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Original Contribution

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 oxygen species (ROS) is regarded as a major contributor to aging and age-related degenerative diseases. ROS have also been shown to facilitate the formation of certain advanced glycation end-products (AGEs) in proteins and DNA 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. In *Sod2*$^{-/+}$ fibroblasts vs wild-type fibroblasts, the CEdG content of mtDNA was increased from 1.90 ± 1.39 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 *Sod2*$^{-/+}$ cells. Similarly, cytosolic proteins did not show any difference in advanced glycation end-products or protein carbonyl contents between *Sod2*$^{+/+}$ and *Sod2*$^{-/+}$. 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.

The accumulation of somatic mutations in mitochondrial DNA (mtDNA) induced by oxidative stress is regarded as a major contributor to aging and age-related degenerative diseases [1]. Mitochondria are extremely susceptible to oxidative damage, as 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 by-product of the respiratory chain. Two mouse lines lacking MnSOD (*Sod2*) have been constructed to further establish the role of mitochondrial reactive oxygen species (ROS) in the pathophysiology of diseases, especially in age-related disorders [4,5]. Reduced activity of MnSOD can be correlated with 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 life span compared to wild-type 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 the *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 non-enzymatic 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 ROS [11,12]. Similar to the 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, and in the human kidney and aorta [16-20]. Very recently, a method

Abbreviations: AGE, advanced glycation end-product; CEdG, N^2-carboxyethyl-2′-deoxyguanosine; CEL, N^{ε}-(carboxyethyl)lysine; CML, N^{ε}-(carboxymethyl)lysine; DNPH, 2,4-dinitrophenylhydrazine; MEF, mouse embryonic fibroblast; 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; T_d, cell population doubling time; XO, xanthine oxidase.

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was developed to allow simultaneous quantification of AGEs bound to proteins, mtDNA, and nuclear DNA (nDNA). Thereby, significantly higher CEdG levels in mtDNA compared to nDNA were detected in NIH 3 T3 fibroblasts [21].

We hypothesize that mitochondrial ROS contribute to the accumulation of somatic mutations by promoting the formation of mtDNA AGEs. Therefore, this 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 *Sod2*$^{-/+}$ 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, 5000 U/ml) were from Fermentas (St Leon-Rot, Germany), and PBS was from Biochrom (Berlin, Germany).

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

In vitro incubation of DNA under oxidative conditions

Incubation tests were performed with herring sperm DNA (1 mg/ml) in phosphate-buffered saline (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. DNA was also incubated in the presence of pyridoxamine alone. Parallel to the DNA/XO/xanthine mixtures, solutions were tested 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 effect 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 days 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 *Sod2*$^{-/+}$ fetuses by standard protocols as described [22]. Animal procedures were approved by the IACUCs at Stanford University and the VA Palo Alto Health Care System. MEFs were cultured in 50% DMEM/50% F12 medium supplemented with 10% fetal bovine serum, 1% PSA, 1% nonessential amino acids, and 15 mM Hepes in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were expanded to passage 1 (P1) and frozen in liquid nitrogen for long-term storage. Frozen stocks of *Sod2* fibroblasts were thawed as P2 and usually split 1:3 every 4 or 5 days when confluent. All experiments were conducted using MEFs within five passages postisolation. The cell population doubling time (T_d) was monitored for passages 3 and 4. The T_d 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 3 T3 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 the 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 (4×) 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 postmitochondrial supernatant, or buffer M alone was analyzed at a concentration of 10% v/v in the assay buffer as described. The absorption at 600 nm was determined in 5-min 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 activity.

Competitive ELISA for CEdG

Before 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 and 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 micrograms of proteins per lane was loaded onto a 12% acrylamide gel and separated at 150 V for 80 min. After electrophoresis was completed, the gels were stained with Coomassie blue as previously described [21].