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Interaction of mitochondrial thioredoxin with glucocorticoid receptor and NF- κ B modulates glucocorticoid receptor and NF- κ B signaling in HEK 293 cells

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Running Title: Trx2 interacts with GR and NF- κ B and modulates their function

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List of Abbreviations: Trx2, mitochondrial thioredoxin; Trx1, cytosolic thioredoxin; GR, glucocorticoid receptor; NF- κ B, nuclear factor-kappaB; TNF α , tumor necrosis factor; OXPHOS, oxidative phosphorylation enzymes; COX I, cytochrome oxidase subunit I; COX III, cytochrome oxidase subunit III; Cyt b, cytochrome b; MnSOD, manganese superoxide dismutase; ANT, adenine nucleotide translocase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SDH, succinate-ubiquinone oxidoreductase

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SYNOPSIS

Mitochondrial thioredoxin (Trx2) is an antioxidant, antiapoptotic factor, essential for cell viability. The cytoplasmic thioredoxin (Trx1) is a cofactor and regulator of redox sensitive transcription factors such as the glucocorticoid receptor (GR) and NF- κ B. Both transcription factors have been detected in mitochondria and a role in mitochondrial transcription regulation and apoptosis has been proposed. Here, we show using surface plasmon resonance and immunoprecipitation that GR and the p65 subunit of NF- κ B are Trx2 interacting proteins. The interaction of Trx2 with GR is independent of the presence of GR ligand and of redox conditions. The p65 subunit of NF- κ B can interact with Trx2 in the oxidized, but not the reduced form. Using HEK 293 cell lines with increased or decreased expression of Trx2, we show that Trx2 modulates transcription of GR and NF- κ B reporter genes. Moreover, Trx2 overexpression modulates the mRNA levels of the mitochondrial COX I, and Cyt b, whose expression is known to be regulated by GR and NF- κ B. Increased expression of Trx2 differentially affects the expression of Cyt b. The glucocorticoid dexamethasone potentiates the expression of Cyt b, whereas TNF α downregulates it. These results suggest a regulatory role for Trx2 in GR and NF- κ B signalling pathways.

INTRODUCTION

Thioredoxins (Trxs) are a class of ubiquitously expressed redox proteins containing a conserved consensus amino acid sequence (Cys-Gly-Pro-Cys) in their catalytic center. Trxs exist either in a reduced dithiol form or in an oxidized form. The oxidized form of thioredoxin is reduced and activated by thioredoxin reductase (TrxR) and NADPH, the so called thioredoxin system. Trxs are involved in many cellular processes, such as cell growth and proliferation, gene regulation, and apoptosis. They act as antioxidants facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Therefore, they serve as electron donors for enzymes such as ribonucleotide reductases, thioredoxin peroxidases (peroxiredoxins) and methionine sulfoxide reductase. Trxs are also regulators of redox sensitive transcription factors, such as nuclear factor κ -B (NF- κ B) and glucocorticoid receptor (GR) [1]. Regarding NF- κ B activation, Trx1 plays dual and opposing roles in its activation. In the cytoplasm, Trx1 blocks the degradation of I- κ B that could lead to nuclear NF- κ B translocation and activation. In the nucleus, however, Trx1 enhances NF- κ B transcriptional activity by reducing a disulfide bond involving Cys 62 in the DNA binding loop of the p50 subunit [2]. Restoration of the glucocorticoid receptor activation in cells under oxidizing conditions is also attributed to Trx1. Trx1 directly interacts with both the ligand and the DNA binding domains of GR, keeping the receptor in a reduced transcriptionally active form [3,4].

In mammalian cells, other, noncytosolic, members of the thioredoxin family have been described, including a mitochondrial thioredoxin system (Trx2 and TrxR2) [5].

Mitochondria are the major source of reactive oxygen species (ROS) generated during physiological respiration and under pathological conditions, such as inflammation, in response to cytokines [6,7]. Therefore, the existence of the mitochondrial thioredoxin system, together with other mitochondrial antioxidant defense systems like mitochondrial glutathione-peroxidase, manganese superoxide dismutase (MnSOD), and mitochondrial peroxiredoxins (Prx) is of utmost importance for cell viability [8]. Trx2 plays a crucial role in protection against oxidative stress and in regulating the mitochondrial apoptosis signalling pathway [8-11]. Moreover, absence of Trx2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice [12]. Trx2 is also involved in the regulation of hypoxia by attenuating hypoxia-evoked HIF-1 α accumulation and NO levels [13]. The exact mechanism by which Trx2 exerts its function in mitochondria and the identity of Trx2-interacting molecules involved in these cellular processes are not known yet.

The mitochondrial localization of orphan, thyroid, and steroid receptors, including GR and recently of other transcription factors such as NF- κ B has been demonstrated in several cell types [14-20]. Novel functions of these factors in mitochondria have been proposed, including involvement a) in mitochondrial transcriptional regulation acting as transcription factors which upon hormone stimulation and binding to mitochondrial DNA hormone response elements regulate the expression of oxidative phosphorylation (OXPHOS) genes expression and b) in mitochondria dependent apoptosis possibly through interaction with proapoptotic or antiapoptotic factors [19,21]. In the present study, we explored the role of Trx2 in the regulation of GR and NF- κ B functions. We demonstrate a direct interaction of Trx2 with GR and the p65 subunit of NF- κ B and we characterize these interactions. In addition, we provide evidence for a direct or indirect involvement of Trx2 in the modulation of GR and NF- κ B transcriptional activation.

EXPERIMENTAL

Chemicals

Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL. Molecular weight protein markers and complete protease inhibitors cocktail were purchased from Fermentas and Roche, respectively. Glutathione 4B Sepharose was from Amersham Pharmacia Biotech, 4-acetoamido-4'-maleimidylstilbene-2-2'-disulfonic acid (AMS) and Lipofectamin 2000 were from Invitrogen. Human GR and p65 or p50 subunit of NF- κ B recombinant proteins were obtained from Panvera and Active Motif, respectively. All other chemicals including protein A sepharose were purchased from Sigma-Aldrich.

Antibodies

The following affinity purified polyclonal antibodies commercially provided by Santa Cruz Biotechnology were used: GR-H300, which recognizes an epitope corresponding to amino acids 121-420 of human GR, and sc-109, an antibody raised against the p65 subunit of NF- κ B. For the Trx2 immunoprecipitation and detection a monoclonal antibody, provided by Abcam, and an affinity purified polyclonal antibody were used [9]. The anti- β -actin antibody and the anti-succinate-ubiquinone oxidoreductase (SDH) antibodies were provided by Sigma and Invitrogen, respectively.

Cell culture–Stable Trx2 underexpressing HEK 293 Cells

Human HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified atmosphere with 5% CO₂. At day 1 before transfection, HEK 293 cells were plated in 60-mm culture dishes so that they reached 50-70% confluency at the time of transfection. To obtain stable expression of the small-interfering RNA sequence and reduced expression of hTrx2, an oligonucleotide (5'CAGTGAGACACCAGTGGTT3') directed against nucleotide 228-246 of the human Trx2 cDNA (GenBank accession no. AF480262) was designed. The duplex, produced by annealing the forward and reverse oligonucleotides gateccccCAGTGAGACACCAGTGGTTtcaagagaAACCACTGGTGTCTCACTGtttttgaaa and agcttttccaaaaaCAGTGAGACACCAGTGGTTtctcttgaaAACCACTGGTGTCTCACTGggg, was inserted between BglII and HindIII sites in the pSUPER vector and transfected into the HEK 293 cells together with the pcDNA3neo plasmid vector (Clontech), carrying the Tn5 aminoglycoside phosphotransferase gene (neo) (pSuper/pcDNA3neo, 3:1) using Lipofectamine 2000, according to the manufacturer's instructions. Control cells were prepared by cotransfection of the empty pSUPER with the pcDNA3neo vectors in a 3:1 ratio. After 24 h incubation at 37°C, cells were trypsinized and passaged at a 1:5 dilution in complete growth containing 1.5 mg/ml Geneticin (G418) (Calbiochem). Next day, media in all plates were replaced with selective media for 2 weeks, and G418 resistant colonies were established in 24 well plate, expanded, cloned independently and analysed by Western blot to confirm the underexpression of Trx2.

Expression of recombinant proteins in bacteria

GST fusion proteins: For the construction of the expression plasmid for GST-Trx2, the DNA fragment encoding the human Trx2, without the mitochondrial signal (nucleotides:181-501),

was amplified by PCR and ligated in frame into the pGEX4T-3 plasmid (Amersham Pharmacia Biotech). GST or GST-Trx2 fusion proteins were expressed in *E. coli* BL21(DE3), by induction with 0.3 mM isopropyl- β -D-thiogalactopyranoside, for 3 h, at 37 °C. Cell pellets were suspended in PBS containing 1% Triton X-100 and 5 mM dithiothreitol (DTT) and subsequently sonicated. Lysates were centrifuged at 12,000 x g for 30 min, at 4 °C and supernatants were incubated with glutathione Sepharose 4B beads for 3 h, at 4 °C. Beads were washed three times with PBS, and bound proteins were eluted with 10 mM glutathione in 50 mM Tris/HCl, pH 8.0.

His Tag fusion proteins: Trx1 and Trx2 recombinant proteins were expressed in *E. coli* as the N-terminal fused form with hexahistidine (6XHis) tag, using the pET-15b expression plasmids (Clontech). Trx2 was expressed without the mitochondrial localization signal. We have previously shown that both the endogenous [5] and the overexpressed Trx2 [13] are targeted to mitochondria where they lose the targeting signal. *E. coli* strain BL21 was transformed with each pET expression vector. *E. coli* were grown at 37°C with vigorous shaking and were treated for 4 hr with 0.5 mM isopropyl β -D-thiogalactoside. Pelleted cells were lysed in phosphate buffer (25 mM sodium phosphate, 150 mM NaCl, pH: 7.0) containing 0.5 mg/ml lysozyme, sonicated and clarified by centrifugation. Supernatant containing 6XHis Tag Trx1 or 6XHis Tag Trx2 was loaded onto a Ni²⁺-NTA (nitrilo-tri-acetic acid) agarose column. The column was washed in PBS and the fusion protein was eluted by PBS containing 150 mM imidazol. The eluted protein was dialysed against PBS, over night, at 4 °C.

Surface Plasmon Resonance interaction studies

Surface Plasmon Resonance was carried out using a BIAcore2000 instrument. Using amino coupling, approximately 2500 resonance units (RU) of His-Trx2, His-Trx1 and GST purified proteins were immobilized onto a CM5 sensor chip (BIAcore). Subsequently, various molar quantities of recombinant human GR and 77 nM of p65, were injected over the surface. All proteins were diluted in 10 mM Hepes pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005 % Tween-20. The GST surface was used for background correction.

GST pull-down experiments with *in vitro* translated GR

Human GR was amplified by PCR using suitable primers carrying T7 polymerase binding sites and kozak sequences and was used as template for *in vitro* translation. Protein was synthesized *in vitro* using the T7 RNA polymerase rabbit reticulate lysate-coupled transcription/translation kit (TNT, Promega) and labeled with [³⁵S] Met (Amersham). Recombinant proteins were subjected to pull down experiments as follows: GST or GST-Trx2 protein were incubated with glutathione Sepharose 4B beads in PBS at 4°C, for 3 h. After being washed in PBS three times, the GST or GST-Trx2 conjugated beads were incubated with rotation for 4 h, at 4°C, with *in vitro* translated GR protein in the presence or absence of ligand (10⁻⁶ M DEX), in incubation pull down buffer (50 mM potassium inorganic phosphate pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 10% glycerol and 0.1% Tween-20) containing 1.5% BSA. Subsequently, the beads were washed with pull down incubation buffer three times for 15 min, resuspended in equal volume of 2XSDS sample buffer, boiled for 5 min and pelleted in a microcentrifuge. 10 μ l of each supernatant was loaded on 10 % SDS-PAGE. The gel was dried under vacuum and subjected to autoradiography.

Immunoprecipitation

HEK 293 cells overexpressing Trx2 (HEK-Trx2) [9] grown in DMEM on 15 cm dishes, were rinsed 2X in PBS, harvested in PBS and centrifuged at 500 x g for 5 min. Pellets were lysed in RIPA buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1mM EDTA, 0.1 % NP-40) supplemented with protease inhibitors and sonicated for 3X20 sec. Insoluble material was removed by centrifugation at 13,000 x g for 15 min and the supernatants were preincubated for 1 h, with 20 µl protein A sepharose, at 4 °C. Samples were centrifuged for 2 min at 1,500 x g, and the supernatants were further incubated with the primary antibodies rabbit anti-Trx2 [9] or mouse anti-Trx2 (Abcam). 20 µl of protein A sepharose was added to each sample and incubated for 3 h, at 4°C. Protein A sepharose was pelleted by centrifugation at 1,500 x g for 2 min and washed three times in PBS. The samples were boiled in SDS-PAGE loading buffer, in the presence of 5% β-mercaptoethanol, for 4 minutes and centrifuged at 1,500 x g. The resulting supernatants were loaded on a 10 % SDS-PAGE gel, electrophoresed and Western blotted with specific antibodies against Trx2 (rabbit polyclonal) and GR. Membranes were reprobbed with antibodies against the p65 subunit of NF-κB.

Subcellular fractionation

Cells were homogenized and subjected to subfractionation as previously described [15]. Briefly, cells grown on 15 cm dishes were washed twice with ice cold PBS harvested in 5 volumes of homogenization buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), containing 250 mM sucrose) with addition of protease inhibitors (cocktail from Roche supplemented with 10 µg/ml chymostatin, 2.5 nM pepstatin), and homogenized at 4° C, with 20 strokes of a glass Potter-Elvehjem homogenizer, with a Teflon pestle. The homogenate (Total Extract, TE) was centrifuged for 5 min, at 1,000 x g, in a Sorvall centrifuge using a SS-34 rotor and the supernatant was further centrifuged at 10,000 x g, for 20 min, to give the crude mitochondrial pellet. The resulting supernatant was centrifuged at 100,000 x g, for 1hr to give the cytosolic fraction (Cytosol, Cyt). The crude mitochondrial pellet was submitted to protease treatment using 1 µg of proteinase K per 10 µg of protein (10 min, 4 °C) in buffer B (20 mM Tris pH 7.5, 0.07 M sucrose, 0.21 M mannitol, 1.5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM EGTA). The reaction was stopped with the addition of 1 mM PMSF, for 10 min. Mitochondria were diluted with 10 volumes of Buffer B and subsequently centrifuged at 12,000 x g, for 20 min. The resulting pellet was washed with buffer B twice at 12,000 x g, for 20 min. Pellets of mitochondria were suspended in RIPA buffer with addition of protease inhibitors, placed on ice for 30 min, sonicated with an ultrasonic homogenizer, 2X20 sec, at level 40. The protein concentration of the subcellular fractions (TE, Cyt and Mito) was determined according to Bradford. For Western blotting, 30 µg of protein were separated on 15% SDS-PAGE gels, transferred to nitrocellulose filters and analysed using specific antibodies against Trx2, SDH and β-actin.

NF-κB and GR activity

HEK 293 cells growing on 24 well plates were cotransfected with either a NF-κB-Luciferase (NF-κB-Luc) or a MMTV promoter driven luciferase construct (GR-luc reporter gene construct) and a β-galactosidase reporter construct. Cells were treated either with TNFα (10 ng/ml – 60 ng/ml) for various time periods (2h, 4h, 6h, 12h) or with 10⁻⁶ M DEX for 4 h, lysed in report lysis buffer (Promega) following the manufacturer's protocol, and assayed for the expression of luciferase and β-galactosidase activity. The light emission was measured in

relative light units (RLU) using a chemiluminometer (LB 9507, Berthold) and adjusted to the β -galactosidase activity of the sample. Transfection efficiency was expressed as relative units (RLU).

Trx2 redox state analysis

Redox state of Trx2 was determined by Western blot analysis of cell extracts preincubated with 4-acetoamido-4'-maleimidylstilbene-2-2'-disulfonic acid (AMS) as described previously [22]. Briefly, cells were precipitated with 10 % ice-cold trichloroacetic acid for 30 min, at 4 °C. Samples were centrifuged at 12,000 x g for 30 min, resuspended in 100 % acetone and incubated at 4°C for 30 min. Following centrifugation at 12,000 x g for 10 min, acetone was removed, and protein pellets were dissolved in 20 mM Tris/HCl pH 8.0, 15 mM AMS and incubated at room temperature, for 3 h. Trx2 redox forms were separated on a 15 % SDS-PAGE gel in the presence of non reducing loading buffer.

Real -Time PCR

Expressed levels of mRNA were quantitated using real-time RCR as previously described [20]. Briefly, total RNA was extracted using Trizol followed by DNase treatment (Promega) and reverse transcription into cDNA, using random primers and superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed after mixing the cDNA with SYBR GreenER qPCR super mix Universal (Invitrogen) and appropriate primers for cytochrome oxidase subunit I (COX I), cytochrome oxidase subunit III (COX III) cytochrome b (Cyt b), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β -actin [20]. Products were quantitated with a Chromo4 Real-Time System (Bio-Rad). Conditions for PCR were: 52 °C for 2 min, 95 °C for 2 min, 35 cycles of 95 °C for 15 sec and 60 °C for 40 sec, followed by 60 °C for 10 min.

Statistical analysis

All results are expressed as mean $\pm$ SD (n=3-5). Data were analysed both by independent t-test and Mann-Whitney test. Statistical analysis was performed with SPSS software, version 11.5 and differences were considered significant at a two tailed P value < 0.05.

RESULTS

Glucocorticoid receptor is a Trx2 interacting protein

Glucocorticoid receptor interaction with cytosolic thioredoxin (Trx1) has been previously reported and further studies have shown that both the ligand and the DNA binding domains of GR could be targets for Trx1 [3,4]. In order to examine whether the mitochondrial thioredoxin could also interact with GR, pull down experiments were performed with *in vitro* translated GR (GR 1-777), with truncated forms carrying either the ligand (GR 527-777) or the DNA binding domain (amino acids 421-526) (GR 1-527) [23] and with human GST-Trx2 or GST immobilized onto glutathione sepharose beads. The GST and GST-Trx2 proteins were expressed and purified as described in Experimental. The purity of the isolated proteins was analysed by SDS gel electrophoresis and silver staining (Fig. 1A). The binding affinity of the different forms of GR with GST and GST-Trx2 was studied in the presence or absence of 10^{-6} M dexamethasone (DEX), the GR agonist. None of the GR variants showed binding to GST (Fig. 1B lanes 4, 8, 12). However, the entire GR (lane 2) as well as its truncated forms

carrying either the DNA binding (lane 6) or the ligand binding domain (lane 10) were able to bind to GST-Trx2. The presence of DEX did not affect the interaction of either GR form with Trx2 (lanes 3, 7, 11) demonstrating that the Trx2-GR interaction is ligand independent.

Characterization of Trx2 and Trx1 interaction with the glucocorticoid receptor

The binding of GR to Trx2 in pull down experiments prompted us to further characterize the GR-Trx2 interaction and to perform comparative studies with Trx1. For that purpose, His-Trx1 and His-Trx2 recombinant proteins were immobilized on a Biacore sensor chip. Various concentrations of GR (0.7 nM – 5.6 nM) were analyzed for their binding affinity to Trx1 and Trx2. Equilibrium dissociation constants (K_D) of the GR-Trx1 or GR-Trx2 associations were calculated using a 1:1 binding kinetic model. Analysis of the BIAcore data showed that Trx2 interacts with GR in the presence of 1 mM DTT ($K_D = 0.5 \text{ nM} \pm 0.03 \text{ nM}$) (Fig. 2A). In agreement with previously reported observations, Trx1 interacts with GR [4]. However, this interaction is weaker under reducing conditions ($K_D = 26 \text{ nM} \pm 0.4 \text{ nM}$) (Fig. 2B). In order to further elucidate whether the redox conditions affect the binding affinity of GR to Trx1 and Trx2, the experiments were repeated under oxidizing conditions (1.2 mM selenite). As shown in Fig. 2C, the binding of GR to Trx2 was not affected by the redox conditions, ($K_D = 0.4 \text{ nM} \pm 0.02 \text{ nM}$), whereas for Trx1 the affinity to GR was higher under oxidizing conditions ($K_D = 11 \text{ nM} \pm 0.2 \text{ nM}$) (Fig. 2D). The interactions of GR with Trx2 were not affected by the presence of DEX or the specific GR antagonist RU 486; $K_D = 0.6 \text{ nM} \pm 0.03 \text{ nM}$ and $K_D = 0.5 \text{ nM} \pm 0.01 \text{ nM}$, respectively (data not shown).

Interaction of Trx2 with the p65 subunit of NF- κ B

The p50 subunit of NF- κ B is a known Trx1 interacting protein [24-26]. Therefore we examined whether the p50 and p65 subunits of NF- κ B bind to mitochondrial thioredoxin as well. Recombinant His-Trx2 was immobilized on a Biacore sensor chip and recombinant p50 and p65 proteins (77 nM) were subjected to reduction or oxidation with 1mM DTT or 1.2 mM selenite, respectively. The oxidized forms of p50 and p65 were dialyzed against 10 mM Hepes pH 7.4 containing 0.15 M NaCl and 3 mM EDTA and subsequently the reduced or oxidized proteins were analyzed for their ability to bind to immobilized reduced Trx2. As shown in Fig. 3, the oxidized but not the reduced form of the p65 subunit of NF- κ B interacts with Trx2. Neither the reduced nor the oxidized form of the p50 NF- κ B subunit showed any binding to Trx2 in the Biacore studies (data not shown).

Trx2 co-immunoprecipitates with GR and p65

In order to explore the possible interaction of Trx2 with GR and p65 in cells, we immunoprecipitated Trx2 from HEK-Trx2 lysates using either rabbit polyclonal or mouse monoclonal antibodies against human Trx2. The immunoprecipitated proteins were separated on a 10% SDS gel, Western blotted and analyzed for the presence of Trx2 and GR using specific polyclonal antibodies against these molecules. The membranes were subsequently reprobed with antibodies against p65. A representative experiment is shown in Fig. 4. The Trx2 immunoprecipitated by either monoclonal (Fig. 4A, B) or polyclonal anti-Trx2 antibodies (Fig. 4 C, D) interacts and coimmunoprecipitates with GR and p65 in HEK 293 cell lysates (Fig. 4, A, B, C, lane 2). Control non-immune mouse or rabbit IgG do not immunoprecipitate Trx2, GR and p65 (Fig 4, A, B, C, lane 1). Western blot analysis of equal amounts (10 ng) of Trx1 and Trx2 recombinant proteins using the rabbit anti-Trx2 antibody

proved the specificity of the antibody and the absence of any cross reactivity with Trx1 (Fig. 4E).

Generation of HEK 293 cells stably underexpressing or overexpressing Trx2 in an active reduced form

To further investigate the role of Trx2 in mitochondrial functions and especially in transcriptional regulation, we applied the siRNA method to produce HEK 293 cells stably underexpressing Trx2 (HEK-siTrx2). Colonies from HEK 293 cells that were stably transfected with the siRNA Trx2-pSuper vector, were selected, expanded, cloned independently and total extracts were analysed by Western blot to estimate the levels of Trx2. Several colonies were found to exhibit a two to four fold lower levels of Trx2 compared to control cells (Fig. 5A), among them the HEK-siTrx2 colony 14 that was selected for further experiments. Trx2 overexpressing HEK 293 cells (HEK-Trx2 cells) showing approximately twenty fold Trx2 overexpression [9] were also used in our studies (Fig. 5B). The *in vivo* redox status of Trx2 in both the HEK-siTrx2 and HEK-Trx2 cell lines was analysed using AMS, a high molecular weight compound that reacts with free SH groups, leading to a higher molecular weight of the bound protein. Total extracts from HEK-siTrx2 and HEK-Trx2 cells that were subjected to reducing (1 mM DTT) or oxidizing conditions (1 mM H₂O₂), or not treated at all, were incubated with AMS and analyzed by Western blotting. As shown in Fig 5C, under oxidative conditions AMS did not principally react with Trx2 (lanes 2, 5), leading to the detection of a lower molecular weight band, whereas in either untreated (lanes 3, 6) or DTT treated cells (lanes 1, 4) Trx2 interacted with AMS leading to a higher molecular weight product. These results indicate that Trx2 predominantly exists in a reduced, active form in both cell lines. Western blot analysis of Trx2 in subcellular fractions of HEK-Trx2 cells, obtained upon homogenization and differential centrifugation, revealed mitochondrial localization of Trx2 in HEK-Trx2 cells (Fig. 5D). The exclusive detection of β -actin (cytosolic marker) and succinate-ubiquinone oxidoreductase (mitochondrial marker) in the cytosolic and mitochondrial fraction, respectively apart from their presence in the total extract (TE), verified the purity and the enrichment of the isolated mitochondria (Fig. 5D). Moreover, the detection of Trx2 in the mitochondrial fraction and its absence in the cytosolic fraction where Trx1 is found further confirmed the absence of any crossreactivity of the Trx2 antibody with the cytosolic Trx1.

Trx2 stimulates the TNF α induced NF- κ B activation and DEX induced GR activation of reporter genes

The effect of Trx2 on mitochondrial ROS levels, known regulators of TNF α induced NF- κ B activation [8,27,28], prompted us to examine the effect of Trx2 on NF- κ B transcriptional activation. For that purpose transfection experiments, using a NF- κ B-luc reporter gene construct, were performed in HEK 293 stably overexpressing or underexpressing Trx2, in the presence or absence of TNF α , in a dose and time dependent manner. HEK-siTrx2, HEK-Trx2 and HEK-293 control cells stably transfected with the empty vectors (HEK-siC, HEK-C) were analyzed for their efficiency to regulate TNF α -induced NF- κ B activation. Treatment with TNF α (10-60 ng/ml) rendered the HEK-Trx2 cells more effective in transactivating the NF- κ B-luc reporter gene compared to control cells (HEK-C) (Fig. 6A). 20 ng/ml TNF α , was sufficient to cause approximately 80 % of the maximum transcriptional activation of NF- κ B achieved by 60 ng/ml TNF α and was further used to analyse the effect of Trx2 expression on

NF- κ B transcriptional activation. After 2 hours of TNF α induction, HEK-Trx2 cells were at least 3 fold more efficient in transactivating the NF- κ B reporter gene than control cells (Fig. 6Ba), whereas the down regulation of Trx2 in HEK-siTrx2 caused approximately 20% decrease in the TNF α induced transactivation of the NF- κ B reporter gene compared to control cells (Fig 6Bb).

The role of GR in the regulation of NF- κ B induced immune response [29] and its physical interaction with mitochondrial thioredoxin suggests a role for Trx2 in the GR induced transcriptional activation. To test this hypothesis, HEK-Trx2 and HEK-C cells were transfected with a GR-luc reporter gene construct and incubated in the presence or absence of 10^{-6} M DEX. Luciferase activity was evaluated in both cell lines (Fig. 6C). Expression of Trx2 resulted in a stimulation of the GR-luc reporter gene transactivation in the nuclear environment, which was significantly increased in the presence of DEX (Fig. 6C).

Effect of Trx2 on mitochondrial gene expression

NF- κ B and GR are known transcription regulators of some mitochondrial genes such as COX I, COX III and Cyt b [reviewed in 19,20] The effect of Trx2 on the transcriptional activation of these genes, in the presence or absence of DEX or TNF α , was examined at the mRNA level by real-time PCR, using specific primers for COX I, COX III and Cyt b mRNA. GAPDH (Fig. 7A) or β -actin (Fig. 7B) mRNA measurements were also performed and used as reference for the quantification of the results. Trx2 expression increased COX I gene expression up to two fold compared to control cells (Fig. 7A,B columns 1-3) but had only slight effects on COX III gene expression (Fig. 7A,B columns 4-6). Trx2 caused an approximately 50 % increase in Cyt b gene expression in the presence of DEX. However, TNF α treatment caused an approximately 40% decrease in the expression of this gene in the same cells (Fig. 7A,B columns 8,9).

DISCUSSION

Mitochondria are key organelles participating in many cellular processes, apart from their role in energy production [30]. They receive, integrate and transmit cellular signals such as hormones, Ca²⁺, cytochrome c, APAF1, Diablo, endonuclease G and reactive oxygen species (ROS), leading to survival or apoptosis [19,31]. Mitochondrial ROS are produced as byproducts of incomplete oxygen metabolism and may cause severe cellular damage by oxidizing lipids, proteins and mitochondrial DNA [32]. ROS have also been identified as second messengers playing a role in receptor signaling and posttranslational modification of signaling molecules [33-35]. The mitochondrion houses a variety of enzymatic antioxidant defense systems, among them the thioredoxin-peroxiredoxin system, utilized for protection from oxidative damage and for the assembly of the organelle [8,35]. Steroid and thyroid hormone receptors and other transcription factors are also localized in mitochondria and affect their functions [reviewed in 19,20,36]. In these complex mitochondrial processes, the existence of additional regulatory molecules is plausible. Mitochondrial thioredoxin is such a likely candidate and its role as a regulatory molecule in this respect is explored.

Accordingly, a direct interaction of Trx2 with GR, as well as with truncated forms of GR carrying either the ligand or the DNA binding domains of the molecule, was shown by pull down experiments. This finding indicates the existence of two binding sites for Trx2 on GR molecule. However, we cannot exclude that the conformational stage of the entire

molecule might be in favor of one binding site. The proposed interaction surface of the Trx2, predicted from its crystal structure [37], as well as the kinetic analysis of the Biacore data, showing 1:1 interaction, are in accordance with the utilization of one GR-Trx2 binding site. These results suggest that thioredoxin may keep the ligand and the DNA binding domains of the receptor in a reduced active form, thus allowing it to interact with its ligand and with DNA. Similar results have been demonstrated by Makino and coworkers [3,4] for the cytosolic thioredoxin. Interestingly, we found that the interaction of GR with Trx2 was stronger than the one observed for Trx1. This interaction was not affected by the presence of specific GR agonists or antagonists, indicating that the conformational changes that GR agonists or GR antagonists induce do not affect Trx2-GR interactions. In contrast to what is known [4] and what we also observed for Trx1, Trx2-GR interactions are not affected by the redox state of Trx2. The differential effect of Trx1 and Trx2 redox status on GR binding could be explained by important differences observed during the transition between the oxidized and the reduced states of human Trx2, compared with human Trx1 [37]. Recently, it has been reported that Trx2 could catalyse protein dinitrosylation [38], which constitutes a regulatory process to control GR ligand binding [39]. Thus, the strong interaction of GR with Trx2 may suggest a potential role of Trx2 not only in the maintenance of the mitochondrial GR in a fully reduced active form but also in its preservation in a conformational stage that enables GR to interact with other regulatory molecules in the mitochondrial environment. Moreover, the presence of other members of the thioredoxin system, such as the mitochondrial thioredoxin reductase, could ensure the prompt and effective reduction of Trx2, enabling it to reduce and activate GR being already in its proximity.

The high affinity of Trx2 with GR indicates a possible Trx2 involvement in GR mitochondrial functions, such as transcriptional regulation, in which mitochondrial GR is proposed to be involved [19,20]. We have recently provided evidence for a mitochondrial GR dependent regulation of several OXPHOS genes among them COX I, COX III and Cyt b [20]. Our data from real-time PCR experiments revealing a Trx2 dependent induction of Cyt b expression, under conditions of DEX induced GR activation further support the hypothesis of a Trx2 involvement in mitochondrial GR activation. An opposite Trx2 effect on Cyt b expression was observed after preincubation of the cells with TNF α , a well-known activator of NF- κ B [28]. GR is known to interact with and attenuate the functions of NF- κ B in the nucleus [29]. The opposing effects of TNF α and DEX on Cyt b expression in the presence of Trx2, not only support and corroborate previously reported observations, showing mitochondrial NF- κ B induced down regulation of Cyt b in HT1080 cells after TNF α treatment [17], but also reveal a crucial role of Trx2 in this process. Moreover, the possibility of opposing functions of GR and NF- κ B in mitochondrial transcription, in similarity to what is observed in the nucleus [29] is also suggested. COX I and COX III are also genes whose expression has been reported to be regulated by GR and NF- κ B [17,18,20]. In this report we showed induction of COX I mRNA synthesis in the presence of Trx2 indicating involvement of Trx2 in its expression.

A Trx2 dependent transcriptional regulation of mitochondrial NF- κ B is also supported by our observations showing direct interactions of Trx2 with the oxidized, but not the reduced form, of the p65 subunit of NF- κ B. The redox status of the protein may be used as a signal for recruitment of Trx2 and subsequent reduction and activation of NF- κ B. Trx2, as an antioxidant enzyme, could reduce and activate mitochondrial NF- κ B and other mitochondrial proteins such as the adenine nucleotide translocase (ANT), a molecule that has been identified as a Trx2 interacting protein [40] and is involved in mitochondrial NF- κ B recruitment and

translocation after apoptotic stimulus, including TNF α treatment [41]. Bottero *et al* [16] reported that induction of apoptosis led to I κ B- α -NF- κ B complex exit from mitochondria, suggesting that this could be a mechanism to increase the number of activated NF- κ B molecules susceptible for nuclear translocation. Thus, Trx2 as a putative regulator in this process could control the transcriptional activation of many antiapoptotic and antioxidant NF- κ B induced genes including Bcl-XL and manganese superoxide dismutase (MnSOD) gene [42]. The finding of the Trx2-p65 interaction is in agreement with our unpublished observations from yeast two-hybrid system analysis, revealing the p65 subunit of NF- κ B as a Trx2 interacting protein. Trx1 has been reported to interact with the p50 subunit of NF- κ B, whereas there are no reports of Trx1 interaction with the p65 subunit [24–26]. The differential preference of Trx1 and Trx2 for the p50 and p65 subunits of NF- κ B may reflect differences in the 3D structure of the two proteins and may have important physiological implications. The redox status of Trx2 does not seem to alter the Trx2-GR interaction. However, we can not exclude that significant local differences between the structures of the reduced and the oxidized Trx2 [37] could affect the Trx2-p65 interaction as is the case for the Trx1-p50 interaction [26]. In addition, the possibility of a direct redox interaction of the active thiols of Trx2 with p65 exists. The coimmunoprecipitation of GR and the p65 subunit of NF- κ B proteins with Trx2 from HEK-Trx2 cell extracts further supports the concept of a direct involvement of Trx2 in the modulation of GR and NF- κ B functions.

A very interesting observation is the ability of Trx2 to control transcription of reporter genes driven by GR or NF- κ B promoter in HEK 293 cells, indicating a Trx2 dependent regulation of the GR- and NF- κ B- induced transcriptional activation in the nucleus. It has been previously reported that mitochondrial reactive oxygen species could regulate the antiapoptotic activation of NF- κ B and evidence has been provided for the involvement of mitochondrial defence systems and mitochondrial targeted antioxidants in this process [27, 43]. It has been observed that increased levels of Trx2 leads to reduced mitochondrial ROS levels even under conditions of oxidative stress [44], whereas decreased levels of Trx2 causes mitochondrial dysfunction, accumulation of intracellular ROS, increased oxidative damage, and apoptosis [10,45,46]. Thus Trx2 could also act as a regulator molecule in GR and NF- κ B signaling pathways through the regulation of mitochondrial ROS levels and control of cellular redox status. Reducing conditions either in the cytosol or in the nucleus facilitates the ligand binding as well as the DNA binding of GR in the respective cellular compartment. Moreover, reducing conditions in the nucleus facilitates the NF- κ B DNA binding and thus transcriptional activation. The cellular ROS levels, the activity of the antioxidant defence systems, as well as the cell type, seem to be critical for the final signalling effect of ROS on NF- κ B activation [34]. This delicate balance and cell type specificity may be the reason for the contradictory results that arise in the literature regarding the effect of ROS on NF- κ B activation [34,47,48]. Such an example is the observed NF- κ B transcriptional inactivation in HeLa cells transiently overexpressing Trx2 [44].

Fluctuations of the Trx2 levels, observed in many physiological and pathological conditions, could also attribute to the fine regulation of GR and NF- κ B signaling pathways. We have previously observed elevated levels of Trx2 mRNA after DEX treatment in specific areas of rat brain [49]. Increased levels of Trx2 have also been observed in many human cancers. Furthermore, alterations in Trx2 levels have been implicated in many diseases such as ischemic diseases, hypertension, cardiovascular disease, diseases of the respiratory system, neurodegenerative diseases, immune response and apoptotic or antiapoptotic functions in

which GR and NF- κ B have a key role [19,29,50].

A putative mechanism by which mitochondrial thioredoxin exerts its antiapoptotic and antioxidant activity is summarized in Fig. 8. According to our hypothesis, mitochondrial thioredoxin could affect mitochondrial and cellular functions not only by protecting against reactive oxygen species generation but also by interacting with steroid receptors and other transcription factors, such as GR and NF- κ B, present in mitochondria and possibly involved in differential regulation of mitochondrial gene expression. Thus, Trx2 could directly or indirectly affect the respiratory chain action and the generation of mitochondrial signals, such as ROS, that subsequently act as second messengers and control nuclear transcription activation by GR and NF- κ B. In the mitochondrial context, Trx2 probably through direct interaction with GR and NF- κ B, could control respiratory chain action by promoting glucocorticoid- or TNF α -regulated mitochondrial genes expression. Moreover, regarding NF- κ B, whose mitochondrial translocation is increased after apoptotic stimulus, Trx2 may promote an increase in activated NF- κ B available for mitochondrial exit and subsequent nuclear translocation to induce antioxidant or antiapoptotic genes expression. Therefore, mitochondrial thioredoxin could act as a prosurvival or proapoptotic factor affecting mitochondria enzyme synthesis and function not only by reducing reactive oxygen species but also by acting as a regulator of transcription factors involved in NF- κ B and GR signalling.

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FIGURE LEGENDS

Figure 1. Binding of GR to Trx2. A. Expression and purification of GST and GST-Trx2 proteins. The Trx2 gene was inserted into the pGEX vector. The GST and GST-Trx2 proteins were expressed into BL21 cells. The cells were lysed and the purified proteins were visualized by electrophoresis and silver staining. B. Pull down experiments with GST and GST-Trx2 recombinant proteins, *in vitro* translated entire molecule of GR (Lanes 1-4) as well as GR truncated forms carrying either the GR DNA- (Lanes: 5-8) or ligand- (Lanes 9-12) binding domain, revealed interaction of Trx2 with all the above mentioned GR forms, in the presence or absence of 10^{-6} M DEX. Lanes: 1, 5, 9, Input (30%); Lanes: 4, 8, 12, Control GST interacting proteins; Lanes: 2, 6, 10, GST-Trx2 interacting proteins in the absence of DEX; Lanes: 3, 7, 11, GST-Trx2 interacting proteins in the presence of DEX.

Figure 2. Characterization of Trx and glucocorticoid receptor interactions by Surface Plasmon Resonance analysis. 0.7 nM to 5.6 nM human recombinant GR were analyzed for their interaction with approximately 2,500 arbitrary units of immobilized His-Trx2 (Panels A, C) and His-Trx1 (Panels B, D) recombinant proteins in the presence of either 1 mM DTT (Panels A, B) or 1.2 mM selenite (Panels C, D). GR-Trx2 interaction is stronger than GR-Trx1 interaction and independent of redox conditions.

Figure 3. Interaction of Trx2 with the p65 subunit of NF- κ B. Equal amounts of recombinant p65 were reduced by 1 mM DTT or oxidized by 1.2 mM selenite, for 20 min, at room temperature. After dialysis, oxidized and reduced forms of p65 were analyzed for their binding affinity with Trx2 by using Surface Plasma Resonance analysis. The oxidized but not the reduced form of p65 showed binding affinity for Trx2.

Figure 4. Trx2 interacts with GR and the p65 subunit of NF- κ B in HEK 293 cell lysates. Trx2 was immunoprecipitated from HEK-Trx2 cell extracts using either monoclonal (A, B) or polyclonal (C, D) antibodies against Trx2. The immunoprecipitated Trx2 was detected using rabbit polyclonal anti-Trx2 antibodies (D). Membranes were probed with anti-GR antibodies (A) and subsequently, were reprobed with anti-p65 antibodies (B, C) for the detection of GR and the p65 subunit of NF- κ B, respectively. Lane 1: Immunoprecipitation with normal IgG; Lane 2: Immunoprecipitation with a-Trx2 antibodies. E) Proof of the specificity of the anti-Trx2 antibody. Equal amounts (10 ng) of His-Trx1 and His-Trx2 recombinant proteins were electrophorised and Western blotted using the anti-Trx2 antibodies.

Figure 5. Generation and characterization of HEK Trx2 under- or over- expressing stable cell lines. A. Applying the siRNA method we generated HEK 293 cells (colonies: 14, 16, 11) stably underexpressing Trx2 (HEK-siTrx2) two to four fold compared to control cells (HEK-siC, colonies 1, 2). Under or over (B) expression of Trx2 in the HEK-Trx2 cells [9] was evaluated by Western blot analysis in comparison to actin expression. C. Redox status of Trx2 in Trx2 overexpressing (Ca) or underexpressing (Cb) HEK 293 cells. HEK 293 cells were subjected to oxidation or reduction after treatment with 1mM H₂O₂ or 1mM DTT, respectively for 30 min at room temperature. AMS treatment was applied for the identification of the redox status of Trx2. Reduced Trx2, binds to AMS and thus migrates more slowly in 15% SDS gel as compared to oxidized Trx2. D) Localization of Trx2 in HEK-Trx2 cells. Western blot

analysis of Trx2, SDH (mitochondrial marker) and β -actin (cytosolic marker) in total extract (TE), cytosolic (Cyt) and mitochondrial fraction (Mito) of HEK-Trx2 cells.

Figure 6. Effect of Trx2 on NF- κ B and GR transcriptional activation. A. Dose dependent (0-60 ng/ml) TNF α induced NF- κ B transcriptional activation in HEK-Trx2 cells compared to control HEK-C cells, treated with the indicated amounts of TNF α for 6 h, at 37°C. B. Time course of TNF α (20 ng/ml) effect on NF- κ B transcriptional activation in Trx2- or siTrx2-HEK 293 cells (Ba, Bb, respectively). Cells expressing vectors without the Trx2 gene (HEK-C) or the siTrx2 (HEK-siC) were used as controls. A NF- κ B luciferase reporter gene construct was used in transfection experiments for the quantification of the NF- κ B transcriptional activation. C. Effect of stably overexpression of Trx2 on the transcriptional activation of a GR-luc reporter gene in the presence or absence of 10^{-6} M DEX. The transcriptional activation of a β -gal reporter gene was used for the normalization of the results. The average of three independent experiments ($\pm$ SD) is presented (* P <0.05 compared to similarly treated control cells).

Figure 7. Effect of Trx2 on mitochondrial encoded genes transcriptional activation. Real-time PCR was applied for the determination of the mRNA synthesis of COX I, COX III and Cyt b in cells overexpressing Trx2 compared to control cells in the presence or absence of TNF α or DEX. GAPDH (A) or β -actin (B) was used as a reference gene. The average of three to five independent experiments ($\pm$ SD) is presented (* P <0.05 when compared to non treated cells).

Figure 8. Putative mechanism of Trx2 involvement in NF- κ B and GR signalling and mitochondrial functions. Trx2 may acts as an antioapoptotic and antioxidant factor through its direct interaction with GR and NF- κ B and by preventing ROS accumulation leading to nuclear GR and NF- κ B activation. Trx2 activates mitochondrial GR and thus increases mitochondrial OXPHOS genes expression and promotes respiratory chain function. After TNF α treatment NF- κ B enters the mitochondria where it is activated by Trx2. Activated NF- κ B could then translocate into the cytoplasm and the nucleus to cause antiapoptotic and antioxidant genes expression. The inhibitory action of NF- κ B on OXPHOS genes expression could be suppressed by GR by a similar mechanism with the one described in the nucleus. Grey lines represent apoptotic processes; black lines represent survival processes.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8