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# Macrophage paraoxonase 2 regulates calcium homeostasis and cell survival under endoplasmic reticulum stress conditions and is sufficient to prevent the development of aggravated atherosclerosis in paraoxonase 2 deficiency/apoE$^{-/-}$ mice on a Western diet

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ABSTRACT

Paraoxonase 2 deficiency (PON2-def) alters mitochondrial function and exacerbates the development of atherosclerosis in mice. PON2 overexpression protects against ER stress in cell culture. In this paper, we examined the role of PON2 in the unexplored link between ER stress and mitochondrial dysfunction and tested whether restoration of PON2 in macrophages is sufficient to reduce aggravated atherosclerosis in PON2-def/apoE$^{-/-}$ mice on a Western diet. ER stress response genes, intracellular calcium levels, and apoptotic nuclei were significantly elevated in PON2-def/apoE$^{-/-}$ macrophages compared to apoE$^{-/-}$ macrophages in response to ER stressors, but not at the basal level. In contrast, PON2-def/apoE$^{-/-}$ macrophages exhibited greater mitochondrial stress at the basal level, which was further worsened in response to ER stressors. There was no difference in ER stress response genes and apoptotic nuclei between apoE$^{-/-}$ and PON2-def/apoE$^{-/-}$ macrophages when pretreated with xestospongin (which blocks the release of calcium from ER) suggesting that PON2 modulates cell survival and ER stress by maintaining calcium homeostasis. Treatment with a mitochondrial calcium uptake inhibitor, RU360, attenuated ER stressor mediated mitochondrial dysfunction in PON2-def/apoE$^{-/-}$ macrophages. CHOP expression (ER stress marker) and apoptotic nuclei were significantly higher in aortic lesions of PON2-def/apoE$^{-/-}$ mice compared to apoE$^{-/-}$ mice fed a Western diet. Restoration of PON2 in macrophages reduced ER stress, mitochondrial dysfunction and apoptosis in response to ER stressors. Furthermore, restoration of PON2 in macrophages reduced lesional apoptosis and atherosclerosis in PON2-def/apoE$^{-/-}$ mice on a Western diet. Our data suggest that macrophage PON2 modulates mechanisms that link ER stress, mitochondrial dysfunction and the development of atherosclerosis.

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## 1. Introduction

Atherosclerosis is a chronic inflammatory condition in which the migration of circulating monocytes into the vessel wall is an important step. According to the lipid oxidation hypothesis of atherosclerosis, low-density lipoprotein (LDL) is a major target of oxidation and minimally oxidized lipids in LDL recruit monocytes into the sub-endothelial space where they differentiate into macrophages. Subsequent uptake of oxidized lipids by macrophages causes the formation of foam cells and atherosclerosis [1,2].

Mitochondrial respiratory chain dysfunction resulting in increased production of superoxides causes the oxidation of low-density lipoprotein (LDL) [3], vascular cell apoptosis, and increased atherosclerotic lesion formation [4]. Unfolded protein response (UPR) leading to endoplasmic reticulum (ER) stress induces oxidative stress, vascular cell apoptosis, and the development of atherosclerosis [5,6]. Emerging evidence suggests that there is an extensive crosstalk between ER and mitochondria under both physiological and pathophysiological conditions [7]. Understanding the key players in the crosstalk between the two organelles is an important area of investigation.

*Abbreviations:* ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein; DCF, dichlorofluorescein; ER, endoplasmic reticulum; ERO1-α, ER oxidase 1 alpha; ETC, electron transport chain; IPT3, inositol 1,4,5-triphosphate receptor; LOOH, lipid peroxide; LDL, low density lipoprotein; IMM, inner mitochondrial membrane; UPR, unfolded protein response; Ox-PAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine; PON2-def, paraoxonase 2-deficiency.

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PON2 is an intracellular membrane- associated protein that is widely expressed in many tissues and vascular cells including macrophages [8,9]. At the subcellular level, PON2 is localized to both the ER [10] and mitochondria [11], and protects against oxidative stress [12]. We have shown that in PON2-def mice, enhanced mitochondrial oxidative stress was accompanied by reduced electron transport chain (ETC) complex I + III activities, oxygen consumption, and adenosine triphosphate (ATP) levels and further exacerbates the development of atherosclerosis [11]. Using cell culture models, we and Altenhofer et al. have shown that overexpression of PON2 prevents the generation of ubisemiquinone-induced mitochondrial superoxide formation [11,13]. In this report, we show that macrophages from PON2-def/apoE$^{-/-}$ mice show altered calcium handling in response to ER stressors, enhanced UPR, and a worsened mitochondrial oxidative stress in a calcium dependent manner. We further demonstrate that restoration of PON2 in macrophages reduces mitochondrial dysfunction, ER stress, ER stress-mediated mitochondrial dysfunction and diet-induced atherosclerosis in PON2-def/apoE$^{-/-}$ mice.

## 2. Materials and methods

### 2.1. Mice and diet

Female PON2-def mice were crossed with apoE$^{-/-}$ mice on a C57BL/6J background to generate PON2-def/apoE$^{-/-}$ and PON2$^{+/+}$/apoE$^{-/-}$ littermate controls. Mice were started on a Western diet containing 0.15% cholesterol providing 42% calories as fat (TD 88137; Harlan Teklad, Madison, WI) at 6 weeks of age, and maintained on this diet for an additional 16 weeks. In separate studies, LysMCre mice were crossed with PON2-def/apoE$^{-/-}$ to obtain PON2-def/LysMCre/apoE$^{-/-}$ mice [14] and were placed on a Western diet for 16 weeks. At the end of the study, the mice were fasted overnight and organs were collected. The Animal Research Committee at UCLA approved all animal protocols.

### 2.2. Atherosclerosis studies

Aortic root lesion analysis, CD68 quantification and en face lesion analysis were carried out in a blinded fashion by light microscopy as described previously [15,16] and lipid peroxide content in the lipoproteins was analyzed as described previously [15].

### 2.3. Macrophage harvest and treatments

Peritoneal macrophages were harvested from mice three days after the animals received an i.p. injection of thioglycolate, and the cells were plated in 10% FBS in DMEM media as previously described [15]. Adherent macrophages were treated with ER stressor namely, tunicamycin or thapsigargin or oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (Ox-PAPC) as described in the figure legends. ER stressor concentrations and treatment times were chosen based on previous reports [17–20]. Higher concentrations (1 mM tunicamycin; 25 μM thapsigargin) were chosen for the short incubation times. The outcomes measured for these experiments were intracellular calcium flux (30 s to 600 s; Figs. 1B [upper panel], 2A, 3A and 6B), mitochondrial calcium (1 h; Figs. 3C and 4A) and UPR gene (1 h; Fig. 3B). Lower concentrations of ER stressors (0.35 μM tunicamycin; 0.25 μM thapsigargin; and 50 μg/ml Ox-PAPC) were chosen for the longer incubation times (5–24 h). The outcomes measured for these experiments were expression of UPR genes (5 h; Figs. 1A, E [left panel], 2B and 6A), mitochondrial superoxide (5 h; Figs. 3G, 4B and 6C), ROS (5 h; Figs. 1C, E (middle panel) and 2C), apoptosis (24 h; Figs. 1D, E (right panel), 2D, 4C and 6D), calcium levels at specified time points (Figs. 1B (lower panel) and 3D) and mitochondrial function (5 h; Figs. 3E–F).

### 2.4. Quantification of mitochondrial superoxide

Mitochondrial enriched fraction was isolated from peritoneal macrophages as described by Graham [21]. Mitochondrial superoxide assay was carried out by incubating 4 μg of mitochondrial protein with 5 μM dihydroethidium in a 96-well plate format at 37 °C for 60 min. The reaction was stopped by the addition of 0.6% Triton X-100 and 10 μg of sonicated salmon sperm DNA and the ethidium bromide/DNA fluorescence was measured by excitation at 544 nm and emission at 612 nm using a BMG Labtech fluorescence microplate reader [22].

### 2.5. Mitochondrial cytochrome oxidase (complex IV) activity assay

Mitochondrial cytochrome oxidase activity assay was carried out as described by the manufacturer's protocol (Sigma Aldrich, St. Louis, USA). Briefly, 0.22 mM of cytochrome c was prepared in water and was reduced (ferrocytochrome c) by adding dithiothreitol (DTT) to the final concentration of 0.5 mM. Mitochondrial suspension (100 μg protein) was added into a cuvette containing 0.95 ml assay buffer (10 mM Tris–HCl, pH 7.0, containing 120 mM KCl) and brought the reaction volume to 1.05 ml with dilution buffer (10 mM Tris–HCl, pH 7.0, containing 250 mM sucrose). Reaction was started by adding 50 μl of 0.22 mM ferrocytochrome c and measured at 550 nm at room temperature using kinetic program (10 second interval; 6 readings) with a spectrophotometer. Enzyme activity was expressed as nmol cytochrome c oxidized/mg protein.

### 2.6. Measurement of calcium level

Intracellular calcium was measured in peritoneal macrophages before and after treatment with tunicamycin or thapsigargin using indo-1 AM as described [23]. Changes in the intracellular calcium concentration were quantified by a shift in the indo-1 emission peak from 485 nm (indo-blue) for unbound dye to 405 nm (indo-violet) when the indo-1 molecule was bound to calcium. Mitochondrial calcium was measured with Rhod-2/AM. Briefly, peritoneal macrophages in phenol red-free medium were incubated with 1 μM Rhod-2/AM at 37 °C for 20 min followed by thapsigargin (25 μM) treatment for 1 h, then mean fluorescence intensity was analyzed by flow cytometry at 549 nm (excitation) and 581 nm (emission) [24].

### 2.7. Intracellular reactive oxygen species (ROS) measurement

A 2′,7′-dichlorofluorescein (DCF) assay was used for the quantification of intracellular ROS as previously described [25]. Briefly, cell viability was assessed with trypan blue on a hemacytometer. Viable cells were plated onto 96-well plates ($7 \times 10^4$ cells/well) and loaded with 100 μM DCF (Invitrogen) for 1 h at 37 °C. Cells were subsequently washed using Krebs–Ringer buffer and treated with ER stressor as described in the figure legends. Fluorescence was measured at the indicated time using a fluorescence microplate reader (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA) with an excitation filter of 485 nm and an emission filter at 530 nm.

### 2.8. Quantitative RT-PCR analysis

Total RNA was isolated from peritoneal macrophages of experimental groups by RNeasy mini kit (Qiagen, Valencia, CA, USA), and 0.1 μg RNA was used for reverse transcription with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). Two microliters of the cDNA was used for PCR reaction with gene specific primers and iQ SYBR Green Supermix (BIO-RAD, Hercules, CA, USA) in a MyiQ Single-Color Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA). mRNA was quantified and normalized to mouse cyclophilin levels. The primers

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