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Bone marrow-derived macrophages exclusively expressed caveolin-2: The role of inflammatory activators and hypoxia

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ABSTRACT

Caveolins are specific proteins involved in regulation of signal transduction to intracellular space. Still, their contribution to immune functions has not been completely clarified. Thus, we decided to characterize the expression of caveolins in bone marrow-derived macrophages (BMDMs) under resting and inflammatory conditions. The effect of classical activators (lipopolysaccharide, LPS; interferon-gamma, IFN-γ) was further potentiated with hypoxic (5% O_2) conditions. The activation of p44/42-extracellular signal-regulated kinases 1 and 2 (ERK1/2) and expression of caveolin-1, -2, and -3, hypoxia inducible factor-1 alpha (HIF-1α), as well as inducible nitric oxide synthase (iNOS) was monitored using the Western blot technique. The production of nitric oxide (NO) and tumor necrosis factor-alpha (TNFα) was analyzed by Griess method or ELISA, respectively. BMDMs were also transfected with siRNA against caveolin-2. Importantly, our study showed for the first time that BMDMs expressed only caveolin-2, and its level decreased after activation of macrophages with LPS, IFN-γ, and/or hypoxia. The expression of caveolin-2 negatively correlates with the iNOS and HIF-1α protein levels, as well as with the LPS/IFN-γ- and hypoxia-induced activation of ERK1/2. We concluded that caveolin-2 is most probably involved in regulation of pro-inflammatory responses of BMDMs, triggered via activation of ERK1/2.

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1. Introduction

Caveolae are small "cave-like" structures, which are located in lipid rafts, composed of cholesterol, sphingolipids and caveolin molecules. The gene family of caveolins consists of three known isoforms: caveolin-1, -2 and -3 (Cohen et al., 2004; Kiss et al., 2002; Parton and Simons, 2007; Razani et al., 2002a). Caveolin-1 and -2 are mostly expressed in adipocytes, endothelial cells, and fibroblasts. In contrast, the expression of caveolin-3 was shown to be dominant in muscle cells (Cohen et al., 2004; Razani et al., 2002a; Hansen and Nichols, 2010; Sowa, 2012). Importantly, caveolins were shown to be involved in regulation of signal transduction to intracellular space (Parton and Simons, 2007; Razani et al., 2002a; Navarro et al., 2004). They act as signalosomes for various molecules, which consequently activate different intracellular signaling pathways (e.g. mitogen activated protein kinases, MAPKs; phospholipase C, etc.) (Parton and Simons, 2007; Razani et al., 2002a; Navarro et al., 2004; Conner and Schmid, 2003; Doherty and McMahon, 2009). Although the functions of caveolins have been intensively studied within the past few years (Cohen et al., 2004; Razani et al., 2002a), their role in regulation of inflammatory processes had not been satisfactorily elucidated. Current studies suggest that caveolins are present in all types of immune cells, although their expression is conditional on the state of cell activation or maturation (Harris et al., 2002a,b). The expression of caveolins was confirmed in mouse peritoneal macrophages, mast cells and neutrophils, human dendritic cells and neutrophils, as well as in bovine monocytes, macrophages, dendritic cells, and lymphocytes (Kiss et al., 2002; Harris et al., 2002a,b; Gargalovic and Dory, 2001; Hu et al., 2008; Zemans and Downey, 2008).

Abbreviations: BMDM, bone marrow-derived macrophage; ERK 1/2, p44/42-extracellular signal-regulated kinases 1 and 2; HIF-1α, hypoxia inducible factor-1 alpha; IFN-γ, interferon-gamma; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; JAK/STAT, JAK kinases/signal transducers and activators of transcription; LPS, lipopolysaccharide; M-CSF, macrophage-colony stimulating factor; MAPKs, mitogen-activated protein kinases; NO, nitric oxide; PM, peritoneal macrophage; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α.

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Caveolin-1 was also shown to act as a potent anti-inflammatory molecule, involved in regulation of intracellular signaling pathways in professional phagocytes (e.g. neutrophils and macrophages), associated with activation of MAPKs and production of an antimicrobial molecule, nitric oxide (NO) (Hu et al., 2008; Zemans and Downey, 2008).

Macrophages represent the front line of defense against infection caused by bacteria, viruses, and other pathogens. Furthermore, they are critically involved in the processes of inflammation, wound healing, and development of various diseases (Burke et al., 2003; Talks et al., 2000). Under inflammatory conditions, when exposed to classical activators (e.g. bacterial lipopolysaccharide, LPS and interferon-gamma, IFN-γ), they effectively up-regulate the expression and activation of enzymes (e.g. inducible nitric oxide synthase, iNOS; NADPH-oxidase; etc.) and are responsible for production of a wide spectrum of pro-inflammatory mediators (e.g. NO and tumor necrosis factor alpha, TNFα) (Burke et al., 2003; Talks et al., 2000; Ben et al., 2013; Laskin et al., 2010; Fakhrzadeh, 2008; Parameswaran and Patial, 2010). Importantly, when recruited to tissue sites of infection or inflammation, macrophages immediately encounter a decreasing oxygen gradient, which leads to stabilization of hypoxia inducible factor-1 alpha (HIF-1α) protein. This protein is then responsible for enhanced expression of innate immune response genes and molecules involved in macrophage recruitment and activation (described above) (Burke et al., 2003; Talks et al., 2000; Angele et al., 1999; Nizet and Johnson, 2009; Murdoch et al., 2005; Wang et al., 2006).

Based on the previously published literature, we decided to study the expression of caveolins in bone marrow derived macrophages (BMDMs) (isolated from mice) under different conditions. BMDMs were exposed to classical macrophage activators (LPS and IFN-γ) in combination with hypoxia (5% of O_2). Besides that, the macrophages were tested for expression of HIF-1α and iNOS, activation p44/42-extracellular signal-regulated kinases 1 and 2 (ERK1/2), as well as for production of TNFα and NO.

2. Materials and methods

2.1. Cell culture and experimental models

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). The experiments were approved by the Animal Care Committee and were in accordance with the EU and NIH Guide for Care and Use of Laboratory Animals. All experiments were conducted in C57BL/6J mice (males, 25–30 g, 12–14 weeks old) (Masaryk University, Brno, Czech Republic) housed in a temperature-controlled animal facility with 12 h light–dark cycle and free access to rodent chow. BMDMs and peritoneal macrophages (PMs) (used as a positive control) were isolated from mice according to standard protocols, described previously in more details (Pekarova et al., 2013a; Rudolph et al., 2010). BMDMs were grown in Dulbecco's Modified Eagle Media (DMEM), which were supplemented with culture medium from CCL-1 cells containing macrophage-colony stimulating factor (M-CSF) (ATCC, Manassas, VA, USA), 20% of fetal bovine serum (FBS, low endotoxin; PAA, Pasching, Austria), and 1% gentamycin. BMDMs were cultured no longer than 7 days before use.

In all experiments, BMDMs were treated with 500 ng/ml of bacterial lipopolysaccharide (LPS, *Escherichia coli* serotype 026:B6), IFN-γ (50 ng/ml, PeproTech, NJ, USA), or with their combination for time periods which ranged from 1 to 24 h. The BMDMs were treated under normoxic (comprising 0–24 h gassed with 21% of O_2, 5% of CO_2 in DMEM + 10% FBS) or hypoxic (comprising 0–24 h gassed with 5% of O_2, 90% of N_2, 5% of CO_2 in DMEM + 10% FBS) conditions (Freeman et al., 2008).

2.2. Cell viability

Cell viability was tested based on total cellular mass of adherent cells using the detergent-compatible protein assay reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard, as described previously (Krejcova et al., 2009). None of the tested compounds or experimental conditions affected the macrophage viability (data not shown).

2.3. Detection of protein expression by Western blot

Equal protein amounts (30 μg) from each cell lysate were subjected to SDS-polyacrylamide gel electrophoresis on a 15% (caveolins and HIF-1α) or 10% (iNOS and β-actin) acrylamide gel. Proteins were firstly transferred to an immobilon polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA) and then incubated with antibodies against HIF-1α (1:1000; Santa Cruz Biotechnology, Santa Cruz, California, USA), iNOS (1:1000; BD Biosciences, San Jose, California, USA), β-actin (1:1000; Santa Cruz Biotechnology), caveolin-1 (1:500; BD Biosciences), caveolin-2 (1:500; BD Biosciences), caveolin-3 (1:500; BD Biosciences) or antibodies against ERK1/2, as well as phospho-ERK1/2 (Thr202/Tyr204), (1:1000; Cell Signaling Technology, USA). Membranes were further incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies for 1 h. The blots were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and exposed to CP-B X-ray films (Agfa, Brno, Czech Republic). The relative levels of proteins were quantified by scanning densitometry using the ImageJ™ program (National Institutes of Health, Bethesda, MD, USA), with the individual band density value expressed in arbitrary units. Equal protein loading was verified by β-actin immunoblotting (Pekarova et al., 2013b).

2.4. Determination of NO production

Changes in NO production were measured indirectly as the accumulation of nitrites, the end product of NO metabolism, in a medium using Griess assay (Pekarova et al., 2013b). Samples (150 μl) were mixed with Griess reagent at the ratio of 1:1 and incubated at room temperature for 15 min. The absorbance was measured at 542 nm using a SPECTRA Sunrise microplate reader (Tecan, Mannedorf, Switzerland).

2.5. Detection of TNFα production by ELISA method

Standard ELISA kit (R&D Systems, Minneapolis, MN, USA) was applied. The plate was coated with 100 μl of capture antibody (0.8 μg/ml) and incubated overnight. Then it was washed with 400 μl of wash buffer, followed by adding of 300 μl of reagent diluent and 100 μl of sample or standard (up to 2.0 ng/ml). The plate was then incubated with 100 μl of the detection antibody (200 ng/ml) and 100 μl of streptavidin (conjugated to horseradish-peroxidase) and substrate solution. The reaction was stopped by adding 50 μl of stop solution; optical density was determined using SPECTRA Sunrise microplate reader (Tecan, Mannedorf, Switzerland) set to 450 nm with reference wavelength 570 nm.

2.6. Transfection of cells with siRNA

Cells were transiently transfected with control siRNA and siRNA against caveolin-2 (all siRNA constructs were purchased from Santa Cruz Biotechnology, USA) using an electroporation system (Gene Pulser II, Bio-Rad Laboratories) as described previously (Pekarova et al., 2013a). There was no change in cell viability when compared

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