivity. It is adapted to 96 wells microplate allowing high-throughput quantitation of MPO in multiple samples concurrently.

One limitation of our assay is the lower sensitivity compared to ELISA or SIEFED techniques [6, 10]. The assay's detection limit is based on a 3-sigma criterion that corresponds to

approximately 8 µg/L, which is about 10 fold higher than immunological methods. However, as the mean MPO concentrations in human plasma of healthy donors were reported to range 18 to 49 µg/L for total level [10, 30, 38, 39], or 14 – 15 µg/l for active level [10, 30], the sensitivity of the probe should be therefore sufficient to measure MPO level in vivo, even when in a low grade of oxidative stress and could serve for investigation of inflammatory pathologies.

In rats, STZ treatment lead to an activation and degranulation of polymorphonuclear neutrophils as revealed by the percentage increase (380 %) of MPO level in plasma of diabetic group when compared to the control. Our results are in accordance with a previous murine study that shows an increase of 192 % of plasma MPO concentration in diabetic animals [40]. A significant increase was also observed (178 % to 342 %) in some tissues of STZ-induced diabetic rats [41-45]. Taken together all these results confirm the assumption of increased inflammation in diabetic animals in this model.

Myeloperoxidase-derived HOCl can react readily with a number of biological molecules. In a complex system such as plasma, proteins are important targets for HOCl, leading to an increase of protein-bound carbonyl levels [46]. Many studies have demonstrated a significant increase of protein carbonyl content in plasma and different cells of diabetic patients resulting from increased myeloperoxidase activity [47-49]. A correlation between protein carbonyl levels and MPO concentrations has been reported previously in preterm infants [50]. Released HOCl is able to penetrate the cell membrane and oxidize intracellular thiols. Glutahione oxidation in erythrocyte by HOCl is considered to be a highly selective process and most of the GSH is converted into GSSG [51]. Glutathione is the most abundant cellular non-protein thiol which plays an efficient role against oxidative stress. It is present in almost all mammalian tissues, at millimolar concentrations in liver, and less than 10% exists in the oxidized form in a normal situation. The ratio GSH/GSSG is considered as a major biomarker of blood redox state or overall health [52]. As a consequence of an increased oxidative status, alterations of GSH and GSSG concentrations are rapidly observed. A decreased GSH level plays a role in the oxidative damage and many works have shown that the GSH/GSSG ratio was much lower in the diabetic subjects than in controls [53-55]. The reduction in GSH concentration observed after STZ treatment is due to its increased utilization and reduced regeneration, and is presumably associated with an increased production of peroxides [56]. In accordance with these findings, we observed in the present study that after 3 weeks of STZ-induced hyperglycemia a strong correlation could be established between MPO concentrations and both protein carbonyls and GSH/GSSG ratio, suggesting that neutrophil

activation is one of the major contributors to the variations of these markers. The increase of lipid peroxidation evaluated by the MDA content and observed in diabetic rats strongly supports these finding and showed a clear unbalance between oxidative stress status and antioxidant defense. Similarities are found between this study's results and those reported earlier in the literature, showing an enhanced oxidative status in diabetes mellitus involving MPO activation [53, 57-61].

5. Conclusions

In summary, it can be concluded that 7-HCCO could be used as a convenient and sensitive fluorogenic probe to detect HOCl generation in biological fluids that allow a reliable high-throughput assessment of active MPO levels in vivo. All reactions were performed in one 96-well microplate by the sequential addition of reagents, and the results obtained were in accordance to those obtained by other methods. In addition, the presence of an inflammatory process and some variations in oxidant-antioxidant balance in STZ diabetic rats is demonstrated.

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Figures captions

Scheme 1. Synthesis of probes 7-HCCO and oxidized 7-HCCO. Reagents and conditions: (a) Ac_2O, pyridine, CH_2Cl_2, 38°C, overnight, quant. yield; (b) hexamethylenetetramine, TFA, reflux, overnight, 37 %; (c) H_2N-OH.HCl, NEt_3, MeOH, reflux, overnight, 69 %; (d) NaOCl, MeOH, rt, 30 min, 44 %.

Figure 1. Normalized absorbance (●) and fluorescence (◆) spectra of 7-HCCO (100 µmol/L) after reaction with HOCl (50 µmol/L) in PBS 1X pH 6.5. The absorption spectrum shows a minimum at 350 nm and the fluorescence spectrum presents a maximum at 470 nm.

Figure 2. Chromatograms of : (a) HOCl (100 µM) ; (b) 7-HCCO (100 µM) ; (c) 7-HCCOox (10 µM); (d) 7-HCCO (100 µM) after 10 minutes incubation at 37°C with HOCl (100 µM) in PBS 1X pH 6.5. 7-HCCOox was prepared as described in section 2.3.

Figure 3. Effect of various amount of H_2O_2 on specific MPO activity in PBS 1X pH 6.5 with 7-HCCO at 100 µM. The specific MPO activity (As) was expressed as U/mg protein. One unit (U) of the enzyme was the activity which formed 1 µmol HOCl per min at 37°C. Data are the means ± standard deviations from 3 independent experiments made in triplicate. Statistics: one-way ANOVA followed by Mann-Whitney test *$p < 0.05$ compared to control without H_2O_2.

Figure 4. Kinetic curves for oxidation of 100 µM 7-HCCO with 100 µM H_2O_2 at 37°C:
1, Oxidation by HL-60 cell lysate 10 times diluted in 1X PBS pH 6.3; **2**, HL-60 cell lysate without H_2O_2 ; **3**, HL-60 cell lysate treated with 1 mM sodium azide. Data are the means ± standard deviations from 2 independent experiments made in triplicate.

Figure 5. Measurements of oxidative stress in streptozotocin-treated hyperglycemic rats (STZ group) compared with non-diabetic controls (A) Myeloperoxidase (MPO) activity in plasma (B) Blood reduced-to-oxidized glutathione ratio (GSH/GSSG) (C) Protein carbonylation (D) Lipid peroxidation measured by the MDA-TBA assay. Data represent means ± SEM of 12 independent experiments made in duplicate or triplicate (MPO assay). One-way ANOVA followed by Mann Whitney test: ** $P<0.01$ and *** $P<0.001$ versus controls.

Figure 6. Myeloperoxidase activity correlates with oxidative stress biomarkers in STZ diabetic rats (A) Plasma protein carbonyl levels and MPO concentrations (A) Blood reduced-to-oxidized glutathione ratio GSH/GSSG and MPO concentrations.

References

[1] M.L. Brennan, S.L. Hazen. Emerging role of myeloperoxidase and oxidant stress markers in cardiovascular risk assessment. Curr. Opin. Lipidol. 14 (2003) 353-359.

[2] E.A Podrez, H.M. Abu-Soud, S.L.Hazen. Myeloperoxidase-generated oxidants and atherosclerosis. Free Rad. Biol. Med. 28 (2000) 1717-1725.

[3] A.J. Kettle, T. Chan, I. Osberg, R. Senthilmohan, A.L.P. Chapman, T.J. Mocatta, J.S. Wagener. Myeloperoxidase and protein oxidation in the airways of young children with cystic fibrosis. Am. J. Respir. Crit. Care Med. 170 (2004) 1317-1323.

[4] J.J. Wiersma, M.C. Meuwese, J.N.I. van Miert, A. Kastelein, J.G.P. Tijssen, J.J. Piek, M.D. Trip. Diabetes mellitus type 2 is associated with higher levels of myeloperoxidase. Med. Sci. Monit. 14 (2008) 406-410.

[5] S. Shetty, N. Suchetha Kumari and L.N. Madhu. Variations in serum myeloperoxidase levels with respect to hyperglycemia, duration of diabetes, Bmi, sex and aging in type 2 diabetes mellitus. Inter. Jour. Res. Pharma. Bio. Sci. 3 (2012) 652-655.

[6] R. Zhang, M.L. Brennan, X. Fu, R.J. Aviles, G.L. Pearce, M.S. Penn, E.J. Topol, D.L. Sprecher, S.L. Hazen. Association between myeloperoxidase levels and risk of coronary artery disease. J. Am. Med. Assoc. 286 (2001) 2136-2142.

[7] A.T Askari, M.L. Brennan, X. Zhou, J. Drinko, A. Morehead, J.D. Thomas, E.J. Topol, S.L. Hazen, M.S. Penn. Myeloperoxidase and plasminogen activator inhibitor 1 play a central role in ventricular remodeling after myocardial infarction. J. Exp. Med. 197 (2003) 615-624.

[8] P. Song, J. Xu, Y. Song, S. Jiang, H. Yuan, and X. Zhang. Association of plasma myeloperoxidase level with risk of coronary artery disease in patients with type 2 diabetes. Disease Markers (2015), Article ID 761939, 5 pages.

[9] B. Pulli, M. Ali, R. Forghani, S. Schob, K.L.C. Hsieh, G. Wojtkiewicz, J.J. Linnoila, J.W. Chen. Measuring Myeloperoxidase Activity in Biological Samples. PLoS One. 8 (2013) e67976.

[10] T. Franck, S. Kohnen, K. Zouaoui Boudjeltia, P. Van Antwerpen, A. Bosseloir, A. Niesten, O. Gach, M. Nys, G. Deby-Dupont, D. Serteyn. A new easy method for specific measurement of active myeloperoxidase in human biological fluids and tissue extracts. Talanta 80 (2009) 723–729.

[11] D. Odobasic, A.R. Kitching, T.J. Semple S.R. Holdsworth. Endogenous myeloperoxidase promotes neutrophil-mediated renal injury, but attenuates T cell immunity inducing crescentic glomerulonephritis. J. Am. Soc. Nephrol. 18 (2007) 760-770.

[12] L.A. Marquez, H.B. Dunford. Mechanism of the oxidation of 3,5,3′,5′ tetramethylbenzidine by myeloperoxidase determined by transient- and steady-state kinetics. Biochem. 36 (1997) 9349-9355.

[13] K. Setsukinai, Y. Urano, K. Kakinuma, H.J. Majima, T. Nagano. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. J. Biol. Chem. 278 (2003) 3170-3175.

[14] A.J. Kettle, C.C. Winterbourn. Assays for the chlorination activity of myeloperoxidase. Methods Enzymol. 233 (1994) 502-512.

[15] J.M. Dypbukt, C. Bishop, W.M. Brooks, B. Thong, H. Eriksson, A.J. Kettle. A sensitive and selective assay for chloramine production by myeloperoxidase, Free Rad. Biol. Med. 39 (2005) 1468-1477.

[16] S.J. Klebanoff, in "Inflammation: Basic Principles and Clinical Correlates" J. I. Gallin, I. M. Goldsteia, and R. Snyderman, eds., p. 391. Raven, New York, 1988.

[17] C.C. Winterbourn, J.J.M. van den Berg, E. Roitman and F.A. Kuypers. Chlorohydrin formation from unsaturated fatty acids reacted with hypochlorous acid. Arch. Biochem. Biophys. 296 (1992) 547-555.

[18] B. Frei, L. England, B.N. Ames. Ascorbate is an outstanding antioxidant in human blood plasma. Proc. Natl. Acad. Sci. 86 (1989) 6377-6381.

[19] A.L.P Chapman, T.J. Mocatta, S. Shiva, A. Seidel, B. Chen, I. Khalilova, M.E. Paumann-Page, G.N. Jameson, C.C. Winterbourn, A.J. Kettlee. Ceruloplasmin is an endogenous inhibitor of myeloperoxidase. J. Biol. Chem. 288 (2013) 6465–6477.

[20] M. Zamocky, S. Hofbauer, I. Schaffner, B. Gasselhuber, A. Nicolussi, M. Soudi, K.F.Pirker, P.G. Furtmüller, C. Obinger. Independent evolution of four heme peroxidase superfamilies. Arch. Biochem. Biophys. 574 (2015) 108-119.

[21] P.G. Furtmüller, U. Burner, C. Obinger. Reaction of myeloperoxidase compound I with chloride, bromide, iodide, and thiocyanate. Biochem. 37 (1998) 17923-17930.

[22] J. Shepherd, S.A. Hilderbrand, P. Waterman, J.W. Heinecke, R. Weissleder P. Libby. A novel fluorescent probe for the detection of myeloperoxidase activity in atherosclerosis-associated macrophages. Chem Biol. 14 (2007) 1221-1231.

[23] S.Y. Yu, C.Y. Hsu, W.C. Chen, L.F. Wei, S.P. Wu. A hypochlorous acid turn-on fluorescent probe based on HOCl-promoted oxime oxidation and its application in cell imaging. Sensors and Actuators B 196 (2014) 203-207.

[24] S.J. Collins. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. Blood. 70 (1987) 1233-1244.

[25] P. N'Guessan, L. Pouyet, G. Gosset, S. Hamlaoui, M. Seillier, C.E. Cano, M. Seux, P. Stocker, M. Culcasi, J.L. Iovanna, N.J. Dusetti, S. Pietri and A. Carrier. Absence of tumor suppressor Tumor Protein 53-Induced Nuclear Protein 1 (TP53INP1) sensitizes mouse thymocytes and embryonic fibroblasts to redox-driven apoptosis. Antiox. Redox Signal. 15 (2011) 1639-1653.

[26] P. Stocker, E. Ricquebourg, N. Vidal, C. Villard, D. Lafitte, L. Sellami, S. Pietri. Fluorimetric screening assay for protein carbonyl evaluation in biological samples. Anal. Biochem. 482 (2015) 55-61.

[27] K.S. Lee, H.J Kim, G.H Kim, I Shin and J.I. Hong. Fluorescent chemodosimeter for selective detection of cyanide in water. Org. Lett. 10 (2008) 49-51.

[28] Y. Ohno and J.I. Gallin. Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils. Studies utilizing the inactivation of myeloperoxidase by hydrogen peroxide and azide. J. Biol. Chem. 260 (1985) 8438-8446.

[29] V. Gorudko, O.S. Tcherkalina, A.V. Sokolov, M.O. Pulina, E.T. Zakharova, V.B. Vasilyev, S.N. Cherenkevich and O.M. Panasenko. New approaches to the measurement of the concentration and peroxidase activity of myeloperoxidase in human blood plasma. Russ. J. Bioorg. Chem. 35 (2009) 566–575.

[30] R.J. Goiffon, S.C. Martinez and D. Piwnica-Worms. A rapid bioluminescence assay for measuring myeloperoxidase activity in human plasma. Nat Commun. 6 (2015) 6271-6280.

[31] http://www.hycultbiotech.com/hk105-02; https://www.alpco.com/pdfs/56/56-MPORT-E02.pdf

[32] D. Lau, H. Mollnau, J.P. Eiserich, B.A. Freeman, A. Daiber, U.M. Gehling, J. Brummer, V. Rudolph, T. Munzel, T. Heitzer, T. Meinertz and S. Baldus. Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. PNAS 102 (2005) 431-436.

[33] M. Whiteman, J.P. Spencer. Loss of 3-chlorotyrosine by inflammatory oxidants: implications for the use of 3-chlorotyrosine as a bio-marker in vivo. Biochem. Biophy. Res. Commun. 371 (2008) 50-53.

[34] C. Delporte, T. Franck, C. Noyon, D. Dufour, A. Rousseau, P. Madhoun, J.M. Desmet, D. Serteyn, M. Raes, J. Nortier, M. Vanhaeverbeek, N. Moguilevsky, J. Nève, L. Vanhamme, P. Van Antwerpen, K. Zouaoui Boudjeltia. Simultaneous measurement of protein-bound 3-chlorotyrosine and homocitrulline by LC–MS/MS after hydrolysis assisted by microwave: Application to the study of myeloperoxidase activity during hemodialysis. Talanta 99 (2012) 603-609.

[35] W. Wu, M.K. Samoszuk, S.A. Comhair, M.J. Thomassen, C.F. Farver, R.A Dweik, M.S. Kavuru, S.C. Erzurum, S.L. Hazen. Eosinophils generate brominating oxidants in allergen-induced asthma. J. Clin. Invest. 105 (2000) 1455-1463.

[36] A.S. Haqqani, J.K. Sandhu, H.C. Birnboim. A myeloperoxidase-specific assay based upon bromide-dependent chemiluminescence of luminol. Anal. Biochem. 273 (1999) 126-132.

[37] D.J. Ormrod, G.L. Harrison, T.E. Miller. Inhibition of neutrophil myeloperoxidase activity by selected tissues. J. Pharmacol. Methods 18 (1987) 137-142.

[38] T.J. Mocatta, A.P. Pilbrow, V.A. Cameron, R. Senthilmohan, C.M. Frampton, A.M. Richards, C.C. Winterbourn, Plasma concentrations of myeloperoxidase predict mortality after myocardial infarction. J. of the Am. Coll. of Cardio. 49 (2007) 1993-2000.

[39] M.L. Brennan, M.S. Penn, F. Van Lente, V. Nambi, M.H. Shishehbor, R.J. Avile, M. Goormastic, M.L. Pepoy, E.S. McErlean, E.J. Topol, S.E. Nissen, S.L. Hazen. Prognostic value of myeloperoxidase in patients with chest pain. N. Engl. J. Med. 349 (2003) 1595-604.

[40] S. Nishat, A. Klinke, S Baldus, L.A. Khan, S.F. Basir. Increased A3AR-dependent vasoconstriction in diabetic mice is promoted by myeloperoxidase. J. Cardiovasc. Pharmacol. 64 (2014) 465-472.

[41] C. Di Filippo, R. Marfella, S. Cuzzocrea, E. Piegari, P. Petronella, D. Giugliano, F. Rossi, M. D'Amico. Hyperglycemia in streptozotocin-induced diabetic rat increases infarct size associated with low levels of myocardial HO-1 during ischemia/reperfusion. Diabetes 54 (2005) 803-810.

[42] S. Ojha, J. Alkaabi, N. Amir, A. Sheikh, A. Agil, M.A. Fahim, A. Adem. *Withania coagulans* Fruit extract reduces oxidative stress and inflammation in kidneys of streptozotocin-induced diabetic rats. Oxid. Med. and Cell. Longev. (2014) Article ID 201436, 9 pages.

[43] R.A Maheshwari, R. Balaraman, A.K. Sen, A.K. Seth. Effect of coenzyme Q10 alone and its combination with metformin on streptozotocin-nicotinamide-induced diabetic nephropathy in rats. Indian J. Pharmacol. 46 (2014) 627-632.

[44] C. Uluoglu, B. Cimen, G. Ozbey, C. Karasu, D.B. Durakoglugil, A. Gunes, N. Turkozkan and H. Zengil. The effect of experimental diabetes on the circadian pattern

of adenosine deaminase and myeloperoxidase activities in rat liver. Gen. Physiol. Biophys. 27 (2008) 25-31.

[45] M. Olukman, E.D. Sezer, S. Ülker, E.Y. Sözmen, G.M. Çınar. Fenofibrate treatment enhances antioxidant status and attenuates endothelial dysfunction in streptozotocin-induced diabetic rats. Exp. Diabetes Res. (2010) Article ID 828531, 10 pages.

[46] J. Himmelfarb, E. McMonagle, E. McMenamin. Plasma protein thiol oxidation and carbonyl formation in chronic renal failure. Kid. Intern. 58 (2000) 2571-2578.

[47] A. Piwowar. Advanced oxidation protein products. Part I. Mechanism of the formation, characteristics and property. P. Merkur. Lekarski. 28 (2010) 166-169.

[48] D. Suzuki and T. Miyata. Carbonyl stress in the pathogenesis of diabetic nephropathy. Internal. Med. 38 (1999) 309-314.

[49] S. Pennathur, J.W. Heinecke. Mechanisms for Oxidative Stress in Diabetic Cardiovascular Disease. Antiox. Redox Signal. 9 (2007) 955-69.

[50] I.H. Buss, B.A. Darlow, C.C. Winterbourn. Elevated protein carbonyls and lipid peroxidation products correlating with myeloperoxidase in tracheal aspirates from premature infants. Pediatr. Res. 47 (2000) 640-645.

[51] M.C.M. Vissers and C.C. Winterbourn. Oxidation of intracellular glutathione after exposure of human red blood cells to hypochlorous acid. Biochem. J. 307 (1995) 57-62.

[52] S.C. Lu. Glutathione synthesis. Biochim. Biophys. Acta 1830 (2013) 3143-3153.

[53] V. Calabrese, C. Cornelius, V. Leso, A. Trovato-Salinaro, B. Ventimiglia, M. Cavallaro, M. Scuto, S. Rizza, L. Zanoli, S. Neri, P. Castellino. Oxidative stress, glutathione status, sirtuin and cellular stress response in type 2 diabetes. Biochim. Biophys. Acta 1822 (2012) 729-736.

[54] R.V. Sekhar, S.V McKay, S.G. Patel, A.P. Guthikonda, V.T. Reddy, A. Balasubramanyam, F. Jahoor. Glutathione synthesis is diminished in patients with uncontrolled diabetes and restored by dietary supplementation with cysteine and glycine. Diabetes Care 34 (2011) 162-167.

[55] Y. Dincer, T. Akcay, Z. Alademir, H. Ilkova. Assessment of DNA base oxidation and glutathione level in patients with type 2 diabetes. Mut. Res. 505 (2002) 75-81.

[56] H. Raza, A. John. Glutathione metabolism and oxidative stress in neonatal rat tissues from streptozotocin-induced diabetic mothers. Diabetes Metab. Res. Rev. 20 (2004) 72-78.

[57] Y. Unlucerci, S. Bekpinar, H. Kocak. Testis glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase activities in aminoguanidine-treated diabetic rats. Arch. Biochem. Biophys. 379 (2000) 217-220.

[58] V.M. Bhor, N. Raghuram, S. Sivakami. Oxidative damage and altered antioxidant enzyme activities in the small intestine of streptozotocin-induced diabetic rats. Int. J. Biochem. Cell. Biol. 36 (2004) 89-97.

[59] T. Miyata, S. Sugiyama, D. Suzuki, R. Inagi, K. Kurokawa. Increased carbonyl modification by lipids and carbohydrates in diabetic nephropathy. Kidney Int. Suppl. 71 (1999) 54-56.

[60] M. Díaz-Flores, S. Angeles-Mejia, L.A. Baiza-Gutman, R. Medina-Navarro, D. Hernández-Saavedra, C. Ortega-Camarillo, R. Roman-Ramos, M. Cruz M, F.J. Alarcon-Aguilar. Effect of an aqueous extract of Cucurbita ficifolia Bouché on the glutathione redox cycle in mice with STZ-induced diabetes. J. Ethnopharmacol. 144 (2012) 101-108.

[61] G. Saravanan, P. Ponmurugan. Ameliorative potential of S-allyl cysteine on oxidative stress in STZ induced diabetic rats. Chem. Biol. Int. 189 (2011) 100–106.