Free Radical Biology & Medicine 51 (2011) 396–405

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

PPARγ stabilizes HO-1 mRNA in monocytes/macrophages which affects IFN-β expression

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ARTICLE INFO

Article history:
Received 29 July 2010
Revised 29 March 2011
Accepted 18 April 2011
Available online 22 April 2011

Keywords:
Innate immunity
Reactive oxygen species
NADPH oxidase
HO-1
mRNA stabilization
Mouse model

ABSTRACT

NADPH oxidase activation in either RAW264.7 cells or peritoneal macrophages (PM) derived from PPARγ wild-type mice increased reactive oxygen species (ROS) formation, caused PPARγ activation, heme oxygenase-1 (HO-1) induction, and concomitant IFN-β expression. In macrophages transduced with a dominant negative (d/n) mutant of PPARγ (RAW264.7 AF2) as well as PPARγ negative PM derived from Mac-PPARγ-KO mice, NADPH oxidase-dependent IFN-β expression was attenuated. As the underlying mechanism, we noted decreased HO-1 mRNA stability in RAW264.7 AF2 cells as well as PPARγ negative PM, compared to either parent RAW264.7 cells or wild-type PM. Assuming mRNA stabilization of HO-1 by PPARγ we transfected macrophages with a HO-1 3′-UTR reporter construct. The PPARγ agonist rosiglitazone significantly up-regulated luciferase expression in RAW264.7 cells, while it remained unaltered in RAW264.7 AF2 macrophages. Deletion of each of two AU-rich elements in the 3′-UTR HO-1 decreased luciferase activity in RAW264.7 cells. Using LPS as a NADPH oxidase activator, PM from Mac-PPARγ-KO mice showed a decreased HO-1 mRNA half-life *in vitro* and *in vivo* compared to PPARγ wild-type mice. These data identified a so far unappreciated role of PPARγ in stabilizing HO-1 mRNA, thus, contributing to the expression of the HO-1 target gene IFN-β.

Introduction

Monocytes/macrophages are cells of the innate immune system [1] and therefore are a first line defense toward pathogens. To guarantee efficient removal of microbes, monocytes/macrophages exhibit several potent killing mechanisms [2]. Rescue mechanisms, which have been developed to protect monocytes/macrophages against their antimicrobial effector systems, including reactive oxygen species (ROS)[3] and nitric oxide (NO), are important to guarantee functional innate immune responses [3,4]. Likely, detoxifying enzymes such as superoxide dismutases (SODs) and catalase contribute to monocyte/macrophage survival during periods of ROS/NO formation [5,6]. However, ROS and NO can act as intracellular signaling molecules as well. ROS are well appreciated as cellular switches for signaling cascades provoking gene regulation. Several transcription factors such as Nrf2, NF-κB, AP-1, or STAT3 [7–10] are activated in response to ROS, contributing to a proinflammatory cellular phenotype. Recently, activation of the NADPH oxidase has been linked to IFN-β expression in the human lung adenocarcinoma epithelial cell line A549 [11]. In these cells a knockdown of NOX-2 expression by an RNAi approach attenuated IFN-β expression in response to Sendai virus infection. Taking into consideration that IFN-β is a HO-1 target gene [12], we were interested to analyze whether in monocytes/macrophages activation of the NADPH oxidase provokes IFN-β expression via a HO-1-dependent mechanism.

HO-1 plays critical roles in defending cells against oxidant-induced injury during inflammatory processes. HO-1 degrades heme to carbon monoxide (CO), biliverdin, and ferrous iron [13]. Biliverdin is converted to bilirubin, a potent endogenous antioxidant. CO has a number of biological functions, including anti-inflammatory properties [14]. Therefore, HO-1 constitutes an intracellular protective system, increasing monocyte/macrophage endurance and contributing to a confined proinflammatory response [15–18]. Although Nrf2-dependent HO-1 expression is established [15,19], it has recently been shown that ligand activation of peroxisome proliferator-activated receptorγ (PPARγ) by thiazolidinediones (TZDs) provokes HO-1 expression in endothelial cells, accounting for an antiproliferative and anti-inflammatory phenotype [20]. Moreover, NO-dependent activation of PPARγ induces HO-1 expression in human umbilical vein and hybrid EA.hy926 endothelial cells. Activation was blocked by the

Abbreviations: 15d-PGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; CRE, causes recombination; CO, carbon monoxide; CHX, cycloheximide; d/n, dominant negative; DPI, diphenyliodonium; HE, hydroethidine; HO-1, heme oxygenase-1; LPS, lipopolysaccharide; LysM, lysozyme M; mac, macrophages; m, mouse; NAC, *N*-actetylcysteine; Nrf2, NF-E2-related factor-2; PPARγ, peroxisome proliferator-activated receptorγ; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; TZD, thiazolidinedione; UTR, untranslated region; ZnPP, zinc-protoporphyrin.

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doi:10.1016/j.freeradbiomed.2011.04.033

p38 MAPK inhibitor SB203580 [21,22]. Although PPARγ agonists such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) are established HO-1 inducers in monocytes/macrophages, this induction has not been attributed to PPARγ [23]. Rather the electrophilic nature of this compound is considered to cause oxidative stress, concomitant Nrf2 activation, and HO-1 expression. Therefore, we were interested to determine how PPARγ activation affects HO-1 expression, consequently altering IFN-β expression in monocytes/macrophages in response to oxidative stress. Using RAW264.7 macrophages expressing either PPARγ wild type or a dominant negative (d/n) PPARγ mutant and macrophages derived from PPARγ wild-type mice as well as conditional PPARγ knockout mice, we observed that activation of PPARγ in response to oxidative stress stabilized HO-1 mRNA *in vitro* as well as *in vivo* as shown in an endotoxin mouse model. A decreased HO-1 mRNA half-life in macrophages lacking functional PPARγ expression is paralleled by an attenuated expression of the HO-1 target gene IFN-β and decreased transactivation of the related transcription factor interferon regulatory factor (IRF) 3 in response to oxidative stress.

Materials and methods

Chemicals

Rosiglitazone (Biomol, Hamburg, Germany), PMA, diphenyliodonium (DPI, Sigma-Aldrich, Deisenhofen, Germany), SB203580 (Calbiochem, Darmstadt, Germany), and actinomycin D (Calbiochem) were dissolved in DMSO. LPS (*Escherichia coli* strain 0127:B8, Sigma-Aldrich), and *N*-acetylcysteine (NAC, Sigma-Aldrich) were dissolved in PBS. Primers were obtained from Biomers, Ulm, Germany. RPMI1640, culture supplements, and fetal calf serum were ordered from PAA Laboratories, Linz, Austria. Appropriate vehicle controls were performed.

Cell culture

The mouse monocyte/macrophage cell lines RAW264.7 and RAW264.7 AF2 were maintained in RPMI 1640 medium, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% heat-inactivated FCS at 37 °C (complete medium) and 5% CO_2. Human PLB985 and PLB985 X-CGD cells [24,25] were kindly provided by Dr. Manual Grez (Molecular Virology and Gene Therapy Unit, Georg-Speyer-Haus, Frankfurt, Germany) and maintained in complete medium as well.

Animals

C57BL/6 LysM-CRE mice (LysM-$CRE^{+/+}$) were bred with C57BL/6 $PPAR\gamma^{fl/fl}$ mice as described [26,27]. Genotyping of LysM-$CRE^{+/+}$ $PPAR\gamma^{fl/fl}$ mice was performed as described [28]. C57BL/6 LysM-$CRE^{+/+}$ $PPAR\gamma^{fl/fl}$ (Mac-PPARγ-KO) and $PPAR\gamma^{fl/fl}$ (PPARγ wild-type) mice (25–35 g) were used for the experiments. Animals had free access to water and pellet food. Animals were exposed to a regular light–dark cycle of 12:12 h.

Experimental design: LPS intoxication

In Mac-PPARγ-KO and PPARγ wild-type mice bacterial burden was mimicked for 24 h by intraperitoneal (ip) injection of 1 mg/kg LPS (Mac-PPARγ-KO-LPS, $n=8$, and PPARγ wild-type-LPS, $n=8$). At the end of the experiment, mice were sacrificed and peritoneal macrophages were isolated by peritoneal lavage with 5 ml PBS. Animal experiments followed the guidelines of the Hessian animal care and use committee (Authorization No. V54-19c20/15-F144/01 and 02).

Flow cytometry of oxygen radical production (HE assay)

Cells (5×10^5) were incubated for 30 min with or without 100 nM PMA followed by two PBS washing steps. After cell stimulation, 3 μM HE was added and incubations went on for 30 min. Cells were harvested, washed with PBS, and resuspended in 200 μl of PBS. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences) and HE was measured through a 575/26 nm filter (FL2). Data from 10,000 cells were collected to reach significance. All incubations were performed at 37 °C.

shRNA knockdown of HO-1

For efficient gene knockdown, a lentiviral shRNA approach was used. A validated Mission shRNA plasmid (TRCN0000234076) for murine HO-1 cloned into the pLKO.1-puro-CMV-TurboGFP backbone and a Mission Non-Target shRNA control vector as a negative control were obtained from Sigma. For generation of lentiviral particles, 3×10^6 HEK293T cells were seeded in 5 ml complete DMEM medium. The following day, cells were transfected using jetPEI (Biomol, Hamburg, Germany) according to the distributor's instructions. Briefly, 3 μg of the shRNA plasmid in combination with 26 μl of the packaging mix (Sigma) and 6 μl jetPEI were used. Afterward, cells were cultured for 2 days. Medium was then changed and after another 2 days, the supernatant containing the infectious lentiviral particles was taken. To transduce target cells, lentivirus containing culture supernatants was filtered through a 0.45-μm filter (Millipore, Schwalbach, Germany) and thereafter directly spun twice onto RAW 264.7 macrophages (90 min, 500*g*, RT). Transduced RAW 264.7 cells were grown for 1 week in complete medium before puromycin was added for antibiotic selection of positive clones. Following 4 weeks of puromycin selection and FACS sorting of EGFP-positive RAW 264.7 macrophages, knockdown efficiency was analyzed at the mRNA level by qPCR.

RNA extraction and quantitative real-time PCR

RNA from RAW264.7 and primary peritoneal macrophages was extracted using peqGold RNAPure (Peqlab Biotechnologie GmbH, Erlangen). Total RNA (1 μg) was transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Munich, Germany). Quantitative real-time-PCR (qPCR) was performed using the MyiQ real-time PCR system (Bio-Rad) and Absolute Blue QPCR SYBR Green fluorescein mix (Thermo Scientific, Karlsruhe, Germany). For HO-1, IFN-β and 18S expression validated QuantiTect primer assays were purchased from Qiagen (Hilden, Germany). Real-time PCR results were quantified using Gene Expression Macro (version 1.1) from Bio-Rad with 18S expression as the internal control.

Western analysis

Cell lysis was achieved with lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, pH 8.0) and sonification (Sonifier; duty cycle 100%, output control 60%). Cell lysates were cleared by centrifugation (10,000*g* for 5 min), and protein concentration was determined with the Lowry method. Eighty micrograms of protein was denatured using 4X sample buffer (250 mM Tris, pH 6.8, 40% glycerol, 10% beta-mercaptoethanol, 8% SDS, 0.02% bromphenol blue), treated at 100 °C for 10 min, and resolved on 12% SDS-polyacrylamide gels. For protein detection, gels were blotted onto nitrocellulose, following standard methodology. To prevent unspecific antibody binding, membranes were blocked with 5% nonfat dry milk in TBST (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h. Blots were then incubated with anti-HO-1 (Biomol), anti-p38MAPK (Cell Signaling, NEB Frankfurt, Germany), anti-p38MAPK-P (Cell Signaling), and anti-$gp91^{phox}$ (Active Bioscience, Hamburg, Germany)