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Endogenous mitochondrial oxidative stress in MnSOD deficient mouse embryonic fibroblasts promotes mitochondrial DNA glycation

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Abstract

The accumulation of somatic mutations in mitochondrial DNA (mtDNA) induced by reactive oxidative species (ROS) is regarded as a major contributor of aging and age-related degenerative diseases. ROS has also been shown to facilitate the formation of certain advanced glycation end-products in proteins and DNAs, and N^2-carboxyethyl-2′-deoxyguanosine (CEdG) has been identified as a major DNA-bound AGE. Therefore, the influence of mitochondrial ROS on the glycation of mtDNA was investigated in primary embryonic fibroblasts derived from mutant mice (*Sod2*−/+) deficient in the mitochondrial antioxidant enzyme, manganese superoxide dismutase (MnSOD). In *Sod2*−/+ fibroblasts *vs.* wildtype fibroblasts, the CEdG content of mtDNA was increased from 1.90±1.39 pg/μg DNA to 17.14±6.60 pg/μg DNA ($p<0.001$). On the other hand, the CEdG content of nuclear DNA did not differ between *Sod2*+/+ and −/+ cells. Similarly, cytosolic proteins did not show any difference in the advanced glycation end-products or protein carbonyl contents between *Sod2*+/+ and −/+. Taken together, the data suggest that mitochondrial oxidative stress specifically promotes glycation of mtDNA and does not affect nuclear DNA or cytosolic proteins. Because DNA glycation can change DNA integrity and gene functions, glycation of mtDNA may play an important role in the decline of mitochondrial functions.

Keywords

Advanced glycation end-products (AGEs); N^2-carboxyethyl-2′-deoxyguanosine (CEdG); DNA glycation; mitochondria; Mn superoxide dismutase (MnSOD); mitochondrial DNA; oxidative stress

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Introduction

The accumulation of somatic mutations in the mitochondrial DNA (mtDNA) induced by oxidative stress is regarded as a major contributor of aging and age-related degenerative diseases [1]. Mitochondria are extremely susceptible to oxidative damage since 2–4% of the oxygen consumed by mitochondria is converted to superoxide anions by the electron transport chain [2]. Additionally, mitochondria have limited protection from oxidative stress [3]. Under normal conditions, the antioxidant enzyme manganese superoxide dismutase (MnSOD) protects mitochondria from superoxide anions that are produced as a byproduct of the respiratory chain. Two mouse lines lacking MnSOD (*Sod2*) have been constructed to further establish the role of mitochondrial ROS in the pathophysiology of diseases, especially in age-related disorders [4, 5]. Reduced activity of MnSOD can be correlated to a decline in mitochondrial function. Whereas complete ablation of MnSOD causes dilated cardiomyopathy, neurodegeneration, and early postnatal death [4, 6], heterozygous mutant mice (*Sod2*−/+) with a 50% reduction in MnSOD have a normal lifespan compared to the wildtype controls [7]. However, young *Sod2*−/+ mice exhibited decreased electron transport activity of Complex I, increased proton leak, reduced respiration rate, decreased respiratory control ratio for substrates metabolized by Complexes I, II, and III, lower mitochondrial membrane potential, and reduced ATP synthesis [8–10]. These findings show that a heterozygous knockout of MnSOD results in mitochondrial respiratory enzyme deficiency and that *Sod2*−/+ mouse is a useful model to study the molecular and pathological consequences of enhanced endogenous mitochondrial oxidative stress.

Advanced glycation end-products (AGEs) are generated by nonenzymatic reactions between sugars or sugar degradation products and free amino groups of proteins. Formation of some AGEs, such as the glycoxidation product N^{ε}-(carboxymethyl)lysine (CML) is greatly favored by reactive oxygen species (ROS) [11, 12]. In a similar way to amino groups of proteins, the exocyclic amino group of 2′-deoxyguanosine can be glycated, and N^{2}-carboxyethyl-2′-deoxyguanosine (CEdG) was identified as a major DNA-bound AGE [13]. CEdG is generated from a range of reactive carbonyl species (RCS), such as sugars, glyceraldehyde, or methylglyoxal [14, 15]. To date, CEdG has been detected *in vitro* in cultured smooth muscle cells and endothelial cells, in human urine, as well as in the human kidney and aorta [16–20]. Very recently, a method was developed to allow simultaneous quantification of AGEs bound to proteins, mtDNA, and nuclear DNA (nDNA). Therewith, significantly higher CEdG levels in mtDNA than in nDNA were detected in NIH 3T3 fibroblasts [21].

We hypothesize that one way mitochondrial ROS contributes to the accumulation of somatic mutations is by promoting the formation of mtDNA-AGEs. Therefore, the present study used primary mouse embryonic fibroblasts (MEFs) isolated from *Sod2*−/+ mice as a model for endogenously elevated mitochondrial oxidative stress, and the induction of intracellular AGEs was monitored by simultaneous quantification of nDNA-, mtDNA-, and protein-AGEs in *Sod2+/+* and −/+ cells.

Experimental Procedures

Chemicals, reagents, and antibodies

Herring sperm DNA was purchased from Fluka (Buchs, Switzerland), sodium chloride, and Roti®-Phenol (phenol/chloroform/isoamyl alcohol [25/24/1]) were obtained from Roth (Karlsruhe, Germany). Aminoguanidine was purchased from Acros Organics (Geel, Belgium) proteinase K (1000 U/mL) and RNase A/T1 mix (RNase A: 2 mg/mL, RNase T1: 5000U/mL) from Fermentas (St Leon-Rot, Germany), and PBS from Biochrom (Berlin, Germany).

DMEM F12 50/50 (with L-glutamine), MEM nonessential amino acids (100 x solution), penicillin, streptomycin, and amphotericin B (PSA) antibiotic antimycotic (100 x), TrypLE express (stable trypsin replacement) and HEPES buffer (1 M) were purchased from Invitrogen (Darmstadt, Germany). Standard fetal bovine serum (collected and processed in USA) was obtained from Thermo Scientific (Logan, USA).

The following commercial kits were used: OxyBlot oxidized protein detection kit, Millipore (Schwalbach, Germany); ECL Western blot system, GE Healthcare (Munich, Germany); DC protein assay, Bio-Rad (Munich, Germany); Complete® protease inhibitor cocktail tablets, Roche Applied Science (Mannheim, Germany).

The following antibodies were applied: for CEdG-ELISA, a monoclonal CEdG antibody developed in mouse (MAb M-5.1.6, [17]) was used. Labeling was performed with a sheep anti-mouse IgG conjugated with horseradish peroxidase conjugate, A5906, from Sigma-Aldrich (Munich, Germany). For AGE Western blot the primary antibody was a rabbit polyclonal antibody to CML 100 μg (1 mg/mL), ab27684, from Abcam (Cambridge, UK), the secondary antibody was a goat anti-rabbit IgG tagged with horseradish peroxidase, A0545, from Sigma-Aldrich (Munich, Germany).

In vitro incubation of DNA under oxidative conditions

Incubation tests were performed with herring sperm DNA (1 mg/mL) in PBS using either solely DNA or reaction mixtures of the DNA with 1 mM H_2O_2 or 25 mU/mL xanthine oxidase (XO) and 0.4 mM xanthine. The H_2O_2 test solutions were varied by adding 5 mM glucose or 10 mM pyridoxamine, respectively. DNA was also incubated in the presence of the pyridoxamine. Parallel to the DNA/XO/xanthine tests, solutions were processed after addition of catalase (275 U/mL) or catalase and SOD (100 U/mL).

Controls containing only H_2O_2, glucose, or pyridoxamine in PBS were prepared to exclude an impact of the reagents on the subsequent ELISA step.

Aliquots of initially 10 mL of each test solution in 50 mL reaction tubes were incubated for 14 d at 37°C in a shaking water bath. Incubations were performed in triplicates. Samples of 350 μL each were drawn every other day and stored at −80°C until analysis by CEdG ELISA.

Cell culture

MEFs were derived from embryonic day 13 *Sod2*+/+ and −/+ fetuses by standard protocols as described [22]. Animal procedures were approved by the IACUC committees at Stanford University and VA Palo Alto Health Care System. MEFs were cultured in 50% DMEM/50% F12 medium supplemented with 10% FCS, 1% PSA, 1% non-essential amino acids, and 15 mM HEPES in humidified atmosphere containing 5% CO_2 at 37°C. Cells were expanded to passage one (P1) and frozen in liquid nitrogen for long-term storage. Frozen stock of *Sod2* fibroblasts were thawed as P2 and usually split 1:3 every 4–5 days when confluent. All experiments were conducted using MEFs within five passages post isolation. The cell population doubling time (Td) was monitored for passage three and four. The Td was calculated from the exponential portion of the growth curve using an online calculator (http://www.doubling-time.com).

Simultaneous isolation of nDNA, mtDNA, and cytosolic proteins from MEFs

The simultaneous isolation of nDNA, mtDNA, and cytosolic proteins was performed as described previously for NIH 3T3 fibroblasts [21] with minor modifications. For cell disruption, only freeze-thaw cycles were performed. The pelleted nuclei and mitochondria

were resuspended in an extraction buffer containing 50 mM Tris (pH 8.0), 50 mM EDTA, 50 mM NaCl and 1% sodium dodecyl sulfate (SDS), and the final supernatant was collected as cytosolic protein fraction. Aminoguanidine (83 mM final concentration) was also added to the resuspended nuclei and mitochondria to prevent artificial glycation reactions during the workup. Nuclei and mitochondria were digested by consecutive additions (4x) of 30 μL proteinase K and incubation at 55°C for 3 h, overnight, 3 h, and 2 h. The DNA quality and concentrations were determined by UV measurements at 260 and 280 nm, and the protein yield was quantified using the DC protein assay.

Assay for succinate dehydrogenase (SDH) activity

The mitochondrial pellet was suspended in 1 mL of buffer M (210 mM D-mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4). The SDH assay was performed as previously described [21]. The suspended pellet, the post-mitochondrial supernatant, or buffer M alone was analyzed in a concentration of 10% v/v in the assay buffer as described. The absorption at 600 nm was determined in five-minute intervals for the first 30 min, and then approximately every 30 min for a total of 160 min. Total optical density changes after 160 min were used to determine SDH activities.

Competitive ELISA for CEdG

Prior to ELISA measurement, DNA was denatured for 5 min at 99°C to render CEdG adducts more accessible to the antibody, and, thus, increase the sensitivity. Denaturation does not lead to artificial CEdG formation (data not shown). The formation of CEdG-modifications of *in vitro* incubated DNA, isolated nDNA and mtDNA was then monitored by ELISA as previously described [23].

Coomassie staining of proteins separated by SDS-PAGE

SDS-PAGE of the cytosolic proteins was performed according to Laemmli [24]. Fifteen μg of proteins per lane was loaded onto a 12% acryl amide gel and separated at 150 V for 80 min. After electrophoresis was completed, the gels were stained with Coomassie blue as previously described [21].

Western blot analysis for AGE-modified proteins

Western blotting for AGE-modified proteins was performed as previously described [21] with minor modifications. Labeling was performed using the horseradish peroxidase-conjugated rabbit IgG diluted 1:1,000 in blocking solution. Specificity of the AGE antibody for CML and N^{ε}-(carboxyethyl)lysine (CEL) was verified by pre-incubation of the antibody with synthesized CML-bovine serum albumin and CEL-bovine serum albumin, respectively, which abolished the signals of the Western blot (data not shown). Simultaneously to each Western blot, an identically loaded acrylamide gel was stained with Coomassie blue, which served as a reference for AGE quantification (see Fig. 4A).

Analysis of oxidative protein modifications

OxyBlot oxidized protein detection kit was applied according to the manufacturer's instructions. Two aliquots of each sample were analyzed; one was subjected to the derivatization reaction, the other aliquot was treated with a control solution and served as negative control. Detection of oxidized bands was performed using the ECL Western blot system with exposure times from 5 s to 5 min.

Results

In vitro glycation of DNA by oxidative species

In order to test if reactive oxidative species (ROS) are able to introduce AGEs in DNA, herring sperm DNA was incubated *in vitro* in PBS under different oxidative conditions at 37°C for up to 14 d. Samples were taken every other day and the CEdG concentration was determined using an established CEdG ELISA method. Incubation was carried out in the presence of air oxygen, hydrogen peroxide (1 mM), and XO/xanthine system. The CEdG concentration increased with incubation time during reaction in the presence of oxygen (Fig. 1A). Starting from day 2, a significant ($p < 0.01$) and, from day 4, a highly significant ($p < 0.001$) increase of CEdG concentration compared to day 0 was measured. Addition of hydrogen peroxide and XO/xanthine further increased CEdG formation significantly compared to the incubation in the presence of air oxygen. Hydrogen peroxide and the XO/xanthine system induced a significant increase of CEdG concentration compared to the unincubated sample (0d) starting from day 2 ($p < 0.01$ at 2d and $p < 0.001$ at ≥ 4d for hydrogen peroxide; $p < 0.05$ at 2d and $p < 0.001$ at ≥ 4d). It was also investigated if co-incubation of DNA with hydrogen peroxide and physiological concentrations of glucose may further enhance CEdG formation. Hydrogen peroxide may liberate reactive carbonyl compounds from glucose which may serve as CEdG-precursors. Further, DNA-oxidation in the presence and absence of hydrogen peroxide was repeated after the addition of the AGE-inhibitor pyridoxamine. As shown in Fig. 1B and 1D, addition of 5 mM glucose did not further increase the CEdG-concentration, whereas pyridoxamine inhibited CEdG-formation in the presence and absence of hydrogen peroxide. Finally, the inhibitory effect of SOD and catalase or catalase alone on XO/xanthine -induced CEdG formation is demonstrated in Fig. 1C.

Standardization of cell culture conditions

To collect MEFs for DNA and protein glycation studies, wildtype (*Sod2*+/+) and *Sod2*−/+ MEFs were cultivated and expanded continuously from P2 to P5. Growth curves were recorded for P3 and P4 and the Td was calculated. The Td for *Sod2*+/+ MEFs was 50.99 h for P3 and 54.86 h for P4, whereas the Td for *Sod2*−/+ MEFs was 39.18 h for P3 and 44.98 h for P4. However, the differences in Td were not significant between *Sod2*+/+ and *Sod2*−/+ cells. At the time of harvest, *Sod2*+/+ and *Sod2*−/+ MEFs were cultured for a total of 18.0 d and 17.7 d, respectively (Fig. 2). The Td and culture time showed that the culture conditions were similar for *Sod2+/+* and *Sod2*−/+ fibroblasts.

Isolation of nDNA, mtDNA, and cytosolic proteins from Sod2 fibroblasts

Table 1 shows the yield of nDNA, mtDNA, and cytosolic proteins per cell. The protein yields amounted to 141.11 pg/cell and 145.23 pg/cell for *Sod2+/+* and *Sod2*−/+ MEFs, respectively. From *Sod2*+/+ MEFs, the average yield was 4.94 pg nDNA and 0.018 pg mtDNA per cell. From *Sod2*−/+ fibroblasts, the average yield was 3.76 pg nDNA and 0.034 pg mtDNA per cell. There was no significant difference in the yield of nDNA, mtDNA, or cytosolic proteins between *Sod2+/+* and *Sod2*−/+ fibroblasts. The ratio of $A_{260/280}$ for nDNA and mtDNA was always greater than 1.8.

Characterization of the mitochondrial fraction

To ensure that the mitochondrial pellet indeed contained the mitochondria, the isolated fraction was analyzed for mitochondrial SDH activity. The post-mitochondrial supernatant was analyzed in parallel as a negative control. Addition of the substrate 2,6-dichlorophenolindophenol (DCPIP) to the mitochondrial fraction resulted in a color

reduction of 72% within 160 min, which verifies SDH activity (data not shown). The post-mitochondrial supernatant, on the other hand, did not show SDH activity.

Determination of the CEdG content of nDNA and mtDNA

In order to measure DNA-AGEs, CEdG modifications in nDNA and mtDNA isolated from *Sod2+/+* and *Sod2*−/+ MEFs were quantified using an established CEdG ELISA method. Nuclear DNA isolated from *Sod2+/+* and *Sod2*−/+ fibroblasts showed CEdG levels of 12.27±4.90 pg/μg nDNA and 12.47±5.13 pg/μg nDNA, respectively. No significant difference in CEdG content was observed in nDNA. Mitochondrial DNA isolated from *Sod2*+/+ fibroblasts showed low levels of CEdG modifications of 1.90±1.39 pg/μg mtDNA. A significant increase in CEdG content to 17.14±6.60 pg/μg mtDNA was observed in *Sod2*−/+ MEFs (n=5, $p<0.001$, Fig. 3). The results indicated that proportionally more mtDNA is glycated in *Sod2*−/+ cells when compared to *Sod2*+/+ controls.

Analysis of protein AGEs and protein carbonyls

The cytosolic protein fractions isolated from *Sod2+/+* and *Sod2*−/+ MEFs were analyzed for their AGE content with Western blot using an antibody that detects both CML and CEL. In the cytosolic protein fraction of both MEFs, AGEs were detected over the whole range of proteins. There was no significant difference in AGE pattern or intensity between wildtype and *Sod2*−/+ MEFs.

Additionally, oxidative modifications of cytosolic proteins were analyzed by Western blot after derivatization with 2,4-dinitrophenylhydrazine (DNPH). Protein samples without DNPH derivatization were used as negative control. As shown in Fig. 4B, there are two dominant bands at 42–43 kDa in both lanes. After quantification of the band intensities and normalization to the corresponding lanes on the Coomassie gel, no significant differences in protein carbonyl content were observed between *Sod2+/+* and *Sod2*−/+ MEFs.

Discussion

In order to investigate whether ROS induces DNA glycation, DNA was incubated under various oxidative conditions *in vitro*. Even air oxygen was sufficient to induce CEdG formation. It is well established that metal traces present in PBS induce oxidation in the presence of oxygen [25]. The addition of hydrogen peroxide as well as of the XO/xanthine system led to a further significant increase in CEdG-concentration. XO produces superoxide and hydrogen peroxide [26]. In a secondary reaction, superoxide can be spontaneously or enzymatically converted into hydrogen peroxide by dismutation. Co-incubation of DNA/XO/xanthine with SOD and catalase significantly suppressed CEdG formation, confirming that the measured CEdG formation was indeed caused by ROS. Co-incubation with catalase alone inhibited CEdG formation to a similar extent as the combination of both enzymes indicating that hydrogen peroxide is the reactive agent leading to DNA glycation. Superoxide, in contrast, must first be converted to hydrogen peroxide which then induces CEdG formation. An increase of CEdG concentration with increasing incubation time could be observed under all tested conditions. Most likely, RCS are formed during the reaction of ROS with DNA by oxidative degradation of deoxyribose. The RCS then reacts with the guanine base to yield CEdG. The addition of glucose did not further enhance CEdG concentration indicating that deoxyribose from the DNA is a considerably more efficient carbonyl precursor than glucose. Pyridoxamine effectively suppressed CEdG formation. Pyridoxamine is a well established AGE-inhibitor, which acts most likely by trapping reactive carbonyl compounds by its amino group [27]. *In vivo*, the administration of pyridoxal phosphate inhibited CEdG accumulation in glomeruli of diabetic rats [28]. Pyridoxal phosphate and pyridoxamine are both B6 vitamers, which can be converted into

each other *in vivo*. Our findings showed that ROS, in particular hydrogen peroxide and superoxide as a precursor of hydrogen peroxide, are able to induce DNA glycation *in vitro*. As a next step, we investigated if endogenous oxidative stress enhances intracellular glycation. Mitochondria are the main source of cellular ROS, because 2–4% of the oxygen consumed by mitochondria is converted to superoxide anions by the electron transport chain [2]. Superoxide anions may then react to generate more reactive ROS, such as hydrogen peroxide and hydroxyl radicals [29].

Partial knockout of MnSOD activity has been established as a suitable model to study the role of endogenous mitochondrial oxidative stress *in vitro* and *in vivo*. Heterozygous MnSOD knockout mice (*Sod2*−/+) show increased levels of ROS and, at the same time, have no compensatory response from other antioxidant enzymes [30]. The detrimental significance of mitochondrial electron transport chain-derived superoxide is well illustrated by the lethal phenotype of mice lacking the mitochondrial matrix superoxide dismutase (i.e. MnSOD) [4]. Therefore, endogenous glycation of nDNA, mtDNA, and cytosolic proteins was analyzed in MEFs with reduced MnSOD activities. Nuclear DNA from both *Sod2*+/+ and −/+ MEFs showed similar CEdG levels (Fig. 3), indicating that reduced levels of mitochondrial MnSOD did not cause increased oxidative stress in the nucleus. This conclusion is supported by a previous study reporting similar levels of 8-OH-deoxyguanosine in wildtype and *Sod2*−/+ mice [10].

Parallel to nDNA analyses, mtDNA was examined for glycation adducts. The CEdG content of mtDNA extracted from *Sod2*−/+ cells was eight times higher than that in *Sod2*+/+ cells, suggesting that the increased mitochondrial oxidative stress in *Sod2*−/+ cells promoted mtDNA glycation. Consistent with this finding, a previous study reported a 30% increase in the 8-OH deoxyguanosine levels of mtDNA from the liver of *Sod2*−/+ mice compared to control animals [10]. The present results demonstrated that DNA glycation is an important second mechanism – in addition to DNA oxidation – that leads to ROS induced damage of mtDNA. It is assumed that elevated superoxide concentration accelerates DNA damage by leaching iron from iron-sulfur cluster containing proteins and subsequent hydroxyl radical generation [31, 32]. The notion is supported by the observation that the activities of Complex I and aconitase, both containing iron-sulfur clusters, are significantly reduced in the liver mitochondria of *Sod2*−/+ mice [33, 34]. Unlike nDNA, mtDNA lacks extensive protection by histones and only possesses a limited capacity for DNA repair [35]. It has been well documented that mtDNA accumulates alterations (deletions, rearrangements, or point mutations) with age in various model systems as well as in humans [36]. Since deletions and point mutations can be a result of glycation reactions [37], the observed accumulation of CEdG triggered by mitochondrial oxidative stress could be involved in the decline in mitochondrial function.

Cytosolic protein AGEs were monitored in *Sod2+/+* and *Sod2*−/+ cells by CML/CEL-Western blot analysis, and cytosolic protein carbonyls by Western blot after derivatization with DNPH. There was no significant increase in AGE modifications as a result of the partial reduction in MnSOD. In contrast, it has been reported that mitochondrial protein carbonyls are increased in *Sod2*−/+ mice compared to wildtype controls [10]. These findings are consistent with an analysis of skin collagen of *Sod2*+/+ and −/+ mice for biomarkers of aging, in which CML and pentosidine were found to change with age to a similar extent in both strains of mice [7]. It has been reported that MnSOD deficiency in *Sod2*−/+ mice does not influence the activity of the cytosolic superoxide dismutase, Cu,ZnSOD. Consequently, the increase in free radical formation is limited to mitochondria.

RCS are generated endogenously by autoxidation of carbohydrates or lipid peroxidation. This process might be accelerated by increased mitochondrial superoxide concentration that

is found in *Sod2*−/+ mice. Therefore, cytosolic protein extracts were further analyzed for oxidative modifications. The cytosolic protein extracts of *Sod2*+/+ and *Sod2*−/+ MEFs did not show a significant difference in the carbonyl content. The results are comparable to a previous study reporting similar levels of carbonyl groups in the cytosolic proteins extracted from the liver of *Sod2*−/+ mice and controls [10].

Conclusion

The present findings indicate that enhanced endogenous mitochondrial oxidative stress in *Sod2*−/+ cells results in an accumulation of glycation products specifically in mtDNA. It is well known that increased DNA glycation can lead to reductions in gene function or alterations in DNA sequence [23]. Since intact mtDNA is important for normal mitochondrial functions, our results suggest that oxidative-stress-induced mtDNA glycation could be involved in a decline in mitochondrial function.

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Abbreviations

AGEs	advanced glycation end products
CEdG	N^2-carboxyethyl-2′-deoxyguanosine
CEL	N^e-(carboxyethyl)lysine
CML	N^e-(carboxymethyl)lysine
DCPIP	2,6-dichlorphenolindophenol
DNPH	2,4-dinitrophenylhydrazine
MEFs	mouse embryonic fibroblasts
MnSOD	manganese superoxide dismutase
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
P	passage
PSA	penicillin, streptomycin, and amphotericin B
RCS	reactive carbonyl species
ROS	reactive oxygen species
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulfate
Td	cell population doubling time
XO	xanthine oxidase

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Highlights

- Reactive oxygen species are important precursors for DNA-glycation in vitro
- Primary embryonic fibroblasts were obtained from mutant mice deficient in MnSOD
- Mitochondrial DNA-glycation is increased in Sod2−/+ compared to Sod2+/+ fibroblasts
- Nuclear DNA-glycation is not affected by MnSOD deficiency
- MnSOD deficiency does not influence glycation and oxidation of cytosolic proteins

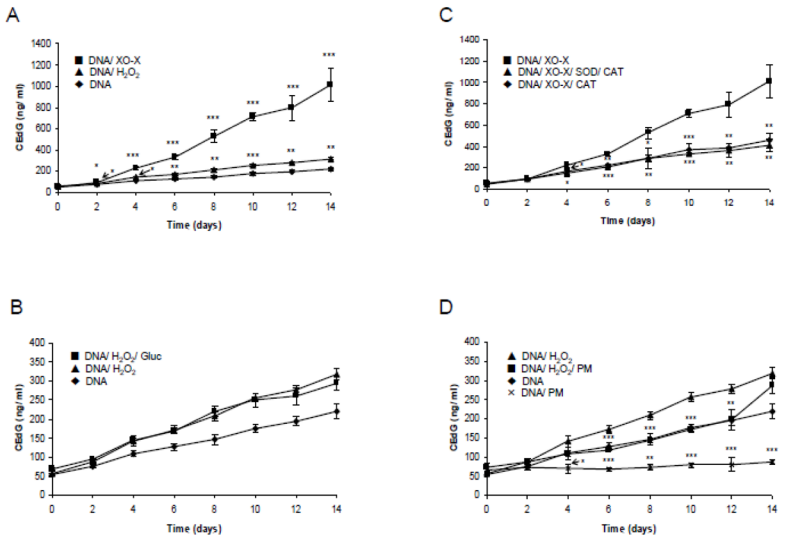

Fig. 1.

CEdG concentration after incubation of DNA under oxidative conditions at 37°C for 14 d: **(A)** herring sperm DNA (1 mg/mL) in the presence of air oxygen, hydrogen peroxide (H_2O_2, 1 mM), or xanthine oxidase/xanthine (XO-X, 25 mU/mL/0.4 mM); **(B)** DNA, DNA/H_2O_2 and DNA/H_2O_2 incubated in the presence of D-glucose (Gluc, 5 mM); **(C)** DNA/XO-X in absence and presence of catalase (CAT) or superoxide dismutase and catalase (SOD/CAT); **(D)** DNA and DNA/H_2O_2 in the absence and presence of pyridoxamine (PM, 10 mM). Concentrations are given as ng CEdG/mL incubation mixture. Data from each time point was measured in triplicates. Mean and standard deviation are shown. Significant differences between DNA and DNA/H_2O_2 or DNA/XO-X **(A)**, between DNA/XO-X and DNA/XO-X/SOD/CAT or DNA/XO-X/CAT **(C)**, between DNA/H_2O_2 and DNA/H_2O_2/PM **(D)** as well as between DNA and DNA/PM **(D)** are shown (* $p<0.05$, ** $p<0.001$,*** $p<0.001$).

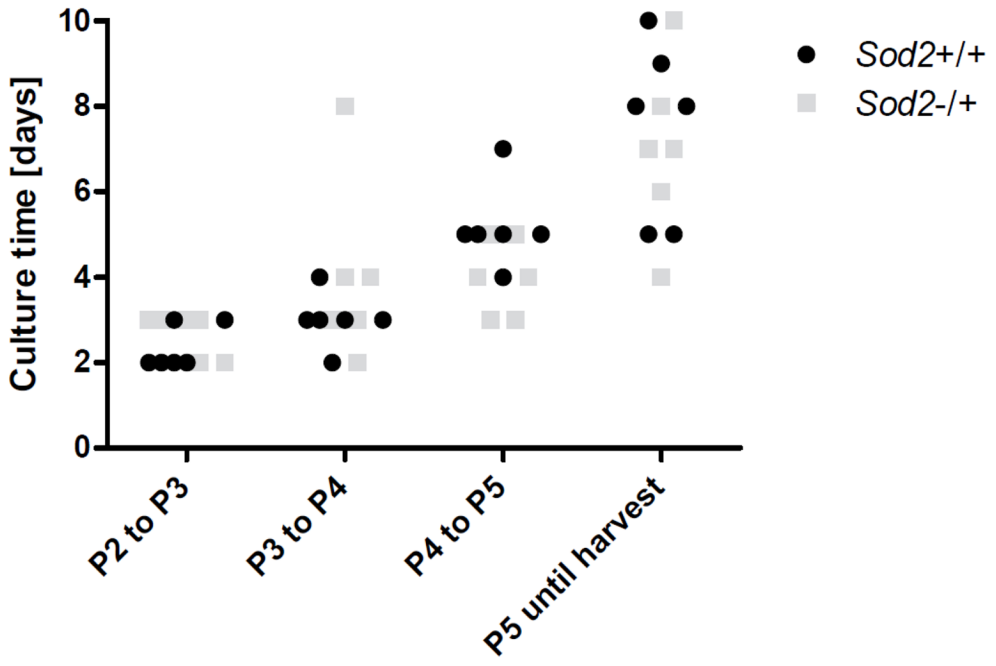

Fig. 2.

Culture time of *Sod2*+/+ and −/+ MEFs. MEFs were thawed at P2 and split 1:3 until harvest at P5. The time required to reach confluency in culture was monitored for P2–P5. With increasing passages post isolation, the time until confluency increased. There was no significant difference between *Sod2*−/+ MEFs and wildtype MEFs (n=6 each).

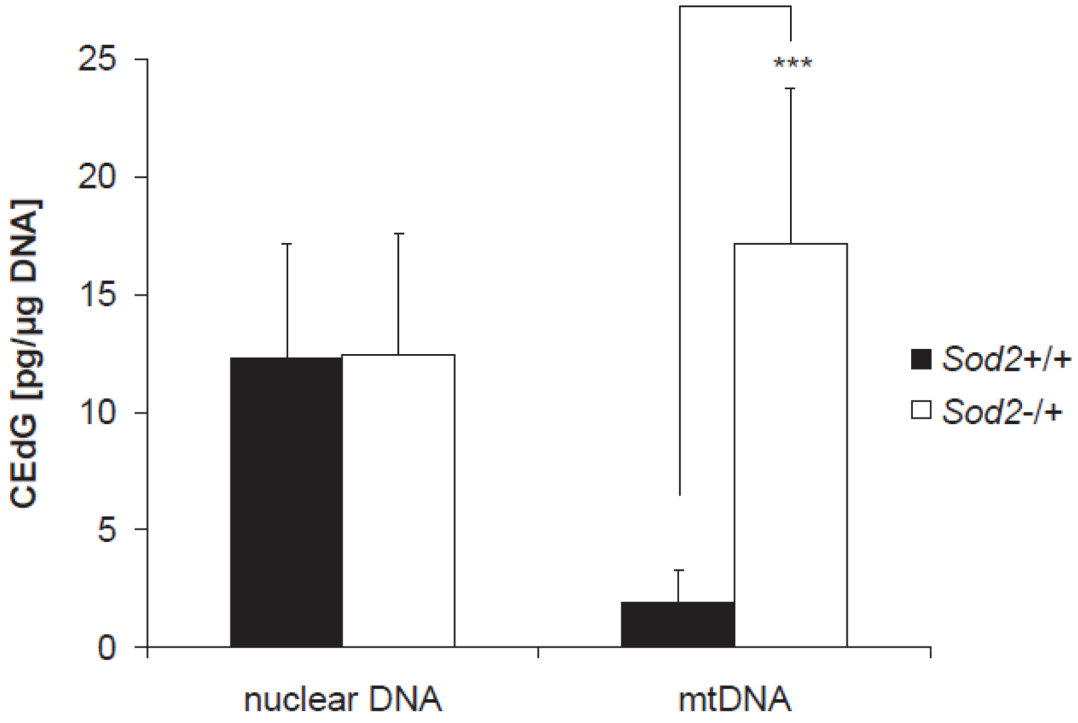

Fig. 3.

CEdG content of nDNA and mtDNA. Nuclear and mtDNA were isolated simultaneously from wildtype and *Sod2*−/+ MEFs. CEdG concentration was determined using an established CEdG ELISA method. Data are presented as mean ± SD. Wildtype cells–nDNA: 12.27±4.90 pg/µg DNA, n=6; mtDNA: 1.90±1.39 pg/µg DNA, n=5; *Sod2*−/+ cells nDNA: 12.47±5.13 pg/µg DNA, n=5; mtDNA: 17.14±6.60 pg/µg DNA, n=5 (*** $p<0.001$)

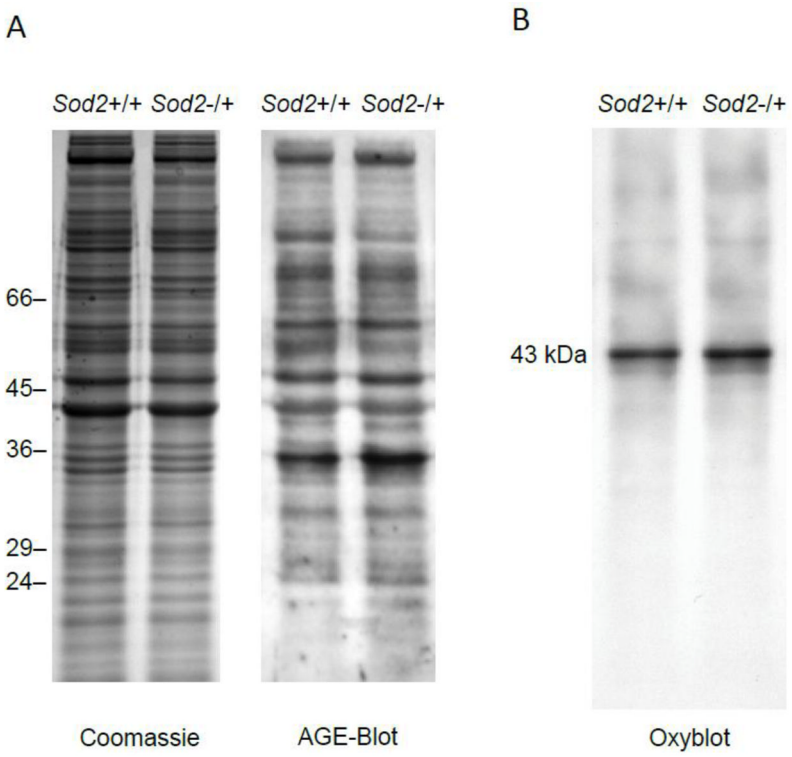

Fig. 4.

Analysis of the cytosolic protein fraction of *Sod2*+/+ and *Sod2*−/+ MEFs. **A)** Protein CML-/CEL analysis. The cytosolic protein fractions were separated on a 12% SDS-PAGE prior to blotting and reaction with a polyclonal CML/CEL-antibody (n=5 each) **B)** Protein carbonyl analysis. The cytosolic protein fraction of *Sod2+/+* and *Sod2*−/+ MEFs was derivatized with DNPH and separated on a 12% SDS-PAGE prior to blotting and reaction with anti-DNPH antibody (n=5 each). Representative Western blot images are shown in A and B.

Table 1

Yield of cytosolic proteins, total nDNA, and mtDNA isolated simultaneously from wildtype and *Sod2*−/+ fibroblasts.

	Protein/Cell [pg]	nDNA/Cell [pg]	mtDNA/Cell [pg]
Sod2+/+	141.11 ± 7.30	4.94 ± 1.91	0.018 ± 0.008
Sod2−/+	145.23 ± 68.26	3.76 ± 1.13	0.034 ± 0.009

Protein yield was measured using the DC protein assay. DNA concentration was determined by absorption at 260 nm. Cells were counted using a Neubauer counting chamber after trypan blue staining. At least three isolation procedures were performed for one data point. Each data point represents the mean value ± standard deviation.