Biochemical and Biophysical Research Communications 384 (2009) 221–225

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Peroxynitrite-induced p38 MAPK pro-apoptotic signaling in enterocytes

Yigit S. Guner [a,b], Christian J. Ochoa [a], Jin Wang [a], Xiaoru Zhang [a], Sarah Steinhauser [c], Lydia Stephenson [c], Anatoly Grishin [a], Jeffrey S. Upperman [a,*]

[a] Childrens Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA
[b] University of California Davis Medical Center, Sacramento, CA, USA
[c] Children's Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

ARTICLE INFO

Article history:
Received 7 April 2009
Available online 23 April 2009

Keywords:
Necrotizing enterocolitis
Nitric oxide
Peroxynitrite
Enterocytes
p38
AKT

ABSTRACT

Enterocyte apoptosis in necrotizing enterocolitis is partly due to the elaboration of toxic intermediates of nitric oxide (NO), such as peroxynitrite (PN). Because p38 mitogen-activated protein kinase (MAPK) and serine–threonine kinase (AKT) are well-characterized pro- and anti-apoptotic mediators, respectively, we hypothesized that PN could induce enterocyte apoptosis via activation of p38 and deactivation of AKT. To test this hypothesis, the rat intestinal cell line, IEC-6, was treated with PN. PN caused phosphorylation of p38, its upstream activator, MKK3/6, and downstream effector, transcription factor ATF-2. PN-induced apoptosis was inhibited by the p38 inhibitor, SB202190, and by p38 siRNA. PN decreased AKT phosphorylation; this effect was abrogated by pre-treatment with SB202190 or p38 siRNA. PN exposure also increased the activity of the protein phosphatase 2A (PP2A). These data demonstrate that PN-mediated apoptosis depends on the p38 pathway and that p38 mediates deactivation of AKT survival pathways possibly by the involvement of PP2A.

Introduction

Necrotizing enterocolitis (NEC) is a devastating inflammation of the intestine seen primarily in pre-term neonates. Epithelial injury caused by perinatal insults such as formula feeding, abnormal bacterial colonization of the gut, hypoxia, and hypothermia leads to bacterial translocation across the epithelium [1]. Bacteria then stimulate sensory cells of the innate immune system such as macrophages and monocytes, as well as enterocytes, to produce pro-inflammatory cytokines/chemokines, and nitric oxide (NO) [2]. NO and its reactive oxidation intermediate, peroxynitrite (PN), play an important role in the pathogenesis of NEC [3]. Physiological levels of NO are relatively innocuous, and at low levels NO is protective by virtue of its ability to increase blood flow [4]. However, inflammatory up-regulation of inducible nitric oxide synthase (iNOS) during NEC leads to dramatic local accumulation of NO and formation of PN, which leads to epithelial injury [4].

Mechanisms leading to cellular injury due to high levels of NO and PN are complex. NO or its derivatives may induce conformational changes in iron–sulfur linkages within the catalytic domain of enzymes involved in the mitochondrial electron transport chain such as NADH: ubiquinone oxido-reductase, NADH: succinate oxido-reductase, and aconitase of the Krebs cycle [5]. In addition, NO may cause DNA damage directly through deamination reactions, or by increasing oxidative stress via formation of PN [6]. We previously demonstrated that intestinal injury in the rat model of NEC is associated with increased immunoreactivity to 3-nitrotyrosine, a molecular footprint of PN [7]. We also showed that PN can induce enterocyte apoptosis through a caspase-mediated pathway in the rat intestinal epithelial cell line IEC-6 [8]. Furthermore, PN decreased epithelial cell proliferation by inhibiting the Src kinase pathway, thus inhibiting tissue repair mechanisms [9].

The mitogen-activated protein kinase (MAPK) pathways regulate various aspects of inflammatory stress responses [10]. p38 is a member of the MAPK family that is activated by stressors and inflammatory factors [10]. p38 activation may lead to enterocyte death by up-regulating proteins responsible for apoptosis. An example of such a protein is tensin homolog deleted on chromosome ten (PTEN), which promotes apoptosis by inhibiting phosphorylation of the serine–threonine protein kinase AKT [11]. The phosphatidylinositol 3′-kinase (PI 3-K)/AKT pathway is critical for cell survival and prevention of apoptosis [12]. We hypothesized that PN-mediated enterocyte apoptosis is governed by the p38 MAPK and AKT pathways. In this study, we show that PN induces enterocyte apoptosis by inhibiting phosphorylation of AKT in a p38-dependent manner.

Materials and methods

Materials. The reagents used were from the following suppliers: SB202190, okadaic acid (OA), 7-amino-actinomycin D (7-AAD), and

* Corresponding author. Fax: +1 323 361 3534.
E-mail address: jupperman@chla.usc.edu (J.S. Upperman).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.04.091

anti-β-actin monoclonal antibody (Sigma–Aldrich, St. Louis, MO); anti-AKT, anti-phospho-AKT, anti-MKK3, anti-phospho-MKK3/6, anti-p38, anti-phospho-p38, anti-activating transcription factor-2 (ATF-2), and anti-phospho-ATF-2 (Cell Signaling Technology, Beverly, MA); gliotoxin and protein phosphatase 2A Inhibitor I (I_1^{PP2A}) (Calbiochem, Gibbstown, NJ).

Cell culture. The rat intestinal IEC-6 cell line was purchased from American Type Culture Collection. Cells (passages 16–29) were grown at 37 °C and 5% CO_2 to 70–90% confluence in Dulbeco's modified Eagle's medium with 4.5 g/l glucose supplemented with 5% FBS, 2 mM glutamine, 0.1 U/ml penicillin, and 100 μg/ml streptomycin. Cells were treated with PN (Alexis, San Diego, CA) or DPN in PBS following two washes with PBS. DPN was prepared by diluting equivalent amount of PN in PBS and incubating the solution at room temperature overnight.

p38α MAPK siRNA. Rat p38α siRNA (sense: 5′-GGACCUC-CUUAUAGACGAAUU-3′) [anti-sense: 5′-UUCGUCUAUAAGGAG-GUCCUU-3′) duplexes or control siRNA (5′-UAGCGACUAAA-CACAUCAAA-3′) were transfected into IEC-6 cells using DharmaFect-4 solution (Dharmacon, Chicago, IL) using manufacturer's protocol. Transfected cells were allowed to recover for 48 h prior to experiments.

Western blot analyses. Cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 10% glycerol, 1% NP-40, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The supernatant was collected after centrifugation at 14000*g* for 10 min. Aliquots of protein (50 μg) were electrophoresed on 4–15% gradient gels (Bio-Rad, Hercules, CA) using a mini-gel system and transferred to nitrocellulose membranes (GE, Minnetonka, MN). Membranes were blocked with 5% milk in PBS with 0.1% Tween 20 for 1 h at room temperature and then incubated with primary and secondary horseradish peroxidase-conjugated antibodies as recommended by antibody manufacturers. Protein bands were visualized with Super Signal chemiluminescence substrate (Pierce, Rockford, IL).

Apoptosis assay. IEC-6 cells were treated with PN, DPN or gliotoxin and incubated in growth medium for 6 h after exposure. Cells were then trypsinized, stained with 4 μg of 7-AAD in PBS, washed with PBS, and analyzed by flow cytometry as previously described [13].

Phosphatase assay. Phosphatase activity was measured using Ser/Thr phosphatase assay kit optimized to detect PP2A (Upstate Signaling, Lake Placed, NY). Cells were exposed to PN or DPN, with or without 30 min pre-treatment with the two inhibitors of PP2A, OA (1 μM) or I_1^{PP2A} (100 nM), or the p38 inhibitor SB202190. Cells were lysed in phosphatase buffer (20 mM imidazole–HCl, 2 mM EDTA, 2 mM EGTA, 10 μg/ml of aprotinin, leupeptin, antipain, soybean trypsin inhibitor, 1 mM of benzamidine and PMSF). Protein concentration was determined (BioRad, Hercules, CA). After clearing by centrifugation at 2000*g* for 5 min, supernatants were equilibrated to 50 mM Tris–HCl, pH 7.0, 0.1 mM $CaCl_2$ on Sephadex G-25 columns (Roche, Indianapolis, IN). PP2A activity was determined using the kit (Upstate, Lake Placid, NY) as recommended by the manufacturer.

Statistical analysis. The data were analyzed for statistical significance by Student's *t*-tests or ANOVA using the SigmaStat software package (Systat Software, San Jose, CA).

Results

PN increases Phospho-p38 (P-p38), phospho-MKK3/6 and phospho-ATF-2 in vitro. Exposure of IEC-cells to PN, but not DPN, caused time-dependent increase in the activating phosphorylation of p38 (Fig. 1). The increase in p38 phosphorylation occurs as early as 5 min of exposure to PN, and persists up to 1 h. MAPK kinases 3 and 6 (MKK3/6) are the known upstream activators of p38 [14,15]. To determine whether MKK3/6 are activated by PN, we examined PN-induced activating phosphorylation of these kinases. As shown in Fig. 1, PN causes an increase in phospho-MKK3/6. PN exposure and persists for as long as 1 h, which is consistent with MKK3/6 mediating the PN-induced p38 phosphorylation. p38 is known to phosphorylate the transcription factor ATF-2, which, as part of the C/EBP transcription complex, activates transcriptional expression of the inflammatory stress response and pro-apoptotic genes, including *Gadd-45* [16]. To test whether PN activates ATF-2, we examined levels of phospho-ATF-2 in PN-treated cells. PN, but not DPN, increased ATF-2 phosphorylation, persisting at least 1 h (Fig. 1). Our data show that PN is likely to activate ATF-2 via a canonical p38 cascade that involves MKK3/6.

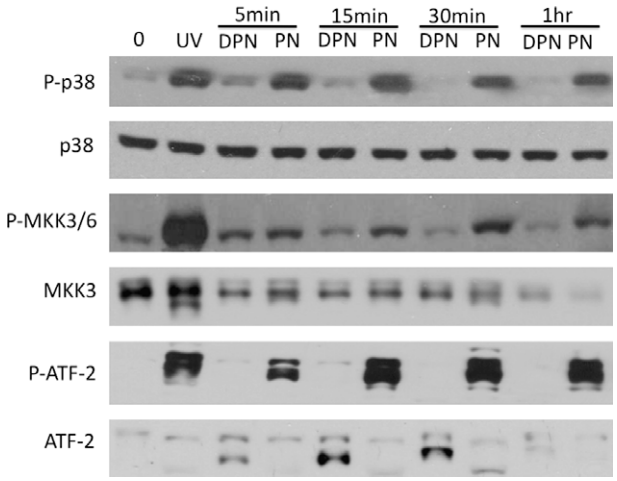

Fig. 1. PN increases phosphorylation of p38, MKK3/6 and ATF-2. IEC-6 cells were exposed to 50 μM PN and DPN for the indicated times. Naive (untreated cells) are indicated by 0 min. Levels of phospho-p38, phospho-MKK3/6, phospho-ATF-2, total p38, total MMK3 and total ATF-2 were determined by Western blotting. UV, cells exposed to 50 mJ/cm^2 UVC and allowed to recover in growth medium for 1 h (positive control for p38 activation). Data shown are representative of at least three independent experiments.

PN-induced apoptosis is prevented by p38 inhibition. We first tested whether transfection with p38 siRNA could decrease the levels of p38 phosphorylation in response to PN exposure (Fig. 2A). Cells transfected with p38 siRNA, but not with control (mock) siRNA, had decreased levels of p38 expression (Fig. 2A). Moreover, siRNA knockdown of p38 expression caused a marked decrease in PN-induced phospho-p38 signal (Fig. 2A). These results show that PN induces phosphorylation of p38, and that levels of phospho-p38 in PN-treated cells can be reduced by transfection with p38 siRNA (Fig. 2A).

To test whether p38 inhibition could prevent PN-induced enterocyte apoptosis, we determined the percentage of apoptotic cells following exposure to PN. Apoptotic cells were identified as cells with sub-G1 DNA content by flow cytometry following staining with 7-AAD (Fig. 2B). Naïve cells had a baseline mean apoptosis rate of 5.6% ± 2.9, compared to 36.8% ± 4.4 in PN-treated cells ($p = 0.001$, $n = 6$). As expected, exposure to PN, but not to DPN (5.9% ± 2.6), increased the percentage of apoptotic cells ($p < 0.001$, $n = 6$). There was no difference between DPN and naïve cells in terms of mean apoptosis rate ($p = 0.9$, $n = 6$). Gliotoxin is a potent inducer of enterocyte apoptosis (positive control) [13]. Gliotoxin caused apoptosis in 54% ± 2.3 cells. Cells pre-treated with the p38 inhibitor, SB202190, and exposed to PN (17.5% ± 0.5) had a twofold decrease in levels of apoptosis compared to cells treated with PN alone ($p < 0.001$, $n = 6$). siRNA-mediated knockdown of p38 has a similar effect: cells transfected with p38 siRNA had significantly lower levels of PN-induced apoptosis (14.1% ± 1.0) than cells transfected with PN ($p = 0.001$, $n = 6$). In addition, cells