Biochemical and Biophysical Research Communications 410 (2011) 643–647

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Impairment of survival signaling and efferocytosis in TRPC3-deficient macrophages

Jean-Yves Tano [a], Kathryn Smedlund [a], Robert Lee [a], Joel Abramowitz [b], Lutz Birnbaumer [b], Guillermo Vazquez [a,*]

[a] Department of Physiology and Pharmacology and the Center for Diabetes and Endocrine Research, University of Toledo College of Medicine, Health Science Campus, 3000 Arlington Av, Toledo, OH 43614, USA
[b] Laboratory of Membrane Signaling, Department of Signal Transduction, National Institute of Environmental Health Science, Research Triangle Park, NC 23709, USA

ARTICLE INFO

Article history:
Received 20 May 2011
Available online 13 June 2011

Keywords:
TRPC proteins
Calcium influx
Calcium channels
Macrophage survival
Apoptosis

ABSTRACT

We have recently shown that in macrophages proper operation of the survival pathways phosphatidylinositol-3-kinase (PI3K)/AKT and nuclear factor kappa B (NFkB) has an obligatory requirement for constitutive, non-regulated Ca^{2+} influx. In the present work we examined if Transient Receptor Potential Canonical 3 (TRPC3), a member of the TRPC family of Ca^{2+}-permeable cation channels, contributes to the constitutive Ca^{2+} influx that supports macrophage survival. We used bone marrow-derived macrophages obtained from TRPC3$^{-/-}$ mice to determine the activation status of survival signaling pathways, apoptosis and their efferocytic properties. Treatment of TRPC3$^{+/+}$ macrophages with the pro-apoptotic cytokine TNFα induced time-dependent phosphorylation of IκBα, AKT and BAD, and this was drastically reduced in TRPC3$^{-/-}$ macrophages. Compared to TRPC3$^{+/+}$ cells TRPC3$^{-/-}$ macrophages exhibited reduced constitutive cation influx, increased apoptosis and impaired efferocytosis. The present findings suggest that macrophage TRPC3, presumably through its constitutive function, contributes to survival signaling and efferocytic properties.

1. Introduction

In several chronic diseases with a clear inflammatory component the ability of macrophages to undergo apoptosis and then be cleared out from the lesion site by resident phagocytes – efferocytosis – plays a determinant role in inflammation resolution. The importance of these processes is well exemplified in atherosclerosis [1] and, more recently, in inflammatory bowel disease [2], in which the balance between generation of apoptotic macrophages and their efferocytic clearance is now recognized as a key determinant in lesion progression. In atherogenesis for example, macrophage apoptosis, but not their survival, during early stages has been shown to be beneficial in that it reduces lesion cellularity and plaque progression [3,4] while increased apoptosis in advanced plaques enlarges the necrotic core promoting plaque instability [5,6]. Therefore, altered expression and/or deregulation of signaling proteins directly or indirectly involved in macrophage survival, apoptosis and/or their efferocytic properties can exert a significant impact on inflammation resolution and lesion progression in those diseases. The phosphatidylinositol-3-kinase (PI3K)/AKT axis and the nuclear factor kappa B (NFkB) route are well recognized survival pathways in macrophages. In other cell types these survival routes are known to depend to a significant extent on Ca^{2+} influx into the cell [7,8], and recently we showed that this is also the case in macrophages. Using THP-1-derived macrophages and a pharmacological approach, we showed for the first time that constitutive, non-regulated Ca^{2+} influx has an obligatory role in compensatory survival mediated by the PI3K/AKT and NFkB pathways, through a mechanism that seems to involve the calmodulin/calmodulin kinase II (CAM/CAMKII) axis as the coupling component between constitutive Ca^{2+} influx and activation of survival events [9]. Members of the Transient Receptor Potential Canonical (TRPC) family of Ca^{2+}-permeable cation channels are expressed in macrophages from both human and rodent origin [10]. Among them, TRPC3 is endowed with significant constitutive function [11,12] and thus emerges as an attractive candidate to mediate the constitutive Ca^{2+} influx that supports macrophage survival. In the present work we directly addressed this possibility using bone marrow-derived macrophages from mice deficient in TRPC3.

2. Materials and methods

2.1. Mice and isolation of bone marrow cells

129SvTrpc3$^{lox/lox}$ (TRPC3$^{+/+}$) and 129SvTrpc3$^{-/-}$ (TRPC3$^{-/-}$) mice were obtained from Dr. Lutz Birnbaumer (NIEHS, NC). Generation and full characterization of these mice has been previously

Abbreviations: CAM, calmodulin; CAMKII, calmodulin dependent kinase II; PI3K, phosphatidylinositol-3-kinase; NFkB, nuclear factor kappa B; TNFα, tumor necrosis factor alpha; TRPC, Transient Receptor Potential Canonical.

* Corresponding author. Address: Department of Physiology and Pharmacology, 3000 Arlington Av, UTHSC Mail stop 1008, Toledo, OH 43614, USA. Fax: +1 419 383 2871.
E-mail address: Guillermo.Vazquez@utoledo.edu (G. Vazquez).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.06.045

described [13]. All animal procedures were approved by University of Toledo IACUC. Bone marrow-derived macrophages were obtained essentially as described by Wooten and colleagues [14,15]. Briefly, femurs and tibias from TRPC3$^{+/+}$ and TRPC3$^{-/-}$ mice were flushed with sterile RPMI (containing 2% FBS + 5 U/ml heparin + 1% penicillin/streptomycin) and cells were plated with L929-conditioned medium for 7 days (37 °C, 5% CO_2 atmosphere); after that, cells were recovered with ice-cold phosphate-buffered saline solution (PBS) and replated in 6-well plates for experiments. Macrophage phenotype was confirmed by cobblestone appearance, F4/80 staining and Ac-LDL uptake, as we described in [16] (not shown). Immunoblotting conditions were as we described in [17]. Briefly, following cell lysis, solubilized proteins were separated in 10% (for detection of IkBα and AKT) or 12% (for detection of BAD) acrylamide gels, electrotransferred to PVDF membranes and immunoblotted with the indicated primary antibody. After incubation with the appropriate HRP-conjugated secondary antibodies, immunoreactive bands were visualized by ECL (Amersham, PA). Because phosphorylation of IκBα is followed by its degradation, immunodetection of phospho-IκBα (Fig. 1A and B) was normalized against GAPDH (see also Section 3). Importantly, basal levels of IκBα and its degradation profile were not significantly different between TRPC3$^{-/-}$ and TRPC3$^{+/+}$ cells (not shown). Primary antibodies used were: phospho-IκBα (Ser32/36, clone 5A5), total IκBα, phospho-AKT (Ser473, clone 587F11), total AKT, phospho-BAD (Ser136, clone D25H8) and total BAD, all from Cell Signaling (MA); anti-GAPDH (clone 0411) was from Santa Cruz (CA).

2.2. Real-time evaluation of cation influx

Fura-2 based measurements of Ba^{2+} influx was performed essentially as described by us in [17,18]. We have previously shown that the use of Ba^{2+} (2 mM) as a surrogate for Ca^{2+} is an efficient and unequivocal way to monitor constitutive, non-regulated cation influx through TRPC3, independently of the cell's Ca^{2+} buffering capacity [17,18].

2.3. Annexin-V binding assay

Binding of annexin-V to apoptotic macrophages was performed using the Alexa-488-labeled Annexin-V kit (Invitrogen). This assay evaluates early/mid-stage apoptosis. Macrophages were grown in Lab-Tek chambers in complete medium (37 °C in humidified air/5% CO_2 atmosphere) and then switched to serum-free RPMI with or without TNFα (10 ng/ml) for 24 h before the assay, which was conducted essentially following manufacturer's instructions except that after incubation with Alexa-488-Annexin-V cells were washed in PBS at room temperature, kept in PBS and immediately analyzed by fluorescence microscopy. Propidium iodide (PI) costaining was used to detect death or late-stage apoptotic cells. Annexin-V-positive cells in five fields were counted (duplicate conditions) and expressed as % of total cells.

2.4. In vitro efferocytosis assay

This was performed essentially as described in [6] with modifications. Briefly, bone marrow-derived macrophages (TRPC3$^{+/+}$ or TRPC3$^{-/-}$) were labeled with 10 μM calcein green/AM ester (Molecular Probes) and made apoptotic by treatment with staurosporine (5 μg/ml, 24 h). Under these conditions staurosporine treatment resulted in ~85% apoptosis, as assayed by Annexin-V binding (not shown). No significant differences were observed in the ability of TRPC3$^{+/+}$ or TRPC3$^{-/-}$ macrophages to incorporate the fluorescent dye calcein green/AM. Apoptotic cells were incu-

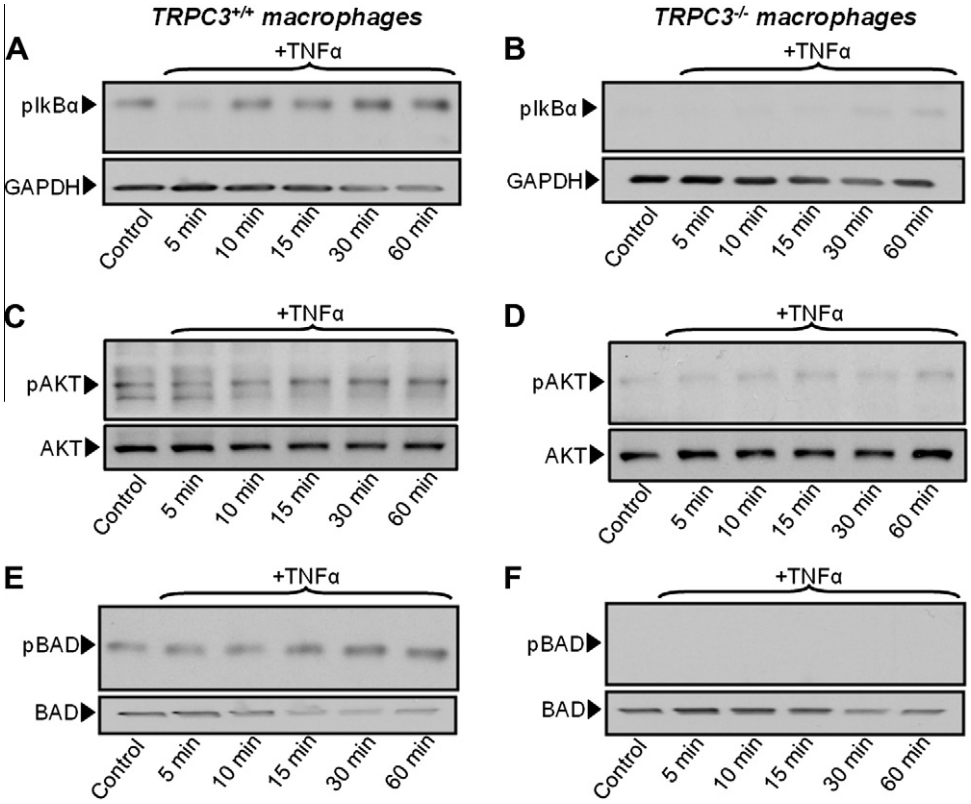

Fig. 1. Bone marrow-derived TRPC3$^{-/-}$ or TRPC3$^{+/+}$ macrophages were treated with TNFα (10 ng/ml) for the indicated times and processed for immunodetection of phospho-IκBα (Ser32/36, 40 kDa, A and B), phospho-AKT (Ser473; 60 kDa, C and D) or phospho-BAD (Ser136, 23 kDa, E and F) in whole cell lysates. Membranes were reprobed for GAPDH, total AKT or total BAD to control for protein loading. Blots are representative from three independent experiments.