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Research Paper

Redox regulation of metabolic and signaling pathways by thioredoxin and glutaredoxin in NOS-3 overexpressing hepatoblastoma cells

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ABSTRACT

Nitric oxide (NO) plays relevant roles in signal transduction in physiopathology and its effects are dependent on several environmental factors. NO has both pro-apoptotic and anti-apoptotic functions but the molecular mechanisms responsible for these opposite effects are not fully understood. The action of NO occurs mainly through redox changes in target proteins, particularly by S-nitrosylation of reactive cysteine residues. Thioredoxin (Trx) and glutaredoxin (Grx) systems are the main cellular controllers of the thiolic redox state of proteins exerting controversial effects on apoptosis with consequences for the resistance to or the development of cancer.

The aim of this study was to ascertain whether Trx and/or Grx systems mediate the antiproliferative effect of NO on hepatoblastoma cells by modulating the redox-state of key proteins.

Proliferation decreased and apoptosis increased in HepG2 cells overexpressing Nitric Oxide Synthase-3 (NOS-3) as a result of multilevel cellular responses to the oxidative environment generated by NO. Enzyme levels and cysteine redox state at several metabolic checkpoints were consistent with prominence of the pentose phosphate pathway to direct the metabolic flux toward NADPH for antioxidant defense and lowering of nucleotide biosynthesis and hence proliferation. Proteins involved in cell survival pathways, proteins of the redoxin systems and phosphorylation of MAPK were all significantly increased accompanied by a shift of the thiolic redox state of Akt1, Trx1 and Grx1 to more oxidized.

Silencing of Trx1 and Grx1 neutralized the increases in CD95, Akt1 and pAkt levels induced by NO and produced a marked increase in caspase-3 and -8 activities in both control and NOS-3 overexpressing cells concomitant with a decrease in the number of cells.

These results demonstrate that the antiproliferative effect of NO is actually hampered by Trx1 and Grx1 and support the strategy of weakening the thiolic antioxidant defenses when designing new antitumoral therapies.

Abbreviations: ACO, aconitase; ASK1, apoptosis signal-regulating kinase 1; Bcl-2, B-cell lymphoma 2; CaM, calmodulin; CD95, cluster of differentiation 95; DTNB, 5,5-dithio-bis-2-nitrobenzoic acid; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Grx, glutaredoxin; HED, 2-hydroxyethyl disulfide; JAKs, Janus protein tyrosine kinases; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MATII, methionine adenosyltransferase II; MM(PEG) 24, Methyl-PEG_{24}-Maleimide; mTOR, mammalian target of rapamycin; NEM, *N*-ethylmaleimide; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate buffered saline; PDK, phosphoinositide-dependent kinase; PKB, protein kinase B; PKM2, pyruvate kinase isozyme M2; PMSF, phenylmethylsulfonyl fluoride; PP2A, protein phosphatase 2A; PrSSG, mixed disulfide between protein and glutathione or glutathionylated protein; PrSSPr, inter- or intra-molecular protein disulfide; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STAT3, signal transducer and activator of transcription 3; TCEP, tris(2-carboxyethyl)phosphine; TKT, transketolase; Trx, thioredoxin; TrxR, thioredoxin reductase; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling; TXNIP, thioredoxin-interacting protein; ROD, uroporphyrin decarboxylase

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1. Introduction

Nitric oxide (NO) is a very small, lipophilic, readily diffusible, chemically unstable molecule with a very short half-life (seconds) that plays a relevant role in signal transduction in physiopathology such as vasodilation, respiration, cell migration, immune response and apoptosis [1]. NO is known to be synthesized in a large number of different tissues by the NO synthases (NOS) using L-arginine as substrate. Three different isoforms of NOS have been identified, products of different genes, with different localization, regulation, catalytic properties and inhibitor sensitivity [2]. Their expression and activity are cellular and tissue specific with differential regulation at transcriptional, translational and post-translational levels [3,4]. These isoforms in mammals are: neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3) [5–8]. The NOS-1 and NOS-3 isoforms are constitutively expressed and can be activated as a result of calmodulin (CaM) binding following a rise in intracellular calcium, and also by phosphorylation/dephosphorylation modifications. The expression of NOS-2 isoform is induced by inflammatory stimuli and is maximally activated by Ca^{2+}/CaM even at basal levels of intracellular Ca^{2+} [1,2,9]. The intracellular localization is relevant for the activity of NOS and accumulating evidence indicates that NOSs are subject to specific targeting to subcellular compartments (plasma membrane, Golgi, cytosol, nucleus and mitochondria) and that this trafficking is crucial for NO production and specific posttranslational modifications of target proteins [10–12].

NO can have opposite biological effects, depending on their local concentration, the target cell type involved and the levels of reactive oxygen species (ROS). The role of NO as a bioregulator of apoptosis is well established, having both antiapoptotic and proapoptotic functions [13]. The molecular mechanisms responsible for its opposite effect are not fully understood but there is strong evidence indicating the involvement of redox changes in key proteins [14].

The cellular redox state plays a critical role in regulating many signaling pathways including activation, differentiation, proliferation, and apoptosis [15–17]. ROS and reactive nitrogen species (RNS), cause irreversible damage when their amounts exceed the cellular antioxidant defense capacity, and are harmful to biomolecules, including genomic and mitochondrial DNA, membrane lipids and proteins. But they can also lead to reversible oxidations that play regulatory roles of protein function. Within proteins, the thiol group (–SH) of cysteine (Cys) can be oxidized in several ways: two thiols can form a disulfide bond as in some proteins (PSSP), or mixed disulfide in glutathionylated proteins (PSSG). Additionally, cysteine can be reversibly oxidized by ROS or RNS to sulfenic acid (–SOH) and nitrosothiol (–SNO). The nitrosylation of reactive cysteine residues in proteins takes part in NO signaling processes and can affect a multitude of intracellular events, beneficial or harmful, depending upon biological context [18–22].

The redox states of Cys residues are controlled by two major cellular systems, the Trx/thioredoxin reductase system and the glutathione (GSH)/Grx system [15,23,24]. The Trx system consists of redox active Trx, thioredoxin reductase (TrxR) and NADPH, which are critical for maintaining DNA synthesis and the cellular redox balance. Human Trx1 and TrxR1 are located in cell cytosol/nucleus. The Grx system consists of Grx, GSH and NADPH-dependent glutathione reductase. Human Grx1 is located in the cytosol [25] and is involved in redox-regulation through the reduction of protein disulfides and mixed disulfides, *e.g.* deglutathionylation of proteins [24,26].

A relationship exists between redoxins levels and apoptosis with consequences for the resistance or the development of cancer. As an antioxidant system Trx/TrxR catalyzes the denitrosylation of SNO-caspase-3 [27] and some experimental data suggested that it also participates in the denitrosylation of SNO-caspase-9 and the reductive reactivation of caspase-8 [28]. But as a pro-oxidant Trx has been described trans-nitrosylating and inactivating caspase-3 thus showing an anti-apoptotic action [29].

It has been shown that Trx1 and TrxR1 are often overexpressed in tumor cells and that high Trx could be linked to drug resistance during cancer treatment [30]. Other studies suggest that high Trx and TrxR may induce apoptosis and reduce the mitotic index of certain tumors linked to p53 dependent cell death [31]. Reduced Trx is a negative regulator of ASK1 (apoptotic-inducing kinase), which relates the Trx system to evasion of apoptosis [32]. Another apoptosis-regulatory enzyme whose nitrosylation status is reversibly regulated by Trx1 is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [33]. Because reduced Trx1 plays a critical role in cellular proliferation and viability, excessive oxidation of Trx will lead to cell death [30,34].

On the other hand, Grx1 plays an important role in protecting cells from apoptosis by regulating the redox state of Akt1, also called protein kinase B (PKB), that has consequences for cell survival and also affect the multiple roles played by Akt1, as in the Akt-mTOR signaling cascade [35]. Mitochondrial Grx2 also exerts a protective effect on mitochondrial mediated apoptosis, preventing cardiolipin oxidation and cytochrome *c* release [36].

The intracellular mechanism regulating cell death and cell proliferation are intimately connected and different studies have shown that NO production has an important role in the regulation of the carcinogenic process. For instance, S-nitrosylation of some proteins, such as GAPDH and CD95, stimulates apoptosis whereas S-nitrosylation of other proteins, such as caspases and Bcl-2, inhibits apoptosis [33]. NO exerts an antineoplastic effect in tumoral cells by increasing cell death [37] and a specific pattern of S-nitrosylation has been observed during induction of apoptosis in hepatocytes [38].

The role of antioxidants in cancer has been controversial for decades. On one hand, ROS could mediate the activation of multiple signaling cascades that promote cell proliferation and on the other hand, the consequent increase in oxidative stress could cause senescence or apoptosis and became a tumor suppressor. Recent evidence indicates that antioxidants such as GSH and Trx can actually contribute to tumorigenesis by preventing ROS accumulation in cancer cells. The cellular response will depend on the levels of ROS and antioxidant status in the cell [31,39,40].

The main objective of this study was to ascertain whether Trx and/or Grx systems mediate the antiproliferative effect of NO on hepatoblastoma cells by modulating the redox-state of key proteins. We demonstrate that Trx1 and Grx1 behave differentially depending on the intracellular oxidative/nitrosative stress in HepG2 cells. They are required for proliferation but they also contribute to the antiproliferative effect of NO, associated with Akt1 redox changes.

2. Material and methods

2.1. Materials

All reagents were of analytical grade and were purchased from Sigma, unless otherwise specified.

HepG2 cell line used in this work was obtained from ATCC LGC Standards Company (Teddington, UK). Cell culture dish and flasks were from TPP (Switzerland). Anti-Trx1 and anti-Grx1 were obtained from rabbit in our laboratory. Antibodies against STAT3, MAPK, Thr^{202}/Tyr^{204}p-MAPK (p-MAPK) and Ser^{473}p-Akt (p-Akt) were from Cell Signaling Technology. Antibodies against ACO1 and