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Activated AMPK boosts the Nrf2/HO-1 signaling axis—A role for the unfolded protein response

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ABSTRACT

In light of the emerging interplay between redox and metabolic signaling pathways we investigated the potential cross talk between nuclear factor E2-related factor 2 (Nrf2) and AMP-activated kinase (AMPK), central regulators of the cellular redox and energy balance, respectively. Making use of xanthohumol (XN) as an activator of both the AMPK and the Nrf2 signaling pathway we show that AMPK exerts a positive influence on Nrf2/heme oxygenase (HO)-1 signaling in mouse embryonic fibroblasts. Genetic ablation and pharmacological inhibition of AMPK blunts Nrf2-dependent HO-1 expression by XN already at the mRNA level. XN leads to AMPK activation via interference with mitochondrial function and activation of liver kinase B1 as upstream AMPK kinase. The subsequent AMPK-mediated enhancement of the Nrf2/HO-1 response does not depend on inhibition of the mammalian target of rapamycin, inhibition of glycogen synthase kinase 3β, or altered abundance of Nrf2 (total and nuclear). However, reduced endoplasmic reticulum stress was identified and elaborated as a step in the AMPK-augmented Nrf2/HO-1 response. Overall, we shed more light on the hitherto incompletely understood cross talk between the LKB1/AMPK and the Nrf2/HO-1 axis revealing for the first time involvement of the unfolded protein response as an additional player and suggesting tight cooperation between signaling pathways controlling cellular redox, energy, or protein homeostasis.

Introduction

Generation and detoxification of reactive oxygen species (ROS) are tightly linked with the nutrient and metabolic status of a cell. Likewise, oxidation of nutrients is linked with superoxide formation. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), mainly derived from the pentose phosphate pathway, is a crucial cosubstrate for enzymes involved in detoxification (e.g., glutathione reductase, thioredoxin reductase) and production (NADPH oxidases, NO synthases) of reactive (oxygen) species or oxidation products. ROS-forming enzymes alter their activity on phosphorylation, glycosylation, or acetylation, all posttranslational modifications lastly dependent on the cellular nutrient status (e.g., [1–3]).

On the level of individual players within the cellular redox balance, nuclear factor E2-related factor 2 (Nrf2) is an accepted master regulator. Nrf2 is a ubiquitously expressed transcription factor of the cap´n ´collar basic leucine zipper family, and constitutes a major part of the cellular defense against oxidative but also other harmful insults. In unstressed cells the level of Nrf2 protein is low which is usually brought about by binding of Nrf2 to its most prominent inhibitor Kelch-like ECH-associated protein (KEAP) 1 in a 1:2 molar ratio. KEAP1, being an adapter for Cul3/Rbx1 E3 ubiquitin ligases, facilitates constant proteasome-dependent degradation of Nrf2. On exposure to oxidative or electrophilic agents cysteine residues of KEAP1 are oxidized or covalently modified. This alters the conformation of the Nrf2/KEAP1 complex, stops ubiquitination and degradation of Nrf2, and favors its accumulation and subsequent nuclear translocation. In the nucleus, Nrf2 heterodimerizes with small musculoaponeurotic fibrosarcoma (Maf) proteins and binds to antioxidant response element

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated kinase; ARE, antioxidant response element; βTrcP1, β-transducin-repeat containing protein 1; CaMKK, calcium calmodulin-dependent kinase kinase; CHO, Chinese hamster ovary; DCF, dichlorofluorescein; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; FCS, fetal calf serum; GSH, glutathione (reduced); GSK3β, glycogen synthase kinase 3β; GSSG, oxidized glutathione; HO-1, heme oxygenase 1; Hrd1, synoviolin/Hrd1 (HMG-CoA reductase degradation)-ubiquitin ligase; KEAP1, Kelch-like ECH-associated protein; LKB1, liver kinase B1; Maf, small musculoaponeurotic fibrosarcoma; MEF, mouse embryonic fibroblasts; mTOR, mammalian target of rapamycin; NADPH, nicotinamide adenine dinucleotide phosphate; Nrf2, nuclear factor E2-related factor 2; OCR, oxygen consumption rate; PERK, protein kinase RNA-like endoplasmic reticulum kinase; ROS, reactive oxygen species; TAK, transforming growth factor β-activated kinase; UPR, unfolded protein response; WT, wild type; XN/Xn, xanthohumol

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(ARE) consensus sequences (TCAG/CXXXGC) in promoters of Nrf2-regulated genes. Those comprise approximately 200 genes including heme oxygenase 1 (HO-1) and promote detoxification of the initial insult. Besides the canonical KEAP1-dependent regulation Nrf2 abundance is further controlled by degradation via the glycogen synthase kinase 3β (GSK3 β)/β-transducin-repeat containing protein (βTrcP) 1 pathway or synoviolin/Hrd1-ubiquitin ligase (Hrd1), by altered transcription of the Nrf2 gene or microRNAs targeting Nrf2. Posttranslational modifications at the Nrf2 protein fine-tune nuclear residence and activity of Nrf2 and include phosphorylation or acetylation of Nrf2 or interaction of the transcription factor with distinct binding partners [4–7]. Thus, the Nrf2-dependent cytoprotection pathway is able to receive and integrate cues from multiple different signal transduction pathways.

AMP-activated kinase (AMPK) is a central hub for cellular energy homeostasis. The heterotrimeric serine/threonine kinase is formed by the catalytic alpha and the regulatory beta and gamma subunits. Its enzymatic activity is highly responsive to the cellular energy load. Binding of AMP to the gamma subunit in cases of low energy alters conformation and favors phosphorylation of Thr172 of the alpha subunit mainly by liver kinase B1 (LKB1), resulting in a massive increase of enzymatic activity of AMPK. Largely AMP-independent phosphorylation and activation of AMPK can be mediated by calcium calmodulin-dependent kinase kinase (CaMKK)β and transforming growth factor β-activated kinase (TAK)1, rendering AMPK also susceptible to stimuli beyond energy stress, such as increased intracellular calcium levels [8–11]. Accordingly, activated AMPK elicits pleiotropic effects including generally increased catabolism and decreased anabolism, improved endothelial function, reduced inflammation, or improved redox balance [12–14]. Notably, those phenotypic readouts were also observed in numerous studies on activation of Nrf2 [15–18], suggesting that Nrf2 and AMPK may cooperate within their signaling networks.

Given the link between the cellular metabolic and redox state [4] and the widely overlapping cellular responses on Nrf2 or AMPK activation we set out to investigate a potential AMPK/Nrf2 cross talk in molecular detail. We used wild-type (WT) and isogenic Nrf2 or AMPK knockout mouse embryonic fibroblasts (MEF) as well as xanthohumol (XN), a prenylated chalcone from hops, as small molecular probe mediating activation of both AMPK and Nrf2 [19–21]. Induction of HO-1, an enzyme decomposing heme to biliverdin, iron, and CO_2 and accounting for many beneficial outcomes on Nrf2 activation (e.g., [22–24]), served as readout for Nrf2 activity.

Materials and methods

Cells, chemicals, and antibodies

Isogenic Nrf2-/- and WT mouse embryonic fibroblasts (MEF) were kindly provided from Thomas Kensler (University of Pittsburgh, PA, USA), isogenic AMPKα-/- and WT MEF from Benoit Viollet (Institute Cochin INSERM, Paris, France), and the LKB1-/- and WT MEF from Reuben J. Shaw (Salk Institute, La Jolla, CA, USA) [25–27]. The clone of Chinese hamster ovary (CHO) cells stably expressing the ARE-dependent luciferase reporter (CHO-ARE-Luc) has been previously described [28]. The primary antibodies directed against AMPKα (No. 2532), phospho-AMPKα (Thr172) (No. 2535), ACC (No. 3662), phospho-ACC (Ser79) (No. 3661), LKB1 (No. 3047), lamin B1 (No. 13435), BiP (No. 3183), and α,β-tubulin (No. 2148) were from Cell Signaling Technology (Danvers, MA, USA). The antibody against Nrf2 was from Santa Cruz (C-20; No. sc-722) (Dallas, TX, USA), the antibody against HO-1 (No. ADI-SPA-896) from Enzo Life Sciences (Farmingdale, NY, USA), and the antibody against actin (No. 69100) from MP Biomedicals (Santa Ana, CA, USA). The secondary HRP-linked antibody against rabbit was from Cell Signaling, the antibody against mouse from MP Biomedicals. Xanthohumol, compound C, chloroquine, phenylbutyrate, tunicamycin, and thapsigargin were purchased from Sigma-Aldrich (St. Louis, MO, USA), rapamycin from Cell Signaling, and CHIR99021 from Santa Cruz. The PERK inhibitor GSK2606414 came from EMD Millipore (Darmstadt, Germany).

Cell cultivation and treatment

MEF and CHO-ARE-Luc were cultured in Dulbecco's modified essential medium (DMEM; Lonza, Braine-L'Alleud, Belgium) supplemented with 10% heat inactivated fetal calf serum (FCS, Lonza) and 2 mM glutamine (Invitrogen, Carlsbad, CA, USA) under a humidified atmosphere of 5% CO_2 at 37 °C. For all experiments cells were reseeded in appropriate plates and grown to approximately 80–90% confluence. During treatment with XN the serum concentration was reduced to 2% in all wells (including control wells) in order to avoid major adsorption of XN to serum proteins [29]. Unless otherwise stated, all compounds were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.2%.

ARE-luciferase reporter gene assay

CHO-ARE-LUC cells were treated as indicated for 8 h before their lysates were subjected to assessment of luciferase activity as previously described [28].

Preparation of whole cell lysates, SDS-PAGE, and Western blot

Cells were seeded in 6-well culture plates. After being treated as indicated cells were lysed, and total cell lysates were subjected to SDS polyacrylamide electrophoresis and immunoblot as described elsewhere [30]. Detailed protocols can be found in the supplemental information.

Fractionated extraction of nuclear and cytosolic proteins

Cells were grown in 6- or 10-cm dishes. Treated as indicated cells were subjected to extraction of cytosolic and nuclear proteins as described previously [16]. Detailed information can be found in the supplemental material. Successful fractionation was confirmed by a lacking signal for lamin B1 in the cytosolic fraction and a missing signal for tubulin in the nuclear fraction in the respective immunoblot.

Real-time quantitative polymerase chain reaction (RTqPCR)

Extraction of total RNA, reverse transcription, and quantitative PCR and evaluation were performed as previously described [30]. Detailed information can be found in the supplemental material. Primers for murine HO-1 and the reference gene, murine hypoxanthine-phosphoribosyl-transferase (HPRT), came as QuantiTect Primer Assays from Qiagen (Venlo, Netherlands).

Determination of the total intracellular ROS load

Levels of intracellular ROS were determined by flow cytometric assessment of dichlorofluorescein (DCF) fluorescence as described previously [30].

Determination of the reduced (GSH) and oxidized glutathione (GSSG)

The cellular ratio of GSSG/GSH was determined with the luminescent GSSG-GSH Glo Assay from Promega (Madison, WI, USA) according to the manufacturers' instructions.

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