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Oxidative stress-dependent p66Shc phosphorylation in skin fibroblasts of children with mitochondrial disorders

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ABSTRACT

p66Shc, the growth factor adaptor protein, can have a substantial impact on mitochondrial metabolism through regulation of cellular response to oxidative stress. We investigated relationships between the extent of p66Shc phosphorylation at Ser36, mitochondrial dysfunctions and an antioxidant defense reactions in fibroblasts derived from five patients with various mitochondrial disorders (two with mitochondrial DNA mutations and three with methylglutaconic aciduria and genetic defects localized, most probably, in nuclear genes). We found that in all these fibroblasts, the extent of p66Shc phosphorylation at Ser36 was significantly increased. This correlated with a substantially decreased level of mitochondrial superoxide dismutase (SOD2) in these cells. This suggest that SOD2 is under control of the Ser36 phosphorylation status of p66Shc protein. As a consequence, an intracellular oxidative stress and accumulation of damages caused by oxygen free radicals are observed in the cells.

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1. Introduction

The 66-kDa isoform of ShcA, the growth factor adapter protein (p66Shc), and its relevance to cell signaling have attracted major interest in aging research. In 1999, Pellici at al. [1] discovered that ROS-mediated signals, generated by p66Shc, favored apoptosis at the cellular and aging at the organismal levels, leading to tissue damage. As a result, p66Shc was proposed to regulate mammalian life span. These authors found that the life span of transgenic mice lacking p66Shc was longer by 30–40% compared to wild-type animals. Interestingly, prolongation of the life span corresponding to the ablation of p66Shc gene had no pathological consequences [1]. Taking into account the free radical theory of aging [2], it can be assumed that this protein can be involved in the cellular response to oxidative stress and clearly explains significantly enhanced resistance to oxidative stress of transgenic animals lacking p66Shc [1,3]. Three proteins of the ShcA family, p66Shc, p52Shc and p46Shc, have been identified up to now [4]. Two mRNAs derive from ShcA gene: p66shc and p46shc/p52shc, the latter one being translated into 2 proteins, p46shc and p52shc, after alternative translation start sites [4,5].

Each protein of the ShcA subfamily (p66Shc, p52Shc and p46Shc) contains three functionally identical domains: C terminal Src-homology domain (SH2), central collagen-homology domain (CH1) and N-terminal phosphotyrosine-binding domain (PTB). p66Shc contains additionally N-terminal proline-rich collagen-homology domain (CH2) with a serine phosphorylation site (Ser36) [3,4]. Numerous studies have demonstrated that p66Shc is phosphorylated mainly at this residue in response to UV exposure or H_2O_2 treatment [1,6]. Such phosphorylation is mediated by one of serine–threonine kinases, protein kinase Cβ (PKCβ), and this event induces a signaling cascade leading to apoptosis [7]. p66Shc phosphorylated at Ser36 (Ser36-P-p66Shc) is a substrate of prolyl isomerase Pin1 and, after isomerisation, it is dephosphorylated by phosphatase A2 (PPA2) [7]. This sequence of events triggers translocation of p66Shc from the cytosol to mitochondria [7]. Recently, we have demonstrated that p66Shc can also be found in plasma membrane-associated membrane (PAM) and mitochondria-associated membrane (MAM) fractions [8–10]. Intracellular distribution of p66Shc seems to be age dependent. MAM fraction isolated from livers of old mice was significantly enriched in p66Shc. Moreover, Ser36-P-p66Shc in the crude mitochondrial fraction (containing the MAM fraction) was more abundant in older mice and its

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higher level correlated well with the enhanced H_2O_2 production [8]. p66Shc, once translocated to mitochondria and/or MAM fraction, perturbs their structure and function, e.g., by modifying mitochondrial Ca^{2+} responses and the mitochondrial metabolism [1,11,12].

The signaling pathway described by Pinton et al. [7] is triggered by extracellular oxidative stress (e.g. H_2O_2 treatment) and involves p66Shc phosphorylation at Ser36. During aging, gradually accumulated mitochondrial and nuclear DNA damages and dysfunctions of the antioxidant defense system lead to an increase of intracellular reactive oxygen species (ROS) level; this in turn activates PKCβ-dependent phosphorylation of p66Shc and initiates a vicious circle of ROS production [13]. A similar situation may occur in the case of mitochondrial dysfunction associated with inherited disorders of oxidative phosphorylation (OXPHOS). The frequency of such mitochondrial diseases is relatively high (1:5000). They are present in neonates, infants, children and adults; they are progressive and not treatable [14,15]. The major pathogenic mechanism is considered to be a shortage in cellular energy supply. The influence of the pathological oxidative stress on the course of mitochondrial disorders and on the antioxidant defense balance has not been elucidated in detail. A higher rate of ROS production by mitochondria has already been described in many genetic mitochondrial defects [16]. The superoxide anion radical ($O_2^{\bullet -}$) is the main reactive oxygen species produced by mitochondria. The defects of respiratory chain caused by genetically inherited mutations in mitochondrial or nuclear DNA, leading to isolated or combined defects in respiratory chain complexes, may result in significantly elevated superoxide production. These extra ROS additionally potentiate mitochondrial damage and, in consequence, cause accelerated ROS production.

In the present work, we investigated whether mitochondrial dysfunctions of different genetic origin lead to an increased phosphorylation of p66Shc at Ser36, modification of the intracellular antioxidant defense system and stress-induced protein carbonylation.

2. Materials and methods

2.1. Ethics

The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committee of Bioethics at the Children's Memorial Health Institute. Informed consent was obtained from the parents before any biopsy or molecular analysis was performed.

2.2. Patients

To study phosphorylation of p66Shc at Ser36 in response to intracellular oxidative stress, five patients with defined mitochondrial disorders were recruited. The patients presented different clinical phenotypes and various OXPHOS abnormalities in muscle biopsies and fibroblast cultures that were derived from these patients. The fibroblast cultures were started from patients at the age of 4 months, 6 months, 3 months, 3 weeks and 14 months, respectively. At the time of the mitochondrial disease suspicion, the patients presented severe mitochondrial encephalopathy (P1), Leigh disease, deafness and retinitis pigmentosa (P2), progressive fatal mitochondrial cardiomyopathy (P3), severe neonatal lactic acidosis (P4) and hypertrophic cardiomyopathy with neutropenia (P5). Hyperlactatemia and hyperlactaturia were present in all patients. Patients 3, 4 and 5 showed additionally increased excretion of methylglutaconic acid in urine.

The probability level of mitochondrial disease determined according to the Nijmegen scale was above the probability threshold respectively: 8, 8, 6, 7 and 4. The overall patient characteristics is shown in Table 1.

Table 1
Clinical, biochemical and molecular characteristics of children with defined or probable mitochondrial disorders.

Patient; gender	Age of onset and the course of the disease	(1) Muscle histology and histochemistry. (2) Spectrophotometry* and BN–PAGE. (3) DNA analysis.	Diagnostic conclusion
Patient 1; M	4 months: vomiting, hypotony, nystagmus, exacerbation episodes, lactic acidemia, hyperalaninemia, respiratory alkalosis, transient liver function failure, MRI—symmetrical changes in basal ganglia and brainstem.	(1) Slight type I fiber predominance, cytochrome oxidase positive. (2) Low activity of complexes I and II + III in muscle homogenate. SC activity 97 nmol/min/mg protein (in reference range). (3) Heteroplasmic m.10191 T>C mutation in ND3 gene.	Mitochondrial encephalomyopathy with respiratory chain complex I deficiency.
Patient 2; F	4–6 months: hypotony, failure to thrive, vomiting, hepatomegaly, deafness, retinitis pigmentosa, dystonia, lactic and methylglutaconic aciduria, Leigh-like MRI changes.	(1) Mild lipid increase in muscles fibers. (2) Low activity of complexes II and II + III in muscle homogenate. SC activity 183 nmol/min/mg protein (in reference range). Low complex V activity. (3) Negative screening for common mtDNA mutations and *SURF1* gene mutations.	Leigh syndrome with methylglutaconic aciduria. Combined respiratory chain defect.
Patient 3; F	3 months: hypotony, hypertrophic cardiomyopathy, severe lactic acidemia (118; 142 mg/dl), hyperalaninemia. Older brother died at the age of 4 months due to progressive cardiomyopathy.	(1) Strong subsarcolemmal activity of cytochrome oxidase activity and s uccinate dehydrogenase activity. Slight type I fiber predominance. (2) Low activity of complexes I and III in muscle homogenate, increased CS activity (392 nmol/min/mg protein). (3) m.3303C>T mtDNA mutation in tRNALeu1.	Mitochondrial cardiomyopathy. Combined respiratory chain defect.
Patient 4; M	2 days: hypotony, cardiomyopathy, clotting abnormalities, rhabdomyolysis (CPK increase), severe metabolic (lactic) acidosis; decreased biotinidase activity. 6 weeks: clinical improvement and normalization, methylglutaconic aciduria.	(1) Decreased cytochrome oxidase activity. Lack of succinate dehydrogenase activity after standard 1 h incubation. Weak reaction appeared after elongation of incubation time (1.5 and 3 h). Moderate to severe lipid accumulation in muscle fibers. (2) Low activity of complexes IV and II + III in fibroblasts. SC activity 130 nmol/min/mg protein (in reference range). Low complex V activity.	Barth disease. Secondary combined respiratory chain defect.
Patient 5; M	Older brother of patient 4. 14 months: dilated cardiomyopathy, failure to thrive, neutropenia, methylglutaconic aciduria, alive at the age of 7.	(1) Low degree of lipid increase in muscle fibers and type I fiber predominance, mild nonspecific changes. Cytochrome oxidase positive. (2) Low activity of complexes II, III and II + III in muscle homogenate, increased CS activity (412.5 nmol/min/mg protein). Low complex V activity.	Barth disease. Secondary combined respiratory chain defect.

*CS reference value for muscle homogenates 97–150 nmol/min/mg protein and for fibroblasts 59–115 nmol/min/mg protein.

2.3. Fibroblast cultures

Human skin fibroblasts were grown from explants of skin biopsies of one healthy individual and five patients in Dulbecco modified Eagle's medium with glucose (4.5 g/l), 5 mM sodium pyruvate and 2 mM L-glutamine (Sigma Aldrich), supplemented with 10% (v/v) fetal bovine serum (Gibco), and 1.2% antibiotic, antimycotic solution (Sigma Aldrich) in an atmosphere of 5% (v/v) carbon dioxide in air at 37 °C. Additionally, NHDF neonatal dermal fibroblasts, Cat. n. CC-2509, and NHDF adult dermal fibroblasts, Cat. n. CC-2511, from Lonza were used as control healthy fibroblasts. The cells were grown in 75 cm^2 culture flasks and used within 6 days after reaching confluence.

2.4. Hispidin treatment

Dulbecco modified Eagle's medium was supplemented with 5 μM hispidin and the cells were cultured for 48 h. In the case of long treatment (28 days), the growth medium supplemented with 3 μM hispidin was changed every two days. The passages (from fourth to eight) were also made in the presence of 3 μM hispidin.

2.5. Enzymatic measurements

Spectrophotometric assays in post-nuclear supernatants obtained from skeletal muscle biopsies and fibroblast cell cultures were used for the measurement of complex I (NADH:ubiquinone oxidoreductase, rotenone-sensitive), complex II (succinate:ubiquinone oxidoreductase, malonate-sensitive), complex III (ubiquinone:cytochrome *c* oxidoreductase, antimycin A-sensitive), complex IV (cytochrome *c* oxidase) and citrate synthase as described previously [17]. Activities of the respiratory complexes were expressed as the ratio to the activity of citrate synthase in order to avoid the influence of different amounts of mitochondria in the material under investigation. Protein content in post-nuclear supernatants was determined by the method of Lowry et al. [18].

2.6. Samples preparation for Western blot

Cell pellets were resuspended in cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton, 0.1% SDS, 1% sodium deoxycholate) containing inhibitors of proteases (1 mM PMSF and the protein protease inhibitor cocktail) and phosphatases (1 mM Na_3VO_4, 10 mM NaF). Samples were incubated on ice for 15 min and centrifuged at 14,000×*g* for 20 min at 4 °C to remove insoluble material. Protein concentration in lysates was determined using Bradford method. Samples for SDS–PAGE were denatured in reducing Laemmli loading buffer at 45 °C or 95 °C for 5 min, depending on the antibody.

2.7. Western blotting

Cell lysates (25 – 50 μg protein) were separated electrophoretically in 8% or 10% SDS–polyacrylamide gel (BioRad) and transferred onto PVDF membrane (BioRad). Membranes were blocked using 2% non–fat

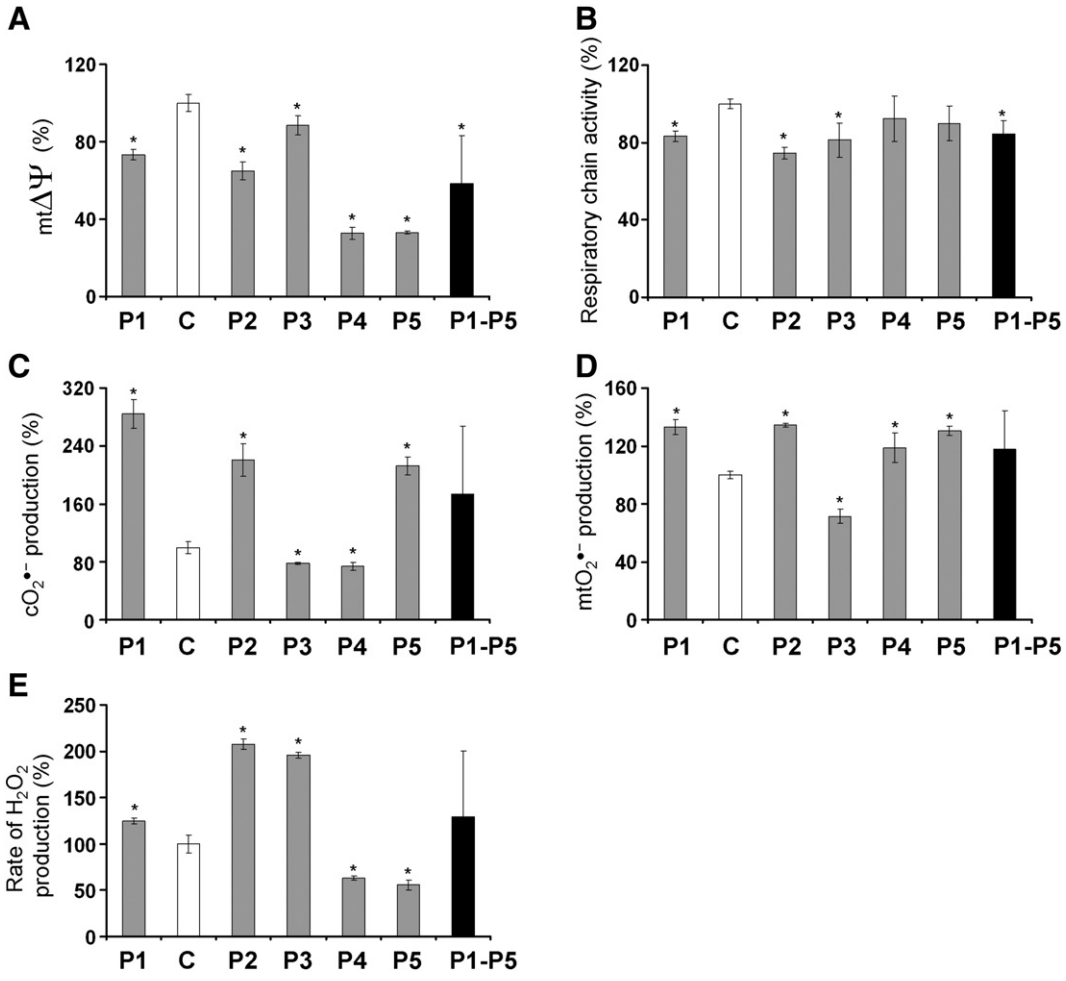

Fig. 1. Bioenergetic parameters (mtΔΨ, respiratory chain activity) and ROS production (cytosolic and mitochondrial $O_2^{\bullet-}$ and H_2O_2) in control and patients' fibroblasts. (A) Mitochondrial membrane potential in cultured fibroblasts. Data shown are means ± SD, $^*p<0.05$ ($n=6$) versus control. (B) Respiratory chain activity in cultured fibroblasts. Data shown are means ± SD, $^*p<0.05$ ($n=6$) versus control. (C) Cytosolic superoxide ($cO_2^{\bullet-}$), (D) mitochondrial superoxide ($mtO_2^{\bullet-}$) and (E) hydrogen peroxide (H_2O_2) production in cultured fibroblasts. Data shown are means ± SD, $^*p<0.05$ ($n=6$) versus control for each parameter.

milk in TBS buffer containing 0.01% Tween 20 (Sigma Aldrich) for 1 h. Proteins were detected with anti-Shc and anti-Ser36-P-p66Shc monoclonal antibodies (1:1000, Abcam), anti-SOD1 rabbit polyclonal antibody (1:5000, Santa Cruz), anti-SOD2 goat polyclonal antibody (1:500, Santa Cruz), anti-catalase monoclonal antibody (1:1000, Santa Cruz) and anti-actin antibody (1:10000, Abcam) followed by secondary HRP–conjugated antibodies (1:5000) (Santa Cruz).

2.8. Measurement of mitochondrial membrane potential ($mt\Delta\Psi$)

Fibroblasts grown in 12-well plates were washed twice with PBS to remove the medium and then incubated in the presence of 10 μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) in PBS containing 5 mM glucose for 10 min at 37 °C. The cells were washed twice with PBS and the green and red fluorescence was recorded in a microplate reader (Infinite M200, Tecan, Austria), respectively, at 485 nm excitation/520 nm emission and at 535 nm excitation/635 nm emission wavelengths.

2.9. Measurement of respiratory chain activity

Fibroblasts grown in 12-well plates were washed twice with PBS and then incubated in PBS containing 5 mM glucose and 6 μM resosurine and the fluorescence was recorded immediately thereafter in a microplate reader at 510 nm excitation and 595 nm emission wavelengths. To determine the unspecific reaction, the cells were preincubated for 15 min with 2 mM KCN added to medium.

2.10. Measurement of cytosolic superoxide ($cO_2^{\bullet -}$) production

Fibroblasts grown in 12-well plates were incubated for 20 min at 37 °C in the presence of 0.5 μM dihydroethidium (DHE) in PBS containing 5 mM glucose. The cells were washed twice with PBS, and the fluorescence was recorded in a microplate reader at 535 nm excitation and 635 nm emission wavelengths.

2.11. Measurement of mitochondrial superoxide ($mtO_2^{\bullet -}$) production

Fibroblasts grown in 12-well plates were incubated for 10 min at 37 °C in the presence of 2.5 μM MitoSox in PBS containing 5 mM glucose. The cells were washed twice with PBS and the fluorescence was recorded in a microplate reader at 510-nm excitation and 595-nm emission wavelengths.

2.12. Measurement of H_2O_2 production

The rate of H_2O_2 production was measured with the ROS-sensitive fluorescent probe 5-(and 6)-chloromethyl-2′,7′-dichlorohydrofluorescein diacetate (CM-H_2DCFDA). The cells grown in 12-well plates were treated with 2 μM CM-H_2DCFDA, and the fluorescence was

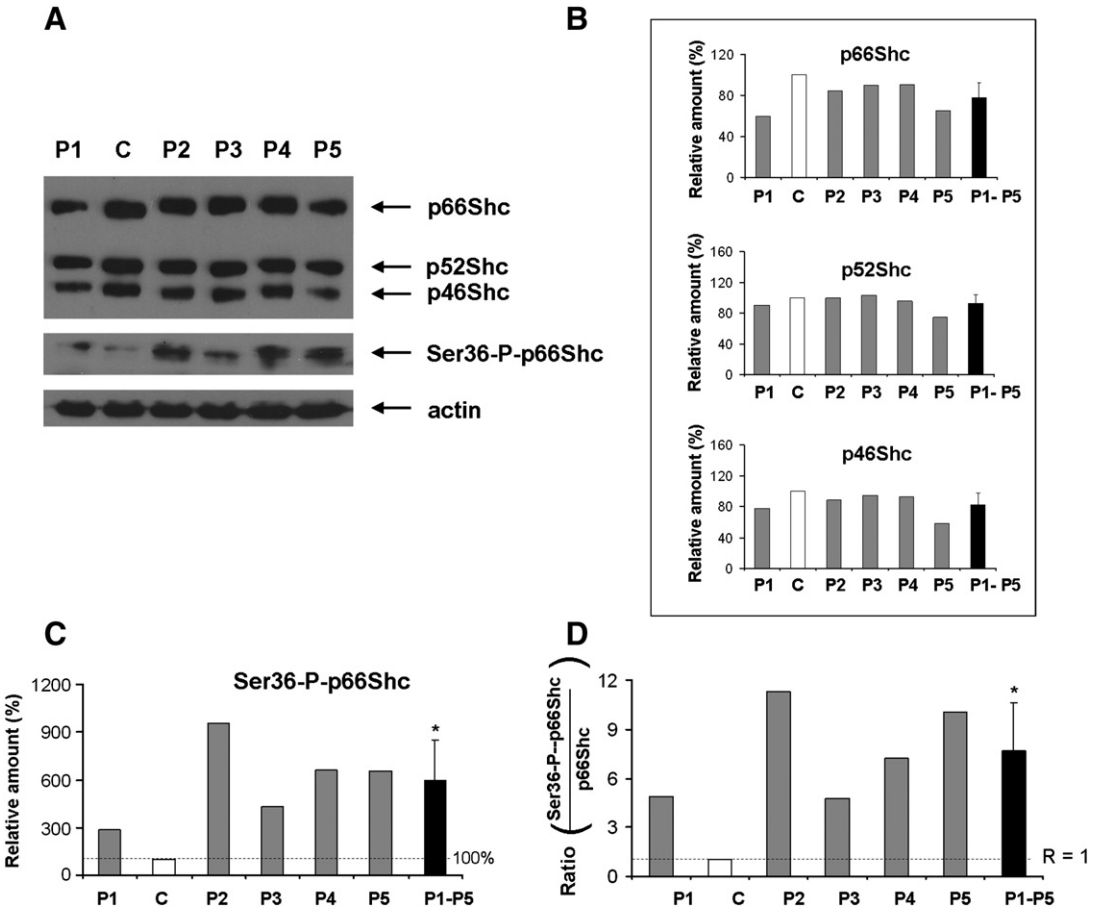

Fig. 2. Levels of p46Shc, p52Shc, p66Shc and its Ser36-fosphorylated form in control and patients' fibroblasts. (A) Representative Western blots of Shc proteins (p66Shc, p52Shc and p46Shc) and Ser36-P-p66Shc. Samples (15–30 μg protein/lane) from a control volunteer and 5 patients were separated on 8% gel (for Shc proteins) or 10% gel (for Ser36-P-p66Shc). (B) Densitometry analysis of Shc protein expression. (C) Densitometric analysis of Ser36-P-p66Shc level. (D) Calculated ratio of Ser36-P-p66Shc to p66Shc protein. The levels of individual proteins were calculated as a ratio to α-actin. The values are means from two independent experiments.

recorded in a microplate reader for 30 minutes at 495 nm excitation and 520 nm emission wavelengths.

2.13. Detection of protein modification by oxygen free radicals

The level of oxidized proteins was estimated with the use of the OxyBlot Protein Oxidation Detection Kit (Chemicon). Aliquots of 25 μg protein were separated on 10% SDS–polyacrylamide gel and the standard protocol of the manufacturer was followed.

2.14. Statistical analysis

Differences in band densities were analyzed using NIH ImageJ software. Data obtained from the Tecan microplate reader were calculated using Microsoft™ Excel 2005 and analyzed for significance by Student's *t*-test.

3. Results

To study phosphorylation of p66Shc at Ser36 in the situation of intracellular oxidative stress, we used fibroblast culture from five patients with genetically defined mitochondrial defects. The diagnosis of mitochondrial disease depended on the clinical phenotype, biochemical and enzymatic studies of muscle biopsies and DNA analysis. There were one severely affected infant with isolated complex I deficiency due to the mitochondrial DNA (mtDNA) mutation in ND3 subunit (P1), one infant with combined OXPHOS defect due to a tRNALeu1 rare mutation (P3) and three children with multi-organ mitochondrial disorders and methylglutaconic aciduria, among them one girl (P2) and two brothers with Barth syndrome (P4 and P5). The patients' characteristic is presented in Table 1. The expression of relevant OXPHOS abnormalities in some patients' fibroblasts was previously detected by spectrophotometric measurement of respiratory chain complex I-IV activities and confirmed by BN–PAGE and SDS (not shown).

3.1. Metabolic characterization of patient fibroblasts

To determine whether a mitochondrial dysfunction was present in the studied fibroblast cultures, mitochondrial membrane potential (mtΔΨ) and respiratory chain activity were measured. The measurement of mtΔΨ with the use of a fluorescent probe JC-1 showed a decrease in the proton-motive force in mitochondria of patients' fibroblasts. In three cases (P1, P2 and P3), mtΔΨ was decreased by 10–20%, while in P4 and P5 it was decreased by as much as 65–70% (Fig. 1A). In three lines of fibroblasts (P1, P2 and P3), the activity of the respiratory chain was reduced by ~20%. The respiratory chain activity in P4 and P5 was only slightly decreased (Fig. 1B). Lower mtΔΨ and decreased activity of the respiratory chain suggest that mitochondria were coupled and that the observed decreased electron flow through the respiratory chain could be a result of OXPHOS dysfunction. Moreover, evaluation of intracellular ROS production confirmed the occurrence of increased intracellular oxidative stress in the studied cells. Both cytosolic and mitochondrial ROS generation by fibroblasts of patients P1, P2 and P5 was increased (Fig. 1C, D), while in fibroblasts from P4 only mitochondrial superoxide generation was enhanced. The rate of H_2O_2 production was higher in P1 and was even doubled in P2 and P3 (Fig. 1E). Oxidative stress in these cells was also demonstrated by increased level of protein carbonylation (Supplementary Fig. 1). This indicated a proper selection of the material for these studies. Additionally, we compared control cell line (C) with human neonatal dermal fibroblasts (Fn) and human adult dermal fibroblasts (Fa). These results show that the control cell line (C) and neonatal dermal fibroblasts (Fn) have similar mitochondrial bioenergetics parameters and ROS production. The adult dermal fibroblasts

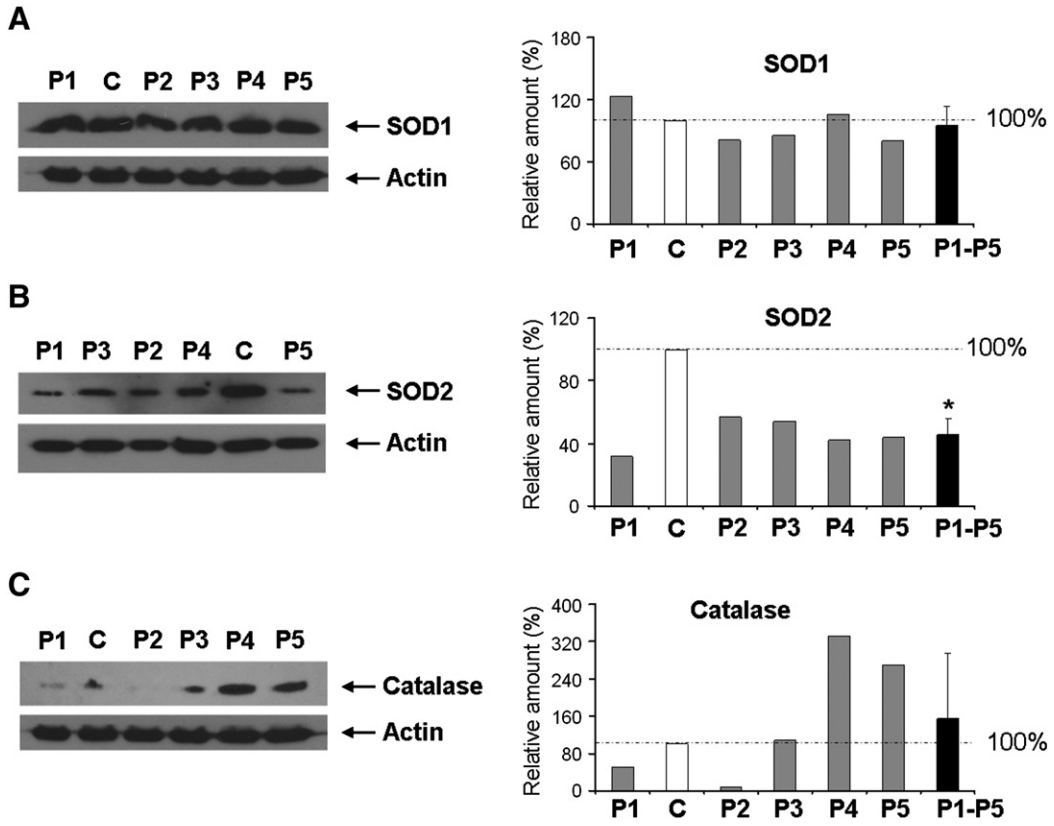

Fig. 3. Expression of SOD1, SOD2 and catalase in control and patients' fibroblasts with mitochondrial dysfunction. (A) Expression profile of cytosolic superoxide dismutase (SOD1). (B) Expression profile of mitochondrial superoxide dismutase (SOD2). (C) Expression profile of catalase. Cell lysates (40 μg protein/lane) were separated on 10% SDS–polyacrylamide gel. Bar charts show the levels of antioxidant enzymes calculated as a ratio to α-actin. The values are means from two independent experiments.

have significantly lower mitochondrial membrane potential and respiratory chain activity (Supplementary Fig. 2). This indicates necessity of the use of cell lines derived from the individuals at the similar age in such comparative studies.

3.2. Evaluation of p66Shc level and Ser36 phosphorylation status of p66Shc in control and patients' fibroblasts

To estimate changes in the levels of p66Shc and Ser36-P-p66Shc, we first measured the level of three ShcA proteins (p46Shc, p52Shc and p66Shc) and determined the ratios of p66Shc/p52Shc and p66Shc/p46Shc in control and patients' fibroblasts. The level of all ShcA proteins in the control and in all five patients' fibroblasts was similar (Fig. 2A and B) Interestingly, in all patients' cells, the level of Ser36-phosphorylated p66Shc was dramatically elevated without any external stimulus (like H_2O_2 treatment) (Fig. 2A and C). Additionally, the ratio of Ser36-P-p66Shc to the p66Shc confirmed a drastic shift in the extent of p66Shc phosphorylation at Ser36 (Fig. 2D). Comparison of control cell lines showed similar low level of Ser36 phosphorylated p66Shc (Supplementary Fig. 3). This points to an activation of PKCβ and p66Shc phosphorylation as a response to the mitochondrial defect-related enhanced oxidative stress in these cells. Antimycin A-induced oxidative stress in neonatal dermal fibroblasts (Fn) (represented as an augmented cytosolic and mitochondrial superoxide production) is connected with the increased phosophorylation status of p66Shc (Supplementary Figs. 4 and 5). Mitochondria-targeted antioxidant SkQ partially protects against antimycin A effect, what indicates direct relation between oxidative stress and Ser36 phosphorylation of p66Shc (Supplementary Fig. 5).

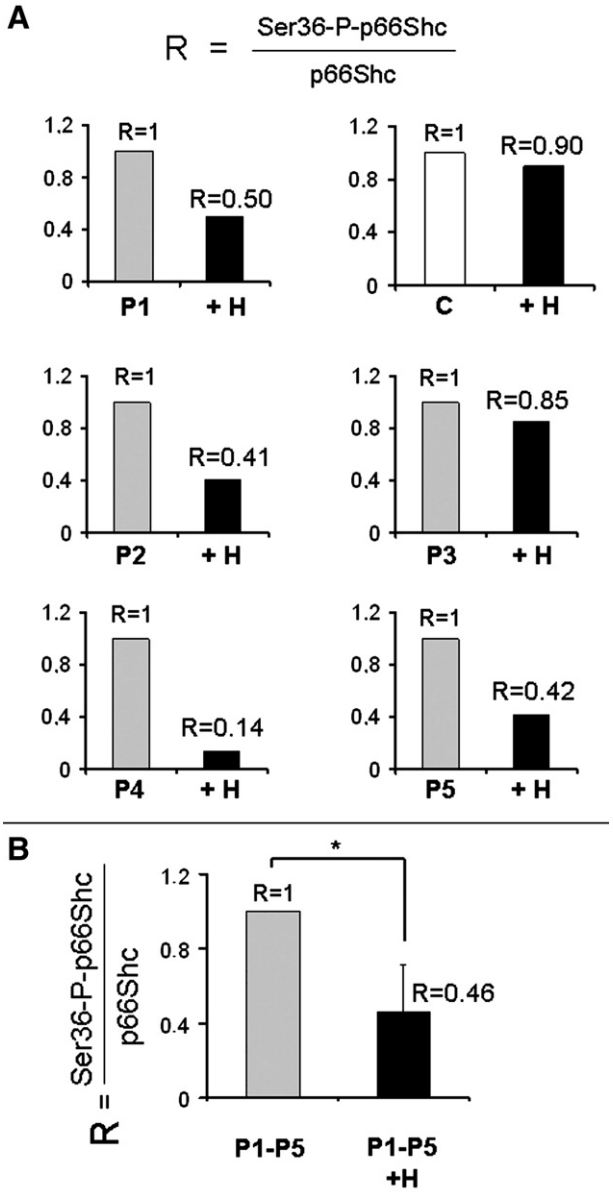

Fig. 4. Effect of long-term hispidin treatment on Ser36-phosphorylation status of p66Shc in control and patients' fibroblasts with mitochondrial dysfunction. Fibroblasts were treated for 28 days with 3 μM hispidin as described in Materials and methods. (A) Bar charts and *R* values show the ratio of Ser36-P-p66Shc to the p66Shc protein. The values are means from two independent experiments. (B) The ratio of Ser36-P-p66Shc to the p66Shc. The values are means from all patients ± SD, $^*p<0.05$ ($n=5$) versus control.

3.3. Antioxidant enzyme levels in patients' fibroblasts

Intracellular oxidative stress may modify the cellular antioxidant defense system by p66Shc phosphorylation at Ser36. We determined the expression profile of cytosolic (Cu/ZnSOD, SOD1) and mitochondrial (MnSOD, SOD2) superoxide dismutases and catalase (Cat). We observed a significant decrease of SOD2 level and unchanged SOD1 level in fibroblasts of all five patients (Fig. 3 B and A). Catalase levels in the cells with mitochondrial dysfunction were variable. In P3 fibroblasts the level seemed to be unchanged; in fibroblasts of two patients (P1 and P2) it was lower, whereas in fibroblasts of P4 and P5 it was greatly increased (Fig. 3C). Supplementary Fig. 3 shows antioxidant enzyme levels in control cell lines.

3.4. Inhibition of the PKCβ pathway and its effect on ROS production and mitochondrial parameters

To check if the inhibition of p66Shc phosphorylation at Ser36 could attenuate intracellular oxidative stress, we incubated the fibroblasts

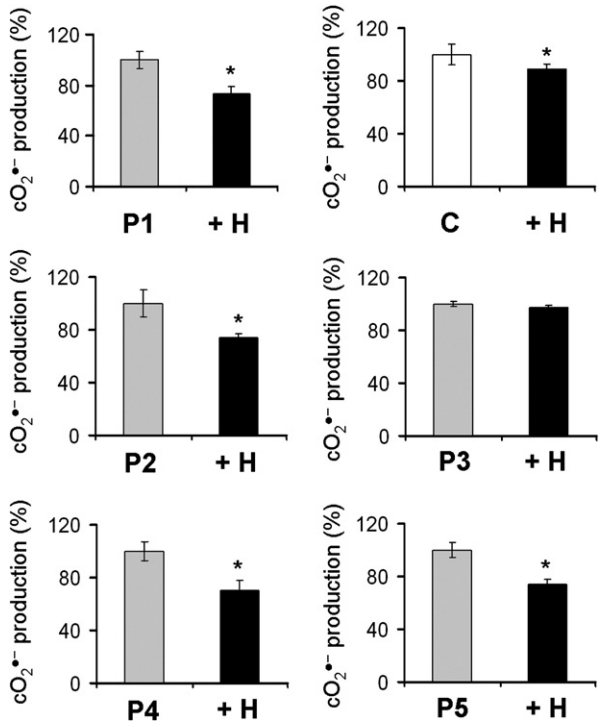

Fig. 5. Effect of long-term hispidin treatment on cytosolic superoxide production in control and patients' fibroblasts cultured conventionally (C) or treated (+H) for 28 days with 3 μM hispidin as described in Materials and methods. Data shown are means ± SD, $^*p<0.05$ ($n=6$) versus control for each parameter.

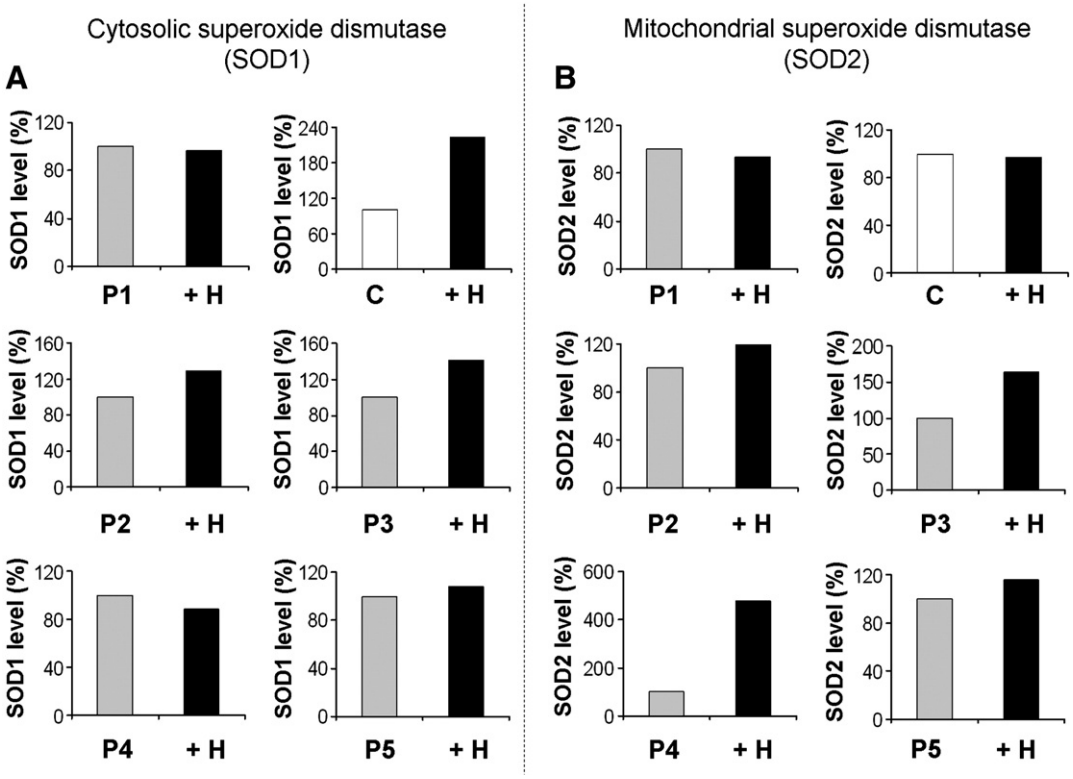

Fig. 6. Effect of long-term hispidin treatment on SOD1 and SOD2 expression in control and patients' fibroblasts. (A) Expression profile of cytosolic superoxide dismutase; (B) mitochondrial superoxide dismutase in control and patients' fibroblasts. Fibroblasts were cultured conventionally (C) or treated (+H) for 28 days with 3 μM hispidin as described in Materials and methods. Bar charts show the level of antioxidant enzymes calculated as a ratio to α-actin. The values are means from two independent experiments.

with hispidin. Such treatment for 48 h resulted in a slight decrease of p66Shc phosphorylation (Supplementary Fig. 2) and had no effect on ROS production and on mitochondrial parameters (data not shown) in both control and patients' fibroblasts. The long-term effect obtained by incubation with 3 μM hispidin for 28 days resulted in the reduced pool of Ser36-P-p66Shc (Fig. 4A). Calculated mean values from all patients show significant (~50%) decrease in the phosphorylation status of p66Shc after hispidin treatment (Fig. 4B). This was correlated with a decrease of cytosolic superoxide production ($cO_2^{\bullet -}$) (Fig. 5). At the same time, the mitochondrial superoxide production ($mtO_2^{\bullet -}$) was not significantly modified. Moreover, mitochondrial bioenergetic parameters ($mt\Delta\Psi$ and the respiratory chain activity) in patients' fibroblasts were also changed insignificantly by hispidin treatment. Only in fibroblasts from the healthy volunteer hispidin treatment increased $mt\Delta\Psi$, simultaneously decreasing the respiratory chain activity (data not shown).

3.5. Effect of the inhibition of p66Shc phosphorylation on the levels of SOD1 and SOD2

To find out whether the increased level of p66Shc phosphorylated at Ser36 affects the antioxidant defense system, we studied the effect of hispidin on the expression profiles of SOD1 and SOD2. We observed that, after inhibition of Ser36-p66Shc phosphorylation by long-term hispidin treatment, SOD1 was up-regulated in the control and in three lines of patients' fibroblasts (P2, P3 and P5), whereas SOD2 was elevated in P3 and P4 and slightly elevated in P2 and P5. In the control cells, SOD1 level was considerably increased by the hispidin treatment

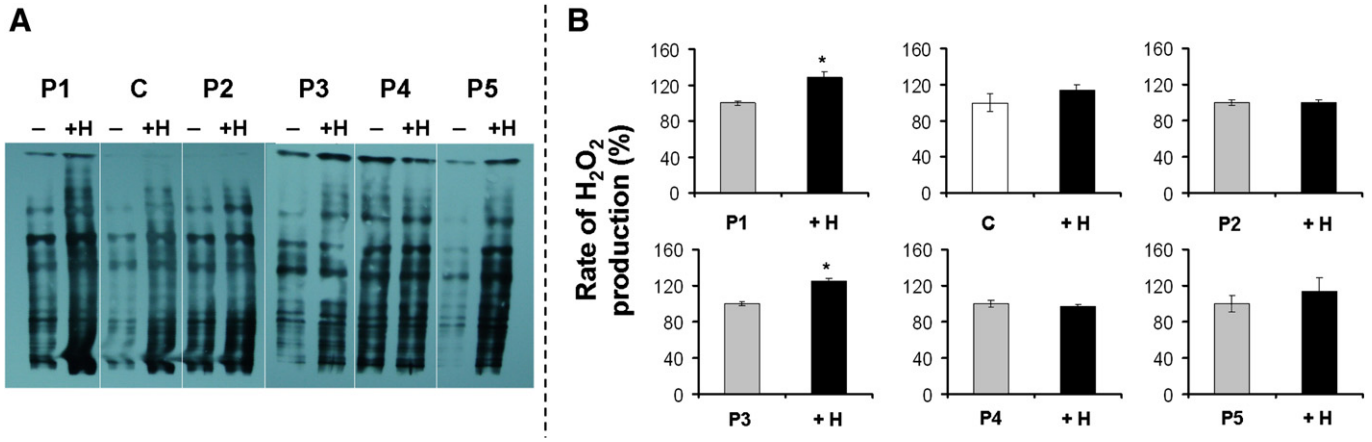

Fig. 7. Effect of long-term hispidin treatment on the degree of protein carbonylation and H_2O_2 production in control and patients' fibroblasts. Fibroblasts were cultured conventionally (C) or treated (+H) for 28 days with 3 μM hispidin as described in Materials and methods. (A) Level of carbonylated proteins was estimated as described in Materials and methods. (B) Hydrogen peroxide production in cultured fibroblasts. Data shown are means ± SD, $^*p<0.05$ ($n=6$) versus control for each parameter.

while SOD2 remained unchanged (Fig. 6A and B). However, under this condition, we observed an increase in protein carbonylation (Fig. 7A) probably as a result of increased H_2O_2 production (Fig. 7B).

4. Discussion

This is the first report showing that mitochondrial dysfunction-related intracellular oxidative stress can trigger phosphorylation of p66Shc at Ser36. As a model of elevated intracellular oxidative stress, we used fibroblasts derived from five patients with defined mitochondrial disorders. Our results clearly demonstrate remarkable abnormalities in all examined bioenergetic and ROS parameters regardless of the origin of genetic defects in the patients' cells. All fibroblasts derived from the patients showed a significant decrease in mtΔΨ and an increase in ROS production. We observed some differences between the patients. The changes were less expressed in the patient with mtDNA tRNALeu1 mutation (P3). Possible elimination of the pathogenic mutation load during fibroblast cultivation (below the heteroplasmy threshold value) were not assessed and might explain this finding. Patients 4 and 5, two brothers with Barth syndrome, differed from other patients by showing respiratory chain activity at the normal range. Mitochondrial dysfunction in Barth syndrome may be caused by abnormal cardiolipin metabolism and its influence on assembly of respiratory chain complexes and carriers [19,20]. Reduced mitochondrial membrane potential and unaffected mitochondrial ATP formation were reported earlier in Barth patient lymphoblasts [21].

Since p66shc protein was related to the cellular response to oxidative stress, its high level was correlated with the increased ROS production [1,8]. We demonstrated that, although the expression of p66Shc was unchanged, Ser36-P-p66Shc/p66Shc ratio was highly elevated in all studied patients' fibroblasts. The presented results clearly indicate that in fibroblasts with mitochondrial dysfunction, SOD2 and SOD1 levels can depend on the p66Shc phosphorylation state. This is in agreement with the recent reports describing similar observations in diabetes, Alzheimer disease, ethanol-induced liver damage and hypoxia/reoxygenation damage of hepatocytes [22–25]. A lower capacity of the antioxidant defense can be a reason for oxidative damage manifested for example by higher protein carbonylation [26]. One of the factors interacting with p66Shc and controlling mammalian intracellular metabolism and antioxidant defense is FKHRL1 (FOXO3a), a transcription factor belonging to the FoxO family and relevant to energy metabolism, proliferation and cell death [27,28]. In response to the mitochondrial dysfunction-related increased intracellular oxidative stress, high Ser36-phosphorylation status of p66Shc promotes an additional increase of intracellular ROS generation. This results in the recruitment of Akt/PKB to the p66Shc-FOXO3a complex and direct inactivation of FOXO transcription factors by their phosphorylation. Thus, decreased level of antioxidant defense and higher ROS production can be observed (Figs. 1 and 3). Moreover, decrease of the p66Shc phosphorylation at Ser36 caused by hispidin, a PKCβ inhibitor, is accompanied by positive modulation of the mitochondrial antioxidant defense in patients' fibroblasts (Fig. 4 and Supplementary Fig. 6). This mechanism is supported by our observations that SOD1 (in control, P2 and P3) and SOD2 (in P2, P3, P4 and P5) levels were up-regulated after inhibition of Ser36-p66Shc phosphorylation (Fig. 6). The up-regulated level of SOD1 (in three cases out of five investigated) and SOD2 (in four cases out of five) in the response to hispidin treatment contributes to an increased production of H_2O_2 and, most probably, to an increased protein carbonylation in the treated cells (Fig. 7). Increased protein carbonylation in response to H_2O_2 addition to cell cultures or enhanced intracellular H_2O_2 production has already been reported [26,29].

Hispidin treatment, apart from improving the antioxidant defense, was manifested in control fibroblasts by an increase in mtΔΨ and a decrease in the rate of respiration. This shows that mitochondria in the treated cells became more tightly coupled. This can explain previously reported higher calcium uptake by fibroblasts treated with hispidin [7]. Increased mtΔΨ is probably a reason of an increased $mtO_2^{\bullet-}$ production observed in control fibroblasts in response to hispidin treatment. Mitochondrial parameters in patients' fibroblasts remained unchanged because hispidin cannot “repair” genetically caused mitochondrial defects.

A growing piece of evidence indicates that low level of p66Shc or its complete ablation (what determines a low level or the absence of Ser36-P-p66Shc) protects against numerous age-related disorders and can partially prevent pathologies caused by ROS. Looking for strategies that could minimize the cellular effect of p66Shc phosphorylation at Ser36, it is necessary to remember that p66Shc also plays an important role in signal transduction pathways and regulates the crucial cellular Ras/MAPK kinase cascade [30]. However, in contrast to the data reported by Migliaccio et al. [1] that the lack of p66Shc protein does not result in any abnormalities, some new data have recently appeared on a low level of p66Shc in autoimmunological disorders [31]. It was found that p66Shc knockout mice developed an age-related autoimmune disease characterized by

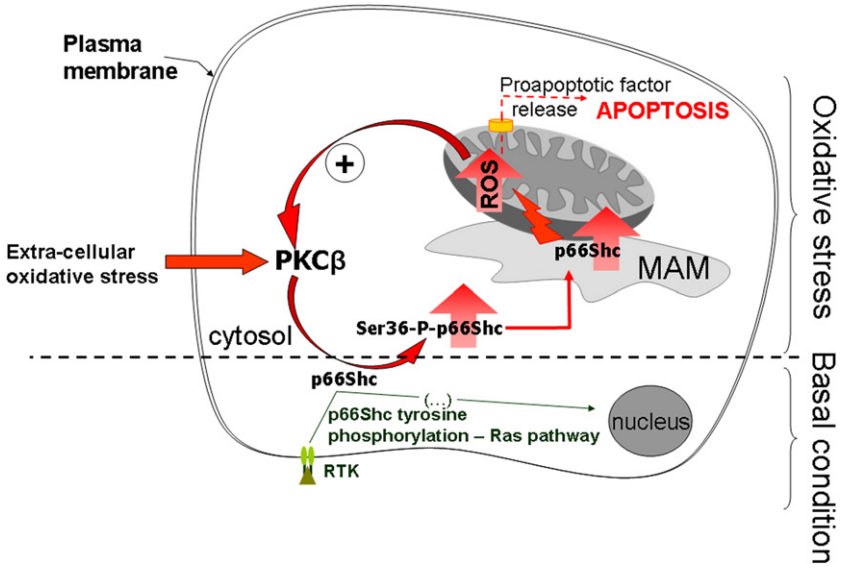

Fig. 8. Scheme showing the effect of p66Shc phosphorylated at Ser36 on the intracellular oxidative stress and antioxidant defense.

enhanced proliferation of B-lymphocytes that spontaneously produce antibodies and T-lymphocytes. In turn, the immunologic tolerance was impaired and resulted in autoimmunological aggression observed in p66Shc knockout mice [31]. In the light of these data, administration of mitochondrialy targeted antioxidants seems to be a promising and safe way to stop the vicious circle of p66Shc phosphorylation at Ser36, responsible for the pro-oxidant properties of p66Shc (see Fig. 8). This may open new possibilities for pharmacological interventions in case of pathological intracellular oxidative stress by such compounds (as SkQ and mitoQ) with documented therapeutic effect [32–34].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2010.03.005.

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