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Original Contributions

Advanced oxidation protein products induce cardiomyocyte death via Nox2/Rac1/superoxide-dependent TRAF3IP2/JNK signaling

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ABSTRACT

Advanced oxidation protein products (AOPPs) are formed during chronic oxidative stress as a result of reactions between plasma proteins and chlorinated oxidants. Their levels are elevated during various cardiovascular diseases. Because elevated AOPPs serve as independent risk factors for ischemic heart disease, and cardiomyocyte death is a hallmark of ischemic heart disease, we hypothesized that AOPPs will induce cardiomyocyte death. AOPP-modified mouse serum albumin (AOPP-MSA) induced significant death of neonatal mouse cardiomyocytes that was attenuated by knockdown of the receptor for advanced glycation end products, but not CD36. Notably, TRAF3-interacting protein 2 (TRAF3IP2; also known as CIKS or Act1) knockdown blunted AOPP-induced apoptosis. AOPP-MSA stimulated Nox2/Rac1-dependent superoxide generation, TRAF3IP2 expression, and TRAF3IP2-dependent JNK activation. The superoxide anion generating xanthine/xanthine oxidase system and hydrogen peroxide both induced TRAF3IP2 expression. Further, AOPP-MSA induced mitochondrial Bax translocation and release of cytochrome *c* into cytoplasm. Moreover, AOPP-MSA suppressed antiapoptotic Bcl-2 and Bcl-xL expression. These effects were reversed by TRAF3IP2 knockdown or forced expression of mutant JNK. Similar to its effects in neonatal cardiomyocytes, AOPP-MSA induced adult cardiomyocyte death in part via TRAF3IP2. These results demonstrate for the first time that AOPPs induce cardiomyocyte death via Nox2/Rac1/superoxide-dependent TRAF3IP2/JNK activation in vitro and suggest that AOPPs may contribute to myocardial injury in vivo. Thus TRAF3IP2 may represent a potential therapeutic target in ischemic heart disease.

Introduction

Reactive oxygen species (ROS) are continuously generated under normal physiological conditions and converted to nontoxic metabolites by the cellular antioxidant defense systems. Whereas low levels of ROS regulate various signal transduction pathways involved in cell survival, differentiation, and growth, chronically elevated production of ROS beyond the antioxidant capacity of the tissue results in oxidative stress; generation and accumulation of deleterious oxidatively modified lipids, proteins, carbohydrates, and nucleic acids; and tissue injury [1–4]. Several chronic inflammatory diseases, including diabetes mellitus, atherosclerosis, and chronic kidney disease, are characterized by increased oxidative stress, enhanced tissue and systemic levels of oxidized lipids, and tissue deposition of immune complexes containing oxidized lipids and antibodies [5–11]. These diseases are also characterized by the accumulation of dityrosine-containing advanced oxidation protein products (AOPPs) in plasma and tissues [5–11].

AOPPs are formed as a result of reactions between plasma proteins and chlorinated oxidants (e.g., hypochlorous acid, HOCl) [9]. AOPPs are mainly carried by albumin in the circulation. Like advanced glycation end products (AGEs), AOPPs signal via the receptor for AGEs (RAGE) in endothelial cells [10] and induce endothelial dysfunction. AOPPs can also induce podocyte death

Abbreviations: AGE, advanced glycation end product; AOPP, advanced oxidation protein product; AP-1, activator protein-1; CD36, cluster determinant 36; CRP, C-reactive protein; dn, dominant negative; CuZnSOD, copper/zinc superoxide dismutase; DPI, diphenylene iodonium; GFP, green fluorescent protein; *gp91phox*, 91-kDa glycoprotein component of the phagocyte NADPH oxidase; IκB, inhibitory κB; IKK, IκB kinase; JNK, c-Jun amino-terminal kinase; LPS, lipopolysaccharide; m.o.i., multiplicity of infection; MSA, mouse serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; NMCM, neonatal mouse cardiomyocyte; Nox, NADPH oxidase; oxLDL, oxidized low-density lipoprotein; PKCα, protein kinase Cα; RAGE, receptor for AGEs; ROS, reactive oxygen species; siRNA, small interfering RNA; shRNA, small hairpin RNA; TRAF, tumor necrosis factor receptor associated factor; TRAF3IP2, TRAF3-interacting protein 2; WT, wild type

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via RAGE [11]. In contrast, AOPPs upregulate the expression levels of almost all components of the renin–angiotensin system (RAS) and enhance angiotensin-converting enzyme (ACE) activity in cultured proximal tubular epithelial cells via the scavenger receptor CD36 [12], suggesting that AOPPs can signal via different receptors in a cell-type-specific manner. AOPPs also inhibit proliferation and differentiation of rat osteoblast-like cells in part via NF-κB activation [13]. However, whether AOPPs affect cardiomyocyte survival has not been reported.

In addition to being products of chronic oxidative stress, AOPPs can also trigger oxidative stress. In earlier reports, AOPPs have been shown to stimulate ROS generation from a variety of cells through a mechanism strongly suggesting a role for the Nox family of NADPH oxidases [11,14,15]. Cardiomyocytes express principally the Nox2 and Nox4 isoforms of NADPH oxidase, which display differential subcellular localizations [3,4,16]. Whereas Nox2 is inducible, is predominantly located in the plasma membrane, and generates mainly superoxide, Nox4 is constitutively active and is found in the nucleus, endoplasmic reticulum, and mitochondria. There is evidence that the ROS generated via NADPH oxidases can perpetuate their own production via a feed-forward mechanism and contribute to chronic oxidative stress and cell injury.

NF-κB and AP-1 are two ubiquitously expressed redox-sensitive transcription factors. In cardiomyocytes, activation of NF-κB has generally been shown to activate cell survival pathways [17], whereas activation of AP-1 is associated with cell injury or death [18]. Under basal conditions NF-κB, comprising mainly p50/p65 heterodimers, is retained in the cytoplasm by virtue of its binding to an inhibitory molecule, IκB. Oxidative stress promotes IκB phosphorylation and subsequent degradation, resulting in translocation of released NF-κB to the nucleus and induction of κB-dependent gene transcription [19]. Phosphorylation of IκB is mediated by the IKK signalosome comprised of the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ [19]. AP-1, comprised of mainly of c-Fos/c-Jun heterodimers, is activated by JNK [20]. Upon phosphorylation by JNK, c-Jun translocates to the nucleus and induces AP-1-dependent gene transcription [20].

TRAF3IP2 is a novel adapter molecule [21,22] that ubiquitinates TRAF6 at Lys63 [23] and activates IKK/NF-κB- and JNK/AP-1-dependent signaling [21–23]. Its critical role in interleukin (IL)-17-mediated autoimmune and inflammatory signaling has been described [24]. IL-17A has recently been shown to play a role in myocardial ischemic injury and induction of cardiomyocyte death [25]. Because TRAF3IP2 physically associates with IL-17RA, and as IL-17A signals mainly via IL-17RA, it is plausible that TRAF3IP2 might contribute to IL-17A-induced cardiomyocyte death. Because AOPPs suppress cell proliferation via activation of NF-κB [13] and induction of cell death [11], and as systemic AOPP levels are increased in various chronic inflammatory diseases with increased oxidative stress and contribute to cardiovascular diseases [26], we hypothesized that AOPPs induce cardiomyocyte death in a TRAF3IP2-dependent manner.

Using pathophysiological concentrations of AOPPs, here we demonstrate for the first time that AOPP-modified mouse serum albumin (AOPP-MSA) induces significant death of neonatal and adult cardiomyocytes, and this effect is reversed by TRAF3IP2 knockdown or gene deletion. Further, in neonatal cardiomyocytes, TRAF3IP2 knockdown blocks AOPP-MSA-induced JNK phosphorylation, c-Jun activation, Bax translocation to mitochondria, and caspase-3 activation and restores the antiapoptotic Bcl-2 and Bcl-xL expression. These results demonstrate that AOPPs exert potent proapoptotic effects on cardiomyocytes, and TRAF3IP2 is a critical player in this response. These results suggest that enhanced AOPPs found during conditions of chronic oxidative stress may exert pathobiological effects in the heart via TRAF3IP2, and targeting TRAF3IP2 could potentially blunt myocardial injury.

Materials and methods

Materials

LPS-EB Ultrapure (tlrl-3pelps) was purchased from InvivoGen (San Diego, CA, USA). Polymyxin B sulfate, fatty acid-free/globulin-free mouse serum albumin, xanthine, xanthine oxidase, bovine liver catalase, hydrogen peroxide, α-sarcomeric actin antibodies, α-tubulin polyclonal antibodies, and lentiviral shRNA for GFP (SHC005V), TRAF3IP2 shRNA (TRCN0000105990), RAGE (TRCN0000071743), and CD36 (TRCN0000066518) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Polyclonal anti-TRAF3IP2 antibodies were purchased from Imgenex (IMG-563; San Diego, CA, USA). Goat anti-mouse RAGE-neutralizing antibodies (AF1179; 20 μg/ml for 2 h) and normal goat IgG (AB-108-C) were purchased from R&D Systems (Minneapolis, MN, USA). CD36-blocking antibodies (ab23680; 20 μg/ml for 2 h) were from Abcam (Cambridge, MA, USA). Anti-mitochondrial protein β-subunit of complex V (V-β; A21351) antibodies and all cell culture supplies were from Invitrogen Corp. (Carlsbad, CA, USA). Affinity-purified rabbit polyclonal antibodies that detect the active (cleaved) p17 form of caspase-3, but not the p35 precursor form (AB3623), and antibodies against gp91phox (Nox2; 07-024), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; MAB374), and TRAF6 (04-451) were purchased from Millipore (Billerica, MA, USA). Antibodies to caspase-3 (9662 and 9664), c-Jun (9165), phospho-c-Jun (Ser63; 9261), JNK (9252), phospho-JNK (Thr183/Tyr185; 9251), α-tubulin (2144), and Lamin A/C (2032) were from Cell Signaling Technology (Beverly, MA, USA). The polyclonal anti-caspase-3 antibodies (9662) detect both the full-length (35 kDa) and the cleaved active forms (17/19 kDa), and the monoclonal antibodies (9664) detect only the cleaved forms. Diphenylene iodonium (DPI; 300260; 10 μM in dimethyl sulfoxide (DMSO) for 30 min), SP600125 (20 μM for 30 min), and their diluent (DMSO) were purchased from EMD4Biosciences (San Diego, CA, USA). Caspase-3 activity assay kit was obtained from EMD Chemicals (Gibbstown, NJ, USA). The caspase-3 inhibitor Z-DEVD-fmk, pancaspase inhibitor Z-VAD-fmk, and negative control Z-FA-fmk were obtained from Enzyme Systems Products (Livermore, CA, USA) and were used at 50 μM in DMSO. QCL-1000 Endpoint chromogenic *Limulus amoebocyte* lysate assay (50-647U) was purchased from Lonza. Enhanced chemiluminescence detection kit was from Amersham Pharmacia Biotech. At the indicated concentrations and for the duration of treatment, the pharmacological inhibitors did not result in detectable modulation of cardiomyocyte morphology, viability, or adherence to culture dishes (data not shown).

Preparation of AOPP-MSA

AOPP-MSA was prepared as previously described [27,28] by exposing fatty acid, globulin, and carbohydrate-free MSA (100 mg/ml) to HOCl/phosphate-buffered saline (PBS) (100 μM) for 30 min at room temperature and dialyzed for 16 h against three changes of PBS. AOPPs comprise several chromophores, including pentosidine, carbonyls, and proteins cross-linked by dityrosine, which shows absorbance at a wavelength of 340 nm [29]. In that study, Capeillere-Blandin et al. reported that the chromophore that absorbs at 340 nm matches pentosidine [29]. To quantify AOPP concentration, 200 ml of AOPP-MSA or MSA in PBS was mixed with 20 ml of glacial acetic acid and measured at 340 nm in a SPECTRAmax Plus microplate reader. A standard curve was generated using 200 ml of chloramine-T solution