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Macrophage endoplasmic reticulum (ER) proteins and reducing elements stabilize paraoxonase 2 (PON2)

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ABSTRACT

Objective: To analyze the ability of macrophage sub-cellular fractions to stabilize paraoxonase 2 (PON2).
Methods: Nuclei, mitochondria, lysosomes, endoplasmic reticulum (ER) and cytosol were isolated from J774A.1 macrophage cell line and incubated with recombinant PON2.
Results: Among the fractions analyzed the ER contains the highest PON2 lactonase activity, and was the most potent one in stabilizing recombinant PON2 (rePON2). Whereas control rePON2 activity was decreased by 40% after 20 h of incubation at 37 °C, in the presence of ER it decreased by only 15%. This effect could be attributed to the ER aqueous phase, and not to the ER lipids. The ER proteins fraction was responsible for PON2 stabilization, since heated ER or proteinase K-treated ER was not able to protect rePON2 from inactivation, while the protein fraction (after ammonium sulfate precipitation) completely prevented rePON2 inactivation. Since in the macrophage ER, there are increased levels of NADPH, secondary to glutathione reductase deficiency, we next studied the effect of the redox environment on PON2 inactivation. Incubation of rePON2 with DTT protected PON2 from inactivation. Similarly, NADPH, but not NADP, significantly increased rePON2 lactonase activity by up to 19%, after 20 h of incubation as compared to control rePON2. Unlike ER from non-treated macrophages, ER harvested from oxidized-, or from cholesterol loaded-macrophages showed a significant lower basal PON2 lactonase activity, and did not protect PON2 from inactivation but rather increased it.
Conclusion: Under normal conditions macrophage ER stabilizes PON2 activity, and this effect could be attributed to ER proteins and redox status.

1. Introduction

Mammalian paraoxonase 2 (PON2) belongs to a unique family of calcium-dependent lactonases, which includes also PON1 and PON3 [1], and among these PONs, PON2 has the greatest lactonase activity against the bacterial quorum-sensing molecules [1,2]. Unlike PON1 which is present in serum as an HDL-associated enzyme, PON2 is not detectable in serum, but it is expressed in most tissues and cells, including macrophages [3,4]. In hypercholesterolemic patients, decreased macrophage PON2 expression was noted [5], and PON2 expression in human carotids was also shown to be decreased during the progression of atherosclerosis [6]. PON2, like PON1, was shown to protect against atherosclerosis development [7,8]. This latter characteristic could be related to PON2 ability to hydrolyze specific oxidized lipids and to inhibit cell-mediated LDL oxidation, oxidized LDL-induced monocytes chemotaxis [3], and also to prevent mitochondrial superoxide formation and apoptosis [9,10]. Furthermore, PON2 was shown to protect macrophages from triglyceride accumulation [11,12]. The regulation of macrophage PON2 is complex and macrophage oxidative stress was shown to affect PON2 expression and activities in a biphasic U-shape pattern [4,13]. Upregulation of macrophage PON2 can occur via several mechanisms, including NADPH-oxidase activation [14], beneficial effect of pomegranate juice polyphenolic antioxidants [15], adverse effect of unesterified cholesterol accumulation [16], and that of urokinase plasminogen activator (uPA) [17].

PON2 appears in two spliced isoforms that predominantly localized to the endoplasmic reticulum (ER), mitochondria and the nuclear envelop [9,15,18,19], but not in the cytosol. Recently, it was shown that PON2 protein is also localized to the inner mitochondrial membrane, where it is found to be associated with respiratory complex III [20]. The redox environment in sub-cellular fractions that contain PON2, as well as the presence of specific sub-cellular constituents which bind PON2, can affect the enzyme expression, and activity. Thus, in the present study we analyzed the effect of macrophage sub-cellular fractions, with spe-

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cial emphasis on specific ER constituents, on recombinant PON2 stability.

2. Methods

2.1. Reagents

Dihydrocumarin (DHC), nitriloacetic acid (NTA), dithiothreitol (DTT), 5,5′-dithio-bis (2-nitrobenzoic) acid (DTNB) and 2′,7′-dichlorofluorescin diacetate (DCFH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2,2′-Azobis, 2-amidinopropane hydrochloride (AAPH) was purchased from Wako, Japan.

PBS, DMEM medium, FCS (heat-inactivated at 56 °C for 30 min), penicillin, streptomycin, nystatin, L-glutamine, and sodium pyruvate were purchased from Biological Industries (Beth Haemek, Israel). Proteinase K was purchased from Epicenter Biotechnologies, Madison, USA.

2.2. Cells

2.2.1. J774 A.1 macrophage cell line

J774A.1 murine macrophage cell-line was purchased from the American Tissue Culture Collection (ATCC, Rockville, MD). The cells were grown in DMEM containing 5% FCS.

2.2.2. Macrophage sub-cellular fractionation

The sub-cellular fractions were prepared from J774A.1 macrophages as previously described [21]. The cells (40×10^6), in 1.5 ml of buffer A (50 mM Tris/HCl, pH 7.4, 250 mM sucrose, mix protease inhibitors), were sonicated for 3×10 s, resulting in about 95% of cell breakage. Step 1-nuclei (centrifugation $600 \times g$ for 5 min), step2-mitochondria (recentrifugation of supernatant $3000 \times g$ for 20 min), step 3-lysosomes (recentrifugation of supernatant $15{,}000 \times g$ for 1 h), step 4-endoplasmic reticulum (ER, recentrifugation of supernatant $100{,}000 \times g$ for 4 h). The final supernatant is the cytosolic fraction.

2.2.3. Isolation of ER lipid and aqueous phases

Macrophage ER lipids were extracted from 500 μl ER + 500 μl PBS by 4 ml of hexane; isopropanol (3:2, v:v). The upper hexane phase was collected and evaporated under nitrogen. The dried lipids were dissolved in 20 μl of 70% ethanol, followed by addition of 480 μL Tris buffer. The final concentration of ethanol in the rePON2–ER lipids incubation system was 0.9%. The isopropanol from the lower phase was evaporated under nitrogen, and the remaining liquid (500 μl at −80 °C) was lyophilized for 20 h. The obtained powder was dissolved in 500 μl of Tris buffer.

2.2.4. Isolation of ER protein fraction

The ER proteins were precipitated from 100 μl of ER with saturated solution of ammonium sulfate [22]. The protein pellet was obtained after 1 h incubation, and centrifugation at $10{,}000 \times g$. The protein pellet was dissolved in 100 μl Tris buffer.

2.2.5. ER total thiols (SH groups) content

The assay procedure determines the amount of protein bound SH groups, as well as of glutathione [23]. An aliquot of 10 μl of whole ER and of ER protein fraction were mixed with 200 μl of Tris–EDTA buffer, and the absorbance at 412 nm was measured. To these samples 8 μl of 10 mM DTNB was added, and after 15 min of incubation at room temperature, the absorbance was measured again together with a DTNB blank. Total SH groups were then calculated.

2.3. Recombinant PON2 (rePON2) preparation

RePON2 was prepared by site directed evolution [24]. The protein is fused to TRX. The protein is kept in (50 mM Tris–HCl pH 8.0, 1 mM $CaCl_2$, 0.1% triton X-100 containing 0.01% NaN3) in a concentration of 13.6 μM (760 μg/ml).

2.3.1. RePON2 lactonase activity towards dihydrocoumarin (DHC)

The assay was performed in 96 well UV plates, in a total volume of 200 μl per well. Lactonase activity was measured using dihydrocumarin (DHC) as the substrate. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm, for 10 min (every 15 s). The assay mixture included 1 mM DHC in 50 mM Tris–HCl, pH 8.0 + 1 mM $CaCl_2$. Nonenzymatic hydrolysis of DHC was subtracted from the total rate of hydrolysis. One unit of lactonase activity equals 1 μmol of DHC hydrolyzed/min/ml [25]. RePON2 lactonase activity with DHC is 3.6 ± 0.4 units/mg.

2.3.2. RePON2 lactonase activity towards thiobuthyl butyrolactone (TBBL)

The assay was performed in 96 well plates, in a total volume of 200 μl per well. Lactonase activity was measured using 5-thiobuthyl butyrolactone (TBBL) as the substrate [26]. Initial rates of hydrolysis were determined spectrophotometrically at 412 nm, for 10 min (every 15 s). The assay mixture included 0.5 mM DTNB and 0.25 mM TBBL in 50 mM Tris–HCl, pH 8.0 + 1 mM $CaCl_2$. Nonenzymatic hydrolysis of TBBL was subtracted from the total rate of hydrolysis. One unit of lactonase activity equals 1 μmol of TBBL hydrolyzed/min/ml.

2.3.3. RePON2 NTA inactivation assay

To rePON2, rePON2 + ER or rePON2 + cytosol an equal volume of inactivation buffer [10 mM Tris, 150 mM NaCl, pH 8.0 supplemented with 2 mM nitrilotriacetic acid (NTA, which is a calcium chelator) and 5 mM β-mercaptoethanol] were added. Then, the samples were incubated at 37 °C for 20 h. Lactonase activity was measured at baseline and after 20 h. RePON2 activity is expressed as percentage of residual activity [27].

2.4. Aggregated LDL (Agg-LDL) preparation

Agg-LDL was prepared by vortexing of LDL for 2 min at room temperature (25° C), using LDL protein concentration of 1 mg/ml.

2.5. Macrophage cholesterol content

J774A.1 macrophages (3×10^6) lipids were extracted with hexane: isopropanol (3:2, v:v). The hexane phase was dried under nitrogen and the amount of cholesterol was measured using a commercial kit (Roche Ltd., Manheim, Germany).

2.6. Total macrophage peroxides (DCFH-DA assay)

Intracellular oxidative stress was assayed by the oxidation of DCFH-DA [28], and monitored by a flow cytometry. J774 A.1 macrophages (1×10^6) were washed with PBS followed by a further incubation with 10 μM of DCFH-DA, for 30 min at 37 °C. The cells were washed (×2) with PBS, and then the adherent cells were detached by gentle scraping. Measurements of cellular fluorescence determined by FACS were performed at 510–540 nm after excitation of the cells at 488 nm with an argon ion laser. Cellular fluorescence was measured in terms of mean fluorescence intensity (MFI).