Available online at www.sciencedirect.com
SCIENCE DIRECT
Toxicology and Applied Pharmacology 210 (2006) 86 – 93
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Activation of transcription factor AP-1 and mitogen-activated protein kinases in aniline-induced splenic toxicity

M. Firoze Khan *, Subburaj Kannan, Jianling Wang

Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555-0609, USA

Received 25 July 2005; revised 16 August 2005; accepted 19 August 2005
Available online 19 September 2005

Abstract

Signaling mechanisms in aniline-induced fibrogenic and/or tumorigenic response in the spleen are not known. Previous studies have shown that aniline exposure leads to iron accumulation and oxidative stress in the spleen, which may cause activation of redox-sensitive transcription factors and regulate the transcription of genes involved in fibrosis and/or tumorigenesis. To test this, male SD rats were treated with 0.5 mmol/kg/day aniline via drinking water for 30 days, and activation of transcription factor AP-1 was determined in the splenocyte nuclear extracts (NEs). AP-1 DNA-binding activity in the NEs of freshly isolated splenocytes from aniline-treated rats increased in comparison to the controls, as determined by electrophoretic mobility shift assay (EMSA). AP-1 binding was also determined in the NEs of cultured splenocytes (2 h and 24 h), which showed even a greater increase in binding activity at 2 h. The specificity of AP-1 binding for relevant DNA motifs was confirmed by competition EMSA and by supershift EMSA using antibodies specific to c-Jun and c-Fos. To further explore the signaling mechanisms in the AP-1 activation, phosphorylation patterns of mitogen-activated protein kinases (MAPKs) were pursued. Aniline exposure induced increases in the phosphorylation of the three classes of MAPKs: extracellular-signal-regulated kinase (ERK 1/2), c-Jun N-terminal kinase (JNK 1/2), and p38 MAPKs. Furthermore, TGF-β1 mRNA expression showed a 3-fold increase in the spleens of aniline-treated rats. These observations suggest a strong association among MAPK phosphorylation, AP-1 activation, and enhanced TGF-β1 gene expression. The observed sequence of events subsequent to aniline exposure could regulate genes that lead to fibrogenic and/or tumorigenic response in the spleen.

Keywords: Aniline; Oxidative stress; Spleen; MAPK; AP-1; TGF-β1

Introduction

Aniline, an aromatic amine that is extensively used in chemical industry, causes selective toxicity to spleen (Bus and Popp, 1987; Khan et al., 1999a, 2003a). The toxicity of aniline is evident from splenomegaly (Jenkins et al., 1972; Khan et al., 1997a, 1999a), increased erythropoietic activity (Jenkins et al., 1972; Bus and Popp, 1987), hyperpigmentation, hyperplasia, fibrosis (Bus and Popp, 1987; Khan et al., 1993, 1999a, 1999b, 2003a), and a variety of primary sarcomas of the spleen after chronic exposure in rats (Ward et al., 1980; Goodman et al., 1984; Weinberger et al., 1984). Among various pathophysiological manifestations, splenic fibrosis appears to be an important initiating preneoplastic lesion of the spleen (Khan et al., 1995, 1999a, 2003b) due to the fact that the splenic sarcomas appear in regions where fibrosis is also highly pervasive (Goodman et al., 1984; Weinberger et al., 1984), suggesting an intricate link between appearance of fibrosis and ensuing development of splenic sarcomas. However, precise molecular mechanisms leading to splenic fibrosis and/or fibrosarcomas after aniline-induced toxic injury remain enigmatic.

Aniline-induced splenic toxicity has been attributed to iron overload (increases in both total and free iron), which subsequently leads to oxidative stress in the spleen evident from increased lipid peroxidation (LPO), protein oxidation, DNA oxidation, nitrotyrosine formation, and malondialdehyde-protein adducts (Khan et al., 1997a, 1997b, 1999a, 2003c; Wu et al., 2005). Aniline-induced oxidative stress is also associated with such morphological changes in the spleen as vascular congestion, increased red pulp cellularity due to increased sinusoidal macrophages and fibroblasts, capsular thickening, and formation of fibrous tissue in the capsule and throughout the parenchyma (Khan et al., 1993, 1997a, 1999a, 2003a). LPO and

* Corresponding author. Fax: +1 409 747 1763.
E-mail address: mfkhan@utmb.edu (M.F. Khan).

0041-008X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2005.08.006

LPO-derived reactive aldehydes are known to induce overexpression of cytokines, and play a pivotal role in fibrogenesis and fibrosclerotic diseases (Parola et al., 1996; Poli and Parola, 1997). It is anticipated that increased oxidative stress could lead to fibrogenic response in spleen as a result of overexpression of fibrogenic cytokines. In fact, we have shown previously that upregulation of TGF-β1, one of the most potent profibrogenic cytokines, precedes the development of fibrosis in the spleen after aniline exposure (Khan et al., 2003b). Also, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), which are not only important proinflammatory cytokines, but also known to stimulate fibroblast proliferation and extracellular matrix production (Elias et al., 1990; Postlethwaite and Seyer, 1990; Chen et al., 1998), are upregulated in aniline-induced splenic toxicity (Wang et al., 2005). Interestingly, the increases in cytokine expressions were also associated with a concurrent increase in the redox-sensitive transcription factor NF-κB binding activity in the spleen, suggesting that upregulation of these cytokines is a redox-dependent mechanism (Wang et al., 2005). However, the precise signaling mechanisms in aniline-induced splenic toxicity, especially the status and role of redox-sensitive transcription factor activator protein-1 (AP-1) and mitogen-activated protein kinases (MAPKs) are not known.

Transcription factors are low-molecular-weight proteins that can bind with the promoter regions of genes and thus regulate gene expression (Baldwin, 1996). AP-1 is a redox-sensitive transcription factor which has been shown to play a pivotal role in the regulation of a variety of downstream target genes including inflammatory, fibrogenic, and cell proliferation genes (Pennypacker, 1998; Kapahi et al., 2000). Mitogen-activated protein kinases (MAPKs) are essential intermediates in signaling events that have been implicitly linked to the activation of the transcription factors and also the gene expression of fibrogenic cytokines (Pestka et al., 2004). We hypothesize that aniline-induced oxidative stress in the spleen leads to phosphorylation of MAPKs, which induce the activation of transcription factors and thus lead to upregulation of cytokine gene expression in the spleen. This study was intended to determine signaling mechanisms in splenic toxicity of aniline, especially the activation of AP-1 and phosphorylation (activation) of MAPKs, which may play a critical role in the activation of AP-1 leading to overexpression of fibrogenic cytokines. Specifically, we have evaluated TGF-β1 gene expression by Northern blot analysis, activation of AP-1 by electrophoretic mobility shift assay (EMSA), competition assays and supershift assays, and phosphorylation of MAPKs (ERK 1/2, JNK 1/2, and p38) by Western blot analysis in the spleens of rats subchronicaly exposed to aniline.

Materials and methods

Animals and treatment. Sprague–Dawley rats (male, ~200 g) were obtained from Harlan Sprague–Dawley (Indianapolis, IN) and housed in wire-bottom cages over absorbent paper with free access to tap water and Purina lab chow. The animals were acclimatized in a controlled-environment animal room (temperature, 22 °C; relative humidity, 50%; photoperiod, 12-h light/dark cycle) for 7 days prior to the treatments. The animals were divided into two groups of six each, and received either 0.5 mmol/kg/day of aniline hydrochloride (Sigma Chemical Co., St. Louis, MO) via drinking water (pH of the solution adjusted to ~6.8) for 30 days (Khan et al., 1993, 1999a, 1999b, 2003a; Wang et al., 2005) or drinking water only to serve as control. Aniline concentration (for treatment through drinking water) was formulated based on average daily water consumption of ~10% of the body weight for rats (Khan et al., 1993, 1999a, 1999b, 2003a; Wang et al., 2005). The drinking pattern of the experimental and control rats was similar and monitored throughout the study which showed exposure to the desired dose. Choice of dose and duration of exposure were based on earlier studies that showed oxidative stress and fibrogenic response in the spleen (Khan et al., 1993, 1999a, 1999b). The animals were euthanized 24 h following the last dose and spleens were aseptically removed immediately, weighed, and divided into several portions for use in various analyses. A portion of the spleen was snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Isolation and culture of splenocytes. A portion of the spleen was passed through a stainless steel mesh in RPMI 1640 culture medium. The cell suspension was centrifuged at 1000 × *g* for 5 min at 4 °C. The cell pellet was resuspended in 6 ml of Hank's solution and laid on to 6 ml of Histopaque-1083 (Sigma). After centrifugation at 400 × *g* for 30 min, the interface (containing splenocytes) was transferred into a fresh tube and washed twice with RPMI 1640 without serum. The cell pellet was suspended in RPMI 1640 medium supplemented with 2 mM glutamine, 50 μg/ml gentamycin, and 10% heat-inactivated FBS, and total splenocyte population was counted. The isolated splenocytes were plated in 24 well plates at a density of 5×10^6/ml/well and incubated at 37 °C with 5%CO_2 for 2 h or 24 h period. Freshly isolated splenocytes (0 h) and cultured splenocytes (2 h and 24 h) were used for the nuclear protein extraction as described below.

Nuclear protein extraction for the analysis of AP-1 activation. Nuclear proteins were extracted from freshly isolated splenocytes (0 h) and from the cultured splenocytes (2 h, and 24 h) essentially as described by Dignam et al. (1983). From the control and aniline-treated groups (6 rats each), splenocytes of two rats were pooled and nuclear extracts (NEs) were prepared. To avoid protein degradation, protease and phosphatase inhibitors (10 μl/ml, Sigma) were added during nuclear extraction. Protein concentration in the lysates was determined by using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Melville, NY).

Electrophoretic mobility shift (EMSA) and supershift assays. Briefly, NEs (8 μg) were incubated with a gel shift reaction mixture containing the binding buffer supplied by the manufacturer (Nushift AP-1 Family Kit, Active Motif, Carlsbad, CA) in the presence of a wild type double-stranded oligonucleotide with the consensus sequence for AP-1 (top strand: 5′-CGCTTGATGAGTCAGCCGGAA-3′; bottom strand 3′-GCGAACTACTCAGTCGGCCTT -5′). After 20 min of incubation at room temperature, 2 ng (20 fmol) of biotin 5′ end labeled double-stranded AP-1 binding oligonucleotide (BioSource Inc., Camarillo, CA) was added and incubation continued for another 20 min. The reaction mixture was subjected to a 6% non-denaturing polyacrylamide gel electrophoresis (PAGE) (6% gel, Novex DNA retardation gels, Invitrogen, Carlsbad CA) with 0.5× TBE (0.045 M tris–borate, 0.001 M EDTA, pH 8.0) as an electrophoresis buffer. The gel was transferred to nylon hybridization transfer membrane (Hybond-N+; Amersham Pharmacia Biotech, Buckinghamshire, England) and cross-linked at 120 mJ/cm^2 using a UV cross linker (Stratagene, La Jolla, CA), and subjected to LightShift stabilized streptavidin-horseradish peroxidase (HRP) conjugate based detection method, essentially as described by the manufacturer (LightShift Chemiluminescent EMSA kit, Pierce Biotechnology, Rockford, IL).

To determine the specificity of AP-1 binding to its consensus motif, competition assays were carried out with 25-, 50-, or 100-fold molar excess of the wild type oligonucleotide or a mutant oligonucleotide (top strand: 5′-CGCTTGATGACCCAGCCGGAA-3′; bottom strand 3′-GCGAACTACTGGGTCGGCCTT-5′). Also, supershift experiments were conducted using specific antibodies (rabbit anti-c-Jun and anti-c-Fos, Santa Cruz Biotechnology, Santa Cruz, CA). Reactions were identical to gel shift reaction conditions except 2 μg of antibody was added to the binding reaction mixtures after the addition of labeled probe and the reaction mixtures were incubated for 30 min at room