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Resolvin D1-mediated NOX2 inactivation rescues macrophages undertaking efferocytosis from oxidative stress-induced apoptosis

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ABSTRACT

Effective clearance of apoptotic cells by macrophages, termed efferocytosis, is pre-requisite for successful resolution of inflammation, and drives macrophage emigration to the draining lymph node, thereby promoting restoration of tissue homeostasis. During efferocytosis, engulfment of apopototic cells induces generation of reactive oxygen species in abundance. Macrophage apoptosis is an important feature of chronic inflammatory diseases including atherosclerosis. In the present study, we found that resolvin D1 (RvD1), one of endogenous pro-resolving lipid mediators derived from docosahexaenoic acid, prevented apoptosis of murine macrophage-like RAW264.7 cells engulfing apoptotic T cells. The inhibitory effect of RvD1 on efferocytosis-induced oxidative burst appears to be mediated by the inactivation of NADPH oxidase (NOX), a key enzyme involved in intracellular ROS production. In RvD1-treated macrophages, efferocytosis-induced phosphorylation of $p47^{phox}$ and association between $p47^{phox}$ and $gp91^{phox}$ were downregulated, resulting in abrogation of generation of superoxide anion and hydrogen peroxide. Furthermore, RvD1-mediated suppression of NOX activation was found to be dependent on cAMP-activated protein kinase (PKA) signaling. Besides inhibiting NOX activation, RvD1 rescued macrophages from oxidative stress-induced apoptosis by upregulating the expression of Bcl-xL and Bcl-2. However, knockdown of the RvD1 receptor, lipoxin A receptor/formyl-peptide receptor (ALX/FPR2), abolished the ability of RvD1 to activate cAMP-PKA signaling, to suppress NOX activation and to increase the expression of anti-apoptotic proteins, suggesting that ALX/FPR2 mediates the protective effect of RvD1 on effeocytosis-induced oxidative stress. Taken together, these findings indicate that RvD1 rescues macrophages from oxidative stress-induced apoptosis during efferocytosis through PKA-mediated repression of NOX activation and upregulation of anti-apoptotic protein expression.

1. Introduction

The successful resolution of inflammation requires a series of processes, including inhibition of inflammatory cell infiltration, stimulation of clearance of apoptotic neutrophils by mononuclear phagocytes and promotion of macrophage egress [1]. At the initial phase of inflammation, a lot of neutrophils are recruited to the inflamed site to remove harmful invaders via phagocytosis, and they subsequently undergo apoptosis. Clearance of apoptotic neutrophils from the inflamed site is important for the resolution of inflammation as it prevents disgorgement of toxic contents from apoptotic cells. Macrophages, which are recruited to the inflamed tissue following influx of neutrophils, play a key role in engulfing apoptotic polymorphonuclear leukocytes (PMNs) [2,3]. Unlike neutrophils which have a short lifespan, macrophages do not undergo apoptosis in the inflamed site, but rather emigrate through local lymphatic vessels and accumulate in draining lymph nodes. Engulfment of apoptotic neutrophils, the process termed efferocytosis, drives macrophages to exit the inflamed site via lymphatics [4]. It has been recently reported that efferocytosis induces the oxidative burst in macrophages, thereby resulting in oxidative stress-induced apoptosis [5]. However, during and after efferocytosis, macrophages normally overcome the oxidative stress-induced apoptosis, and leave the inflamed site together with engulfed apoptotic neutrophils. In several chronic inflammatory disorders such as atherosclerosis, the accumulation of dead macrophages is frequently observed, indicating that macrophage death impairs resolution of inflammation [4]. However, the mechanism responsible for macrophage survival after efferocytosis remains largely unresolved.

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During efferocytosis, a large amount of reactive oxygen species (ROS) is generated through the activation of the phagocyte NADPH oxidase (NOX2) complex [6]. This enzyme is composed of a membrane bound flavocytochrome b558 (comprising $p22^{phox}$ and $gp91^{phox}$) and four cytosolic subunits ($p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and the small GTPase Rac1/2). The activation of NOX2 occurs through phosphorylation of $p47^{phox}$ which is considered as the organizer subunit of NOX2 and also activation of Rac1/2, followed by assembly of all membrane-bound and cytosolic components on the membrane of intracellular vesicles [7–9]. Once activated by efferocytosis, NOX2-containing granules are fused with the phagosomal membrane and NOX transfers electrons from NADPH to phagosomal oxygen to produce superoxide, which is rapidly converted to hydrogen peroxide by superoxide dismutase [10]. Overactivated NOX2 generates excessive amounts of ROS, thereby triggering oxidative stress-induced damage and cell apoptosis. It has been reported that patients with Alzheimer's disease and Parkinson's disease show NOX overactivation [11,12]. Under physiological conditions after efferocytosis, however, NOX2-mediated ROS generation is controlled in a manner that the resulting oxidative stress is not intense enough to induce apoptosis [9]. However, the mechanism involved in regulation of NOX2 activity is largely unraveled.

Resolution of inflammation is an active process tightly regulated by several endogenous lipid mediators which possess anti-inflammatory and pro-resolving effects. Resolvin D1 (RvD1), one of the endogenous lipid mediators derived from docosahexaenoic acid (DHA), has been shown to promote non-phlogistic phagocytosis of apoptotic neutrophils and exfiltration of macrophages which complete efferocytosis [13]. It is well known that RvD1 exerts its pro-resolving effects via specific G protein-coupled receptors such as lipoxin A receptor/formyl-peptide receptor (ALX/FPR2) [14]. Although the role of RvD1 in promoting resolution of inflammation has been extensively investigated, little is known about whether RvD1 could affect macrophage survival after efferocytosis. It has been reported that neutroprotectin D1 can protect human retinal pigment epithelial cells from oxidative stress-induced apoptosis by inactivating pro-apoptotic signaling while stimulating anti-apoptotic signaling [15]. This prompted us to speculate that RvD1 helps macrophages overcome oxidative stress-induced apoptosis during and after efferocytosis. In the present study, we hypothesized that RvD1 downregulates efferocytosis-induced NOX2 activation, thereby protecting macrophages against oxidative stress.

2. Materials and methods

2.1. Materials

RvD1 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). Primary antibodies against $p47^{phox}$, $gp91^{phox}$, ALX, Bcl-xL, Bcl-2 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against cleaved caspase-3 and cleaved PARP were obtained from Cell Signaling (Beverly, MA, USA). Phosphoserine antibody was the product of Abcam (Cambridge, UK). The anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Zymed Laboratories (San Francisco, CA, USA).

2.2. Cell culture

Murine macrophage-like RAW264.7 cells and Jurkat T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM (for RAW264.7 cells) and RPMI 1640 (for Jurkat T cells) with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin in humidified 5% CO_2 at 37 °C.

2.3. Efferocytosis

Apoptosis of Jurkat T cells was induced by UVB (180 mJ/cm^2) irradiation, followed by incubation for 8 h at 37 °C in 5% CO_2 atmosphere. The apoptotic Jurkat T cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V (Invitrogen, Carlsbad, CA, USA), followed by co-incubation with RAW264.7 cells. To remove the non-engulfed apoptotic Jurkat T cells, RAW264.7 cells were washed three times with PBS.

2.4. Flow cytometry analysis

Apoptotic cell death was measured by Annexin-V-FITC staining according to the manufacturer's instructions. After efferocytosis, RAW264.7 cells were treated with RvD1, N-acetyl-cysteine (NAC; Sigma–Aldrich, St Louis, MO, USA) or apocynin (Calbiochem, San Diego, CA, USA) for 8 h and collected with suspended cells. Washed cell pellets were resuspended in 100 μl of annexin V binding buffer and incubated with 5 μl of FITC-conjugated annexin V and 1 μl of propidium iodide (Invitrogen) for 15 min in the dark. Annexin V binding buffer (400 μl) was then added, and cells were analyzed by flow cytometry.

2.5. Protein extraction and Western blot analysis

Cell extracts were prepared by suspending the cells directly in the radioimmunoprecipitation assay (RIPA) buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leuptin, 1 mM phenylmethanesulphonylfluoride (PMSF)] for 1 h on ice, followed by centrifugation for 15 min at 12,000 × *g*. Protein lysates (15 μg) were electrophoresed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and the separated proteins were transferred to polyvinyl difluoride (PVDF) membrane (0.22 μm thickness; Gelman Laboratory, Ann Arbor, MI, USA). To block the non-specific binding of proteins with primary antibodies, the blots were incubated in a 5% non-fat dry milk-PBST buffer [PBS containing 0.1% Tween-20] for 1 h at room temperature. The membranes were then incubated with the primary antibody suspended in 3% non-fat milk PBST buffer overnight at 4 °C. This was followed by washing with 1X PBST and incubation using appropriate secondary antibody coupled to horseradish peroxidase. Proteins tagged with specific primary antibodies were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.6. Measurement of intracellular accumulation of reactive oxygen species (ROS)

The intracellular accumulation of hydrogen peroxide and superoxide was assessed by fluorescence microscopy using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) (Molecular Probe, Carlsbad, CA, USA) and dihydroethidium (DHE) (Santa Cruz). Cells were washed twice with Hanks balanced salt solution (HBSS; Cellgro, Herndon, VA, USA) and incubated with 10 μM of DCF-DA or DHE in humidified 5% CO_2 at 37 °C. After 30 min, cells were washed twice with HBSS solution, suspended in the complete media and they were examined under a microscope.