

Thioredoxin-Glutathione and Lipoamide Systems Act as A Backup in Supporting Ribonucleotide Reductase Catalysis in Absence of Thioredoxin Reductase

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Abstract

Ribonucleotide reductase (RNR) catalyzes the rate-limiting step in the conversion of ribonucleotides (NTPs) to deoxyribonucleotides (dNTPs) for the nucleotide biosynthesis. The thioredoxin system have been widely identified as hydrogen group donor for RNR catalysis. Herein, we report that glutathione (GSH) acts as a backup in supporting RNR catalysis *via* the Trx-GSH system in absence of TrxR. Our data also shows that the lipoamide system is capable of sustaining RNR activity in the complete absence of either Trx or glutaredoxin systems. Thus, our data corroborately supports the potential redundancy between the various antioxidant systems might seemingly emerge as a novel strategy utilized by cancer cells to sustain DNA synthesis and necessitate simultaneously targeting multiple antioxidant systems as a promising therapeutic strategy.

1. Introduction

Recent years have witnessed a widespread redundancy between the cell's most versatile antioxidant systems such as the Thioredoxin (Trx) and glutathione (GSH)/Glutaredoxin (Grx) systems. The Trx system comprising of thioredoxin (Trx), seleno-enzyme thioredoxin reductase (TrxR), and NADPH works in concerted action where Trx upon reducing the substrate, itself gets reduced by TrxR accepting electrons from NADPH, in order to maintain the catalytic cycle [1, 2]. Trx is often referred to as a lethal gene, whose genetic ablation is expensive for the cell resulting in cell death. Glutathione (GSH), being the most predominant antioxidant system in the cell with huge substrate diversity has been noted to behave as a backup within the cell in several ways, one very relevant way is by complementing the job of TrxR in its absence and preventing cellular death [3]. These widespread redundancies and overlapping substrates between Trx and GSH help in maintaining an overall reducing environment within the cell. The classical functionality of Trx underlies donating hydrogen group to ribonucleotide reductase (RNR) for catalysis and conversion of nucleotides to deoxynucleotides [4, 18, 19]. The disulfide formed at the active site (R1 subunit) of the RNR during catalysis is reduced by the C-Terminal swinging arm of the R1 subunit *via* accepting electrons from Trx [4, 5]. Thus, to support this catalytic cycle Trx needs to remain in its reduced form during each catalytic cycle with the help of TrxR. It has been found that cancer cells over-expresses Trx and TrxR, thus inhibiting TrxR activity might show a therapeutic promise in cancer [6, 7]. However, this is not the case and the classical TrxR inhibitors ATG (aurothioglucose) and auranofin (AF) that have been extensively used in numerous studies as pharmacological inhibitors of TrxR was not successful in killing cancer cells [3]. The study conducted by Du et al., 2012 [3] showed that physiological concentration of GSH, NADPH and glutathione reductase reduced Trx-1, which was further enhanced upon addition of glutaredoxin (Grx) to the system. This irrefutably supports that survival of TrxR1^{-/-} tumors were strictly dependent on a functional GSH system to reduce oxidized Trx1 and thus TrxR1^{-/-} cells were susceptible to pharmacological GSH deprivation [7-9]. However, no such study showed to date if GSH-mediated Trx1 reduction can aid in electron donation to support RNR catalysis in absence of TrxR1. Herein, we present a first cell-free study report that GSH-mediated Trx reduction can bypass the

need of TrxR and support RNR catalysis in absence of TrxR1. Likewise, the cytosol is endowed with other direct and indirect antioxidant systems such as the lipoic acid (LA) and lipoamide system (LAM) comprising of Lipoamide (LAM), lipoamide dehydrogenase (LD), and reducing equivalent NADH [10, 13, 14]. It is well noted that TrxR and NADPH can also reduce oxidized LAM independent of LD and NADH [11,12]. Here, we have found for the first time that LAM is also capable of directly reducing the disulfide formed at the catalytic subunit of RNR and support RNR activity in absence of TrxR/Trx by utilizing LD and NADH. Thus, we rationalize that these widespread redundancies between the various antioxidant systems in the cell might potentially constrain chemotherapeutic treatments and contribute to cancer resistance. Thus, our data behold on concomitantly targeting/inhibiting multiple antioxidant systems within the cell might synergize in killing tumor cells and rule out to have a promising future implication in cancer.

2. Materials And Methods:

2.1 Materials and reagents

All chemicals and reagents were purchased from Sigma-Aldrich Chemicals, unless stated otherwise. The [³H] Cytidine 5'-monophosphate was from Moravsek Biochemicals and Radiochemicals. The cation exchanger resin AG® 50W was purchased from Bio-Rad.

2.2 Expression and purification of RNR subunits

Mouse R1 and R2 subunits were cloned in *E.coli* BL21 (DE3). The plasmids were a kind gift from Dr. Lars Thelander (Umeå University, Umeå, Sweden). These plasmids containing mouse R1 and R2 were transformed in BL21 (DE3) and the protein purification was done as described previously [15, 16, 17, 21].

2.3 Expression and purification of recombinant mouse Trx

Similar to the above, expression and purification of recombinant mouse Trx1 (6x-His tagged) was performed as previously described [15, 16, 17, 21].

2.4 Conversion of [³ H] CMP to [³ H] CDP by CMP Kinase

E. coli CMP kinase plasmid was provided by Prof. AnneMarie Gilles (Laboratoire d'Enzymologie et de Biochimie Structurales, France). The plasmid was expressed, purified, and used to convert [³H] CMP to [³H] CDP as described by Bucurenci et al. [22]. The [³H] CDP was separated from the reaction mixture by a C18 HPLC column using a mobile phase comprising 20 mM ammonium acetate buffer, pH 5, and 5% acetonitrile [21]. The obtained [³H] CDP was used in RNR activity assays.

2.5 RNR activity assay

Determination of RNR activities were carried out as described previously [21]. Briefly, the RNR system was reconstituted by mixing recombinant 200 µg/ml R1 and 100 µg/ml R2 proteins. Activity was assayed following the conversion of [³H] CDP into [³H] dCDP. The amount of radioactivity derived from [³H]dCMP

was quantified by liquid phase scintillation counting after ion exchange chromatography on Dowex-50 columns [21].

3. Results

3.1 Trx-GSH system supports RNR activity-

The experiment was carried out using R1-R2 complex in presence of Mg^{2+} and ATP, followed by the addition of [3H]CDP to the reaction mixture as mentioned in detail in the materials and methods. To this mixture Trx1 was added in presence of GSH as the reducing system. It was found that physiological concentrations of GSH along with 5 or 10 μM Trx was able to support RNR activity. RNR activity was measured by the nanomoles of dCDP formed after 30 mins of incubation (Figure 1). It is introspective from the data that the physiological concentration of GSH can reduce oxidized Trx and support Trx mediated RNR catalysis without the presence of TrxR in the system. From our previously reported data Sengupta et al., 2019 [21] it is noteworthy to point out that although Trx-TrxR is much more efficient in supporting RNR catalysis, Trx-GSH astonishingly supports intermediate levels of RNR catalysis presumably at higher concentrations of GSH (7-10 mM, physiologically relevant) in absence of TrxR.

3.2 Lipoamide system directly supports RNR catalysis-

To the RNR assay system, neither Trx nor Grx was added as electron donor which was substituted with the lipoamide system comprising of 1 unit/ml LD, 1 mM NADH, and the indicated concentration of LAM. Our data showed us for the first time that LAM is also capable of reducing RNR disulfide along with LD and NADH in the system and support RNR activity without the need for Trx/Grx systems. It was observed that in presence of the LAM system, RNR activity increased in a concentration-dependent manner. Impact of different concentrations of LAM on the RNR activity was monitored as the nanomole of dCDP produced after 30 min of reaction (Figure 2). Our data congruently provides a first report that the LAM system supports RNR activity in absence of the Trx/Grx system to sustain nucleotide synthesis.

Discussion

We wanted to investigate if RNR activity was sustained in absence of TrxR and have performed the RNR assay with the Trx-GSH system. According to our findings, GSH can compensate for TrxR and reduce Trx1 which in turn donates electrons to support RNR activity, thus GSH is acting as a backup in supporting RNR catalysis (Figure 3). However, as compared to the traditional Trx-TrxR system (figure 3A of ref [21]), Trx-GSH system is relatively less efficient in supporting RNR activity and is strictly GSH concentration-dependent. Nonetheless, it was seen that both Trx and GSH at physiologically relevant concentrations in the assay system supported RNR activity. This profoundly brings into notice the possible mechanism that might take place, where GSH act as a backup that keeps the RNR activity uninterrupted following TrxR inhibition or ablation and facilitate cell survival. Moreover, as GSH concentration gets enhanced in cancer cells which can also put together amenable evidence that elevated GSH plays a vital role in tumorigenesis

[9, 20]. We have also found for the first time that the LAM system is capable of directly reducing RNR that supported intermediate levels of RNR activity without the involvement of either Trx/Grx systems. Moderate levels of RNR activity were detected in presence of LAM which directly reduced the disulfide formed at the catalytic subunit of RNR. We rationally anticipate that cells treated with TrxR inhibitors/ TrxR-knockout utilize these backup antioxidant systems to cumulatively sustain RNR activity and cell survival that by-passes the effect of these chemo/cytotoxic treatments. Figure 3 represents a schematic model based on our study. This also reasonably supports the notion why nature has kept these antioxidant systems to be simultaneously present within the cells with widespread redundancies that could be a strategy utilized by cancer cells to turn the fate towards cell survival. Our data will open new avenues for research which could be further supported by *in situ/in vivo* studies that would aid in better understanding the complexities and cross-talk between potential antioxidants, paving the route towards guiding effective treatments by concomitantly targeting multiple antioxidant systems to overcome treatment resistance.

Abbreviations

RNR, ribonucleotide reductase; GSH, glutathione; Trx, Thioredoxin; TR, Thioredoxin Reducatse; LAM, Lipoamide; LD, Lipoamide Dehydrogenase.

Declarations

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Author Contribution: AC and RS wrote the manuscript. RS provided resources and funding acquisition. RS contributed to the experimental design, performed the experiments. RS and AC performed the experiments. RS, AC and KS provided critical discussion of the manuscript. RS, AC and KS reviewed the results and approved the final version of the manuscript.

Conflict of Interest Disclosure: The authors declare no competing interest.

Compliance with Ethical Standards: Not applicable.

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Ethical approval: This article does not contain any studies with animals and human participants performed by any of the authors.

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Figures

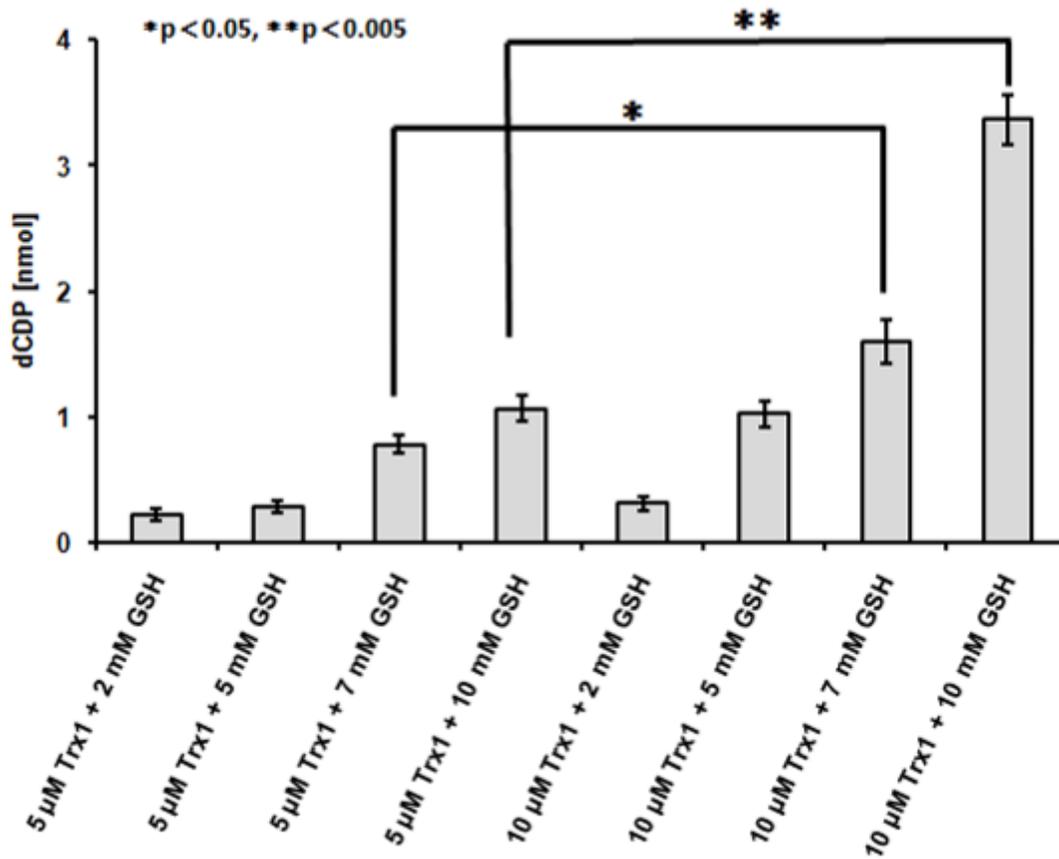


Figure 1

Trx-GSH system supports ribonucleotide reductase activity. The RNR activity was studied with reconstituted recombinant RNR system (see methods for more details) in presence of 5 or 10 μM Trx1 with the indicated concentration of GSH. RNR activity increased in a concentration dependent manner of both GSH and Trx1, where the activity was detectable at 5 μM Trx and 7-10 mM GSH; and was increased at 10 μM Trx and 7-10 mM GSH (* $P < 0.05$, ** $P < 0.005$). Thus, physiological concentration of Trx and GSH supports RNR activity in absence of TrxR.

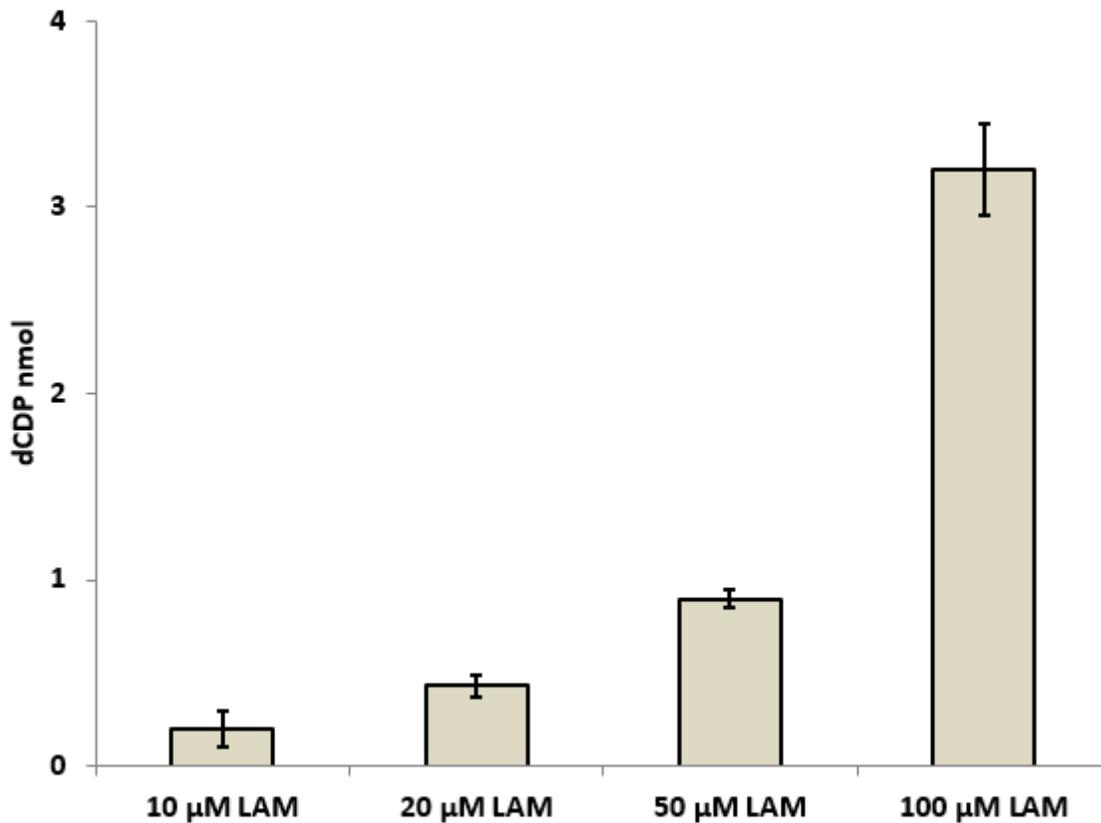


Figure 2

Lipoamide system supports ribonucleotide reductase activity. The RNR activity was studied with reconstituted recombinant RNR system (see methods for more details) in presence of 1unit/ml LD, 1mM NADH, and indicated concentration of LAM. The x-axis plotted lipoamide concentration in μM against y-axis plotted ribonucleotide reductase enzyme activity in nmol dCDP formed shows that 20-50 μM LAM supports basal level RNR activity, which drastically increases with an increase in LAM concentration to 100 μM .

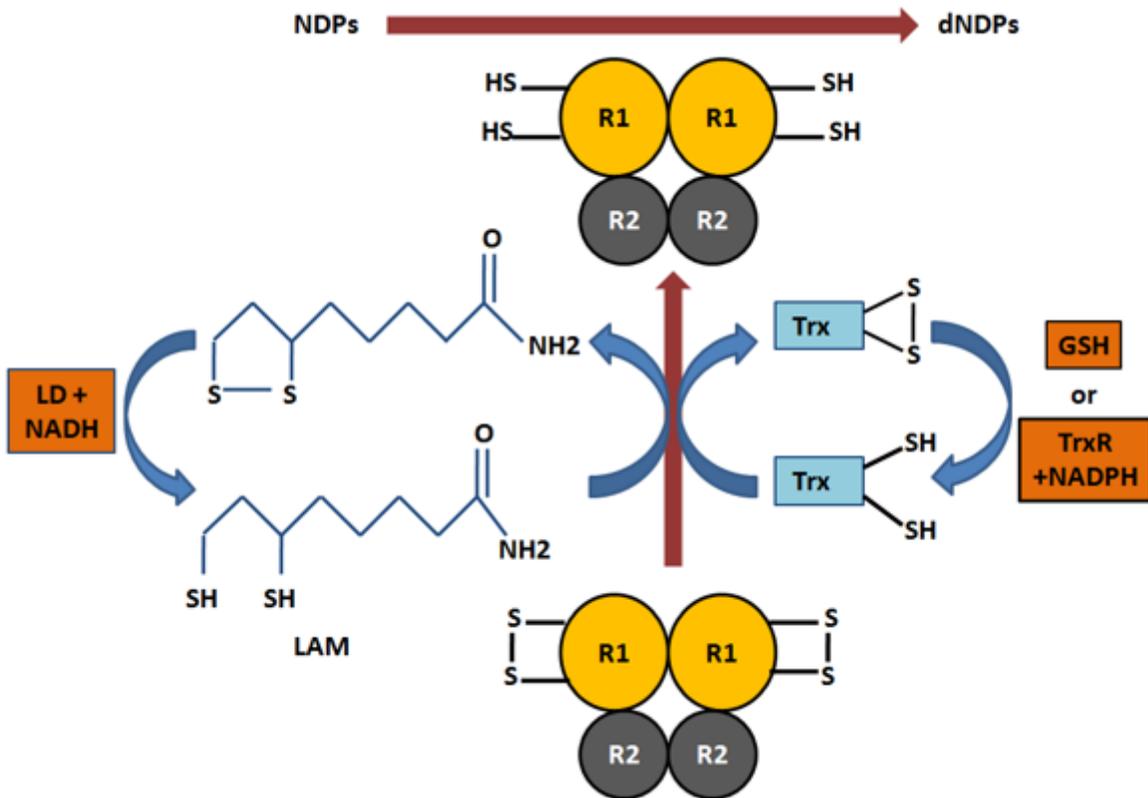


Figure 3

Model of RNR catalysis supported by Trx-GSH and Lipoamide system: We rationalize a possible mechanism that is imparted by the two systems to support RNR catalysis. There for simplicity of the figure and better understanding two disulfide bonds on R1 dimer have been shown. Left side of the figure shows Lipoamide system mediated RNR catalysis. Here, Lipoamide (LAM) directly reduces the disulfide formed at the R1 subunit of RNR resulting in formation of a disulfide in LAM. Following which it itself gets reduced by lipoamide dehydrogenase (LD) accepting electrons from NADH. This mechanism is able to support RNR activity during each catalytic cycle without the involvement of thioredoxin or glutaredoxin systems. Right side of the figure shows Thioredoxin-glutathione (Trx-GSH) mediated RNR catalysis. Here, the disulfide formed at the active site R1 subunit of RNR is reduced by C-Terminal swinging tail of R1 subunit, following accepting electrons from Trx-(SH)₂. This results in formation of oxidized Trx (Trx-(S)₂) which is further reduced by GSH. This mechanism points out a possible backup role of GSH system in reducing oxidized Trx, complementing the job of TrxR. We hypothesize that GSH reduces Trx, which helps in RNR catalysis in absence of TrxR.