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Research paper

Age-dependent regulation of antioxidant genes by p38α MAPK in the liver

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ABSTRACT

p38α is a redox sensitive MAPK activated by pro-inflammatory cytokines and environmental, genotoxic and endoplasmic reticulum stresses. The aim of this work was to assess whether p38α controls the antioxidant defense in the liver, and if so, to elucidate the mechanism(s) involved and the age-related changes. For this purpose, we used liver-specific p38α-deficient mice at two different ages: young-mice (4 months-old) and old-mice (24 months-old). The liver of young p38α knock-out mice exhibited a decrease in GSH levels and an increase in GSSG/GSH ratio and malondialdehyde levels. However, old mice deficient in p38α had higher hepatic GSH levels and lower GSSG/GSH ratio than young p38α knock-out mice. Liver-specific p38α deficiency triggered a dramatic down-regulation of the mRNAs of the key antioxidant enzymes glutamate cysteine ligase, superoxide dismutase 1, superoxide dismutase 2, and catalase in young mice, which seems mediated by the lack of p65 recruitment to their promoters. Nrf-2 nuclear levels did not change significantly in the liver of young mice upon p38α deficiency, but nuclear levels of phospho-p65 and PGC-1α decreased in these mice. p38α-dependent activation of NF-κB seems to occur through classical IκB Kinase and via ribosomal S6 kinase1 and AKT in young mice. However, unexpectedly the long-term deficiency in p38α triggers a compensatory up-regulation of antioxidant enzymes via NF-κB activation and recruitment of p65 to their promoters. In conclusion, p38α MAPK maintains the expression of antioxidant genes in liver of young animals via NF-κB under basal conditions, whereas its long-term deficiency triggers compensatory up-regulation of antioxidant enzymes through NF-κB.

1. Introduction

p38α is a redox sensitive mitogen activated protein kinase (MAPK) activated by environmental, genotoxic and endoplasmic reticulum stresses, and by pro-inflammatory cytokines, in addition to oxidative stress [1,2]. It is one of the major MAPKs critically involved in the regulation of cell proliferation, differentiation, migration, apoptosis, and senescence as well as in inflammation [2,3]. The family of p38 MAPKs comprises four isoforms (p38α, p38β p38γ, and p38δ), and p38α is ubiquitously expressed in mammalian cells [2,4–6]. It is activated by the upstream MAPKKs MKK3 and MKK6, and under certain conditions by MKK4 [7]. It may be also activated by autophosphorylation [8] or by oxidative-induced inactivation of certain protein phosphatases [9]. Substrates of p38α comprise transcription factors, such as p53 and activating transcription factor 2 (ATF2), and protein kinases, such as MAPK-activated protein kinase 2 (MK2) [10,11]. There is evidence that p38α may activate nuclear factor kappa B (NF-κB), being involved in the up-regulation of pro-inflammatory cytokines, such as TNF-α and IL-1β [12].

Hydrogen peroxide activates p38α in endothelial cells and alveolar epithelial cells [13–15]. Under pathological conditions, an excess of reactive oxygen species (ROS) may activate p38α leading either to cell dysfunction or cell death by apoptosis [7,16–19]. In contrast oxidative stress triggers dephosphorylation of p38α in the metabolic syndrome by

Abbreviations: Akt, Protein kinase B; AP-1, Activator protein-1; ASK1, Apoptosis signal-regulating kinase 1; ATF2, activating transcription factor 2;; ChIP, Chromatin immunoprecipitation;; DEN, Diethyl nitrosamine;; EGFR, Epidermal growth factor receptor; G6PDH, Glucose-6-phosphate dehydrogenase; GCLc, Glutamate cysteine ligase catalytic subunit; GSH, Reduced glutathione;; GSSG, Oxidized glutathione;; Gstm1, Glutathione S-transferase mu 1; HPLC, High-performance liquid chromatography; Hsp, Heat shock protein; IKK, IƙB Kinase; IL, Interleukin; JNK, c-Jun N-terminal kinase; LPS, Lipopolysaccharide;; MAPK, mitogen activated protein kinase; MEF, Mouse embryonic fibroblasts; MK2, MAP-activated protein kinase 2;; MKK, MAPK kinase; MKP-1, MAPK phosphatase-1; NEM, N-ethyl maleimide;; NF-ƙB, Nuclear factor kappa B; Nrf2, Nuclear factor erythroid 2-related factor-2; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ROS, Reactive oxygen species;; RSK1, Ribosomal S6 kinase1; SOD1, Cu/Zn-superoxide dismutase; SOD2, Mn-superoxide dismutase; TBP, TATA-binding protein; TNF-α, Tumor necrosis factor-alpha; Trx, Thioredoxin

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JNK-induced upregulation of phosphatase MKP-1, which exhibits a great affinity for p38α as substrate [20].

The major mechanism responsible for the activation of p38α MAPK by oxidative stress is through the MAP3K5 apoptosis signal-regulating kinase 1 (ASK1) [21], which dissociates from its inhibitory binding protein thioredoxin (Trx) upon thiol oxidation [21,22]. Moreover, mitochondrial ROS may activate p38 MAPK by inducing dissociation of the complex Trx-ASK1 [23]. Aging is associated with chronic oxidative stress and over-activation of p38α [24,25]. Indeed, mitochondrial chronic oxidative stress is considered a hallmark of cellular aging [24,26–28]. The ASK1/Trx-ASK1 ratio increases with age in mouse liver, which would explain the enhanced age-related p38 MAPK activity in the liver [23,29]. The intracellular redox state is essential for the control of cell fate and seems to be tightly regulated during aging. It has been reported that p38α inhibition down-regulates the expression of two anti-oxidant enzymes, glutathione peroxidase and thioredoxin, in breast and colon cancer cells in *vitro* [30], but the regulation of the antioxidant defense system by p38α under physiological conditions has been scarcely studied.

p38α mediates the increase in peroxiredoxin I activity induced by lipopolysaccharide (LPS) in microglial cells [31]. On the other hand, liver-specific p38α deficiency leads to decreased phosphorylation of Hsp27 (Hsp25 in the murine model) in old mice triggering severe impairment of the actin cytoskeleton [32], and to decreased Hsp25 expression in diethyl nitrosamine (DEN)-treated mice, which was responsible for glutathione depletion and accumulation of ROS in this experimental model [33]. Although p38α lowers ROS accumulation during liver injury, the role of p38α in the regulation of the antioxidant defense in absence of liver injury has not been investigated yet. Hence, our aim was to assess whether p38α controls the antioxidant defense in the liver; and if so to elucidate the mechanism(s) involved and the age-related changes.

2. Materials and methods

2.1. Animals

Liver- specific p38α-deficient mice were generated by combining p38α floxed alleles [34] and the Afp-Cre transgene as described [35]. Animals were distributed in wild-type and knock-out mice at two different ages: young (4 months-old) and old (24 months-old). Four to six mice were used for each experimental group.

All mice received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication 86–23 revised 1985). The study was approved by the Ethics Committee of Animal Experimentation and Welfare of the University of Valencia (Valencia, Spain). Mice were euthanized under anesthesia with isoflurane 3–5% and once they were unconscious they were exsanguinated. Death was confirmed by cervical dislocation.

2.2. Determination of GSH and GSSG

Frozen liver samples were homogenized in phosphate saline buffer (PBS) with 10 mM N-ethyl maleimide (NEM). Perchloric acid (PCA) was then added to obtain a concentration of 4% and centrifuged at 15,000 g for 15 min at 4 °C. The concentrations of GSH and GSSG were determined in the supernatants by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). The chromatographic system consisted of a Micromass QuatroTM triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Zspray electrospray ionization source operating in the positive ion mode with a LC-10A Shimadzu (Shimadzu, Kyoto, Japan) coupled to the MassLynx software 4.1 for data acquisition and processing. Samples were analyzed by reverse-phase HPLC with a C18 Mediterranea SEA column (Teknokroma, Barcelona, Spain). The mobile phase consisted of the following gradient system (min/%A/%B) (A, 0.5% formic acid; B, isopropanol/acetonitrile 50/50; 0,5% formic acid): 5/100/0, 10/0/100, 15/0/100, 15.10/100/0, and 60/100/0. The flow rate was set at 0.2 ml/min. Positive ion electrospray tandem mass spectra were recorded using the following conditions: capillary voltage 3.5 kV, source temperature 120 °C, nebulization and cone gases were set at 500 and 30 L/h, respectively. Argon at 1.5610–3 mbar was used as the collision gas for collision-induced dissociation. An assay based on LC-MS/MS with multiple reaction monitoring was developed using the transitions m/z, cone energy (V), collision energy (eV) and retention time (min) for each compound that represents favorable fragmentation pathways for these protonated molecules (Table 1). Calibration curves were obtained using twelve-point (0.01–100 mmol/l) standards (purchased from Sigma-Aldrich, St Louis, USA) for each compound. The concentrations of metabolites were expressed as nmol/mg of protein.

Table 1
Transitions and retention times for analytes determined by LC–MS/MS.

Analyte	Cone (V)	Collision (eV)	Transition (*m/Z*)	Retention time (min)
GS-NEM	30	15	433 > 304	4.32
GSSG	30	25	613 > 355	1.46

2.3. Determination of malondialdehyde

Lipid peroxidation was assessed by the measurement of malondialdehyde levels in liver tissue according to Wong et al. [36] by HPLC. This method is based in the reaction of malondialdehyde with thiobarbituric acid (TBA) to yield the adduct MDA-TBA, which is determined specifically by HPLC.

Liver tissue was homogenized in phosphate buffer. Derivatization was performed mixing homogenized sample with 2 M sodium acetonitrile buffer pH 3.5 with TBA 0.2%. The mixture was heated in a boiling-water bath for 60 min at 95 ^{0}C. Then, 50 mM potassium phosphate pH 6.8 was added. The samples were centrifuged at 13000 rpm for 5 min at 4 ^{0}C and the supernatant was mixed 1:1 with 50 mM potassium phosphate pH 3.5.

Kromasil® 100–5C8, 15 × 0,46 cm column (Teknocroma, Barcelona, España) and 50 mM potassium phosphate pH 6.8 with acetonitrile (83/17) were used as stationary and mobile phase, respectively. The flow rate was 1 ml/minute and 532 nm was fixed for the peak detection for 4.5 min.

2.4. Western-blotting

Liver tissues were frozen at − 80 °C until homogenization in Hepes lysis buffer (100 mg/ml) on ice. The Hepes lysis buffer contained 75 mM NaCl, 750 µM magnesium chloride, 25 mM Hepes (pH 7.4), 500 µM EGTA, 5% glycerol, 0,5% Igepal, 1 mM dithiothreitol, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, and 1 mM sodium orthovanadate. A protease inhibitor cocktail (Sigma) containing AEBSF, aprotinin, bestatin, leupeptin, pepstatin A, and E-64 was added just before its use at a concentration of 5 µl/ml. The homogenates were sonicated with a Branson Sonicator SLPe for 30 sec (2 sec each pulse) at 30% of amplitude and centrifuged at 15,000 g for 15 min at 4 °C. In case of nuclei isolation, a slight modification of the nuclei isolation method described by [32] was used. Chemiluminescence was detected with a charge-coupled device camera (Biorad ChemiDoc XRS + Molecular Imager and LAS-3000, Fujifilm) using the Luminata Classico Western HRP Substrate (Millipore, Billerica, USA).

The following antibodies were used: antibody against Nrf2 (1/1000) (Abcam, Cambridge, UK), antibody against PGC-1α (Cell Signaling Technology, Danvers, USA), antibody against AP-1 (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against p-p65 Ser536 (1/1000) (Cell Signaling Technology, Danvers, USA), antibody against