SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

8-Oxoguanine DNA glycosylase-1 links DNA repair to cellular responses via the activation of the small GTPases, Ras and Rac1

by

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The examination takes place at Room 3.506, Life Science Building, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, at 11:00 a.m. on 11th September, 2015.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen,
at 1:00 p.m. on 11th September, 2015.

1. Introduction

Oxidative stress is an evolutionary driving force that often defined as an imbalance between the prooxidative and the antioxidative sources. It has been connected to serious health disorders like Parkinson's disease, Alzheimer's disease, cancer, myocardial infarction, chronic fatigue syndrome. On the other hand, organisms wouldn't be able to survive without the controlled production of reactive oxidative molecules. Redox sensitive amino acids like methionine play an important role in cellular signaling. The reversible oxidation of methionine can inhibit the phosphorylation of adjacent Tyr/Ser/Thr site influencing main signaling pathways.

1.1 Oxidative stress

The environment is becoming richer and richer source of prooxidants because of the increasing amount of pollutants, chemicals, ionizing and ultraviolet radiation. These sources act directly or via activation of oxido-reductases and/or induction of mitochondrial dysfunction. When the antioxidant system of the cells cannot balance out the increased concentration of reactive oxygen species (ROS) molecules those indiscriminately modify proteins, lipids, and DNA and disrupt normal cellular signaling processes.

As a part of their normal physiological activity cells produce ROS molecules. Notable cellular sources of ROS are: mitochondrial leakage during oxidative phosphorylation, xanthine oxidase, cytochromes P450 and NADPH oxidases (NOX1-5, DUOX1-2). These enzymes have the ability to transport electrons across the plasma membrane and to generate superoxide and other downstream reactive oxygen species. The most common ROS molecules are: superoxide anion, hydroxyl (OH•), alkoxy (RO•), peroxy-radicals (ROO•), hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$).

The free-radicals can damage lipids via oxidation, which often referred to as lipid peroxidation. Lipid peroxides can participate in chain reactions that increase further damage to biomolecules like proteins. Protein molecules are frequently damaged either at specific side chains or amino acids (i.e. by hydrogen peroxide) or non-specifically throughout the backbone (i.e. by hydroxyl radicals). Most protein damage results loss of

function (enzyme activity, signaling) modified structure (unfolding, aggregation), altered interactions with other molecules and in some cases, they may contribute to multiple human pathologies.

One of the most common reactive oxygen species, the hydroxyl radical (•OH) reacts with DNA by addition to double bonds of DNA bases and by abstraction of an H atom from the methyl group of thymine and each of the C-H bonds of 2′-deoxyribose. In case of purines hydroxyl radical adds to the C4, C5, and C8 positions, generating OH adduct radicals. Depending on their redox properties, the redox environment and the reaction partners, radicals are reduced or oxidized. Product types and yields depend on absence and presence of oxygen and on other conditions. So far more than 20 base lesions have been identified. The consequences of these DNA lesions are diverse: they can cause mutations, conformational changes in DNA, deletions, epigenetic changes among others.

1.1.1 8-Oxo-7,8-dihydroguanine (8-oxoG)

The most susceptible base among the DNA and RNA bases is guanine (Gua), due to its lowest reduction potential (midpoint potential is -1.29 mV vs. nickel hydrogen electrode: NHE). In vivo, Gua in DNA and RNA can be modified not only by •OH but also by other reactive species, including reactive oxygen (superoxide anion: O2•–), nonradical (ozone: O_3; singlet oxygen: $^1O_2$; hydrogen peroxide: H_2O_2), and nitrogen species (nitric oxide: NO•; peroxinitrite: ONOO–), as well as nitrosoperoxycarbonate (ONOOCO2–), carbonate anions (CO_3^-) and the UVA component of solar light. Its presence as a free base in extracellular fluids is one of the most reliable gauges of the oxidative stress load of an organism. Estimates show that under physiological conditions, several hundred 8-oxoG lesions could be formed in DNA per eukaryotic cell daily. 8-oxoG is one of the most abundant DNA lesions formed in oxidative stress conditions, such as those that exist in diseased and aged cells/tissues In mammals, the intra-helical 8-oxoG is recognized by its unique electronic properties and excised by the E. coli Fpg homolog 8-oxoguanine DNA glycosylase 1 (OGG1) from nuclear and mitochondrial genomes during base excision repair (BER) processes. As regards RNA molecules, which are found mostly in single stranded forms without protecting proteins, they are even more prone to oxidative damage. It is estimated that 30-70% of messenger

RNA contains 8-oxoG because of the low redox potential of guanine and the lack of repair systems. As the amount of RNA is approximately four times higher than DNA, and both guanine and 8-oxoG are susceptible to further oxidation, an antioxidant protective role has been hypothesized for the RNA pool.

1.1.2 Defense mechanisms against ROS

Cells have enzymatic and non-enzymatic antioxidants as protection against ROS. Non enzymatic antioxidants are often reducing agents such as glutathione, ubiquinone, tocopherols (vitamin E), thiols (cysteine), ascorbic acid (vitamin C), beta carotene (vitamin A) or polyphenols. Hydrophilic antioxidants react with oxidants in the cell cytosol, while lipophilic antioxidants protect cell membranes from lipid peroxidation. Many of them is synthesized in the cells, others must be acquired from outer sources.

Cells also have interacting network of antioxidant enzymes such as glutathione enzymes (glutathione reductase, glutathione peroxidase and glutathione S-transferase), catalases, superoxide dismutase (SOD) and various peroxidases that protects against oxidative stress by metabolizing oxidative intermediates.

Oxidative stress activates the expression of a battery of defensive genes in order to eliminate ROS and to prevent free radical generation and further damage. The Nrf2 (NF-E2 related factor 2) pathway is regarded as the most important in the cell to protect against oxidative stress. Nrf2 binds to antioxidant responsive elements (ARE) that regulates the basal and inducible expression of antioxidant genes in response to UV light, xenobiotics, antioxidants, heavy metals. ROS molecules can cause DNA damage and start DNA damage response (DDR) networks. These DNA damage sensing and signaling pathways act via cell cycle checkpoints, apoptosis and cellular senescence. DDR networks preserve genome integrity and prevent tumor growth while DNA repair mechanisms help to restore the damaged DNA to its original form.

1.2 DNA repair mechanisms

ROS can generate over a hundred oxidative DNA adducts such as single/double-strand brake, deoxyribose oxidation, DNA-protein cross-links and base modifications. Majority of DNA damage has endogenous origin and one of the most common among them is

spontaneous hydrolysis, because of the N-glycosidic bond between the DNA base and the deoxyribose. The nucleobase loss generates an apurinic/apirimidic site (AP site), which is estimated to occur at a rate of ten thousand per cell per day. Among the ROS generated DNA adducts 8-oxoG is the most extensively studied and generally used as an indicator of DNA damage. Environment serves with numerous forms of damaging agents. UV light may induce atypical covalent bond between adjacent pyrimidine bases. Another external damaging source is ionization radiation, which can be both artificial (X-rays) and natural (gamma radiation). Chemical agents are very potent at damaging DNA. Topoisomerase I or II inhibitors are used for treating cancer (camptothecin, etoposide, respectively) by inducing single or double strand breaks. Depending on the type of damage, organisms developed multiple pathways to correct DNA lesions. There are five major DNA repair mechanism that mammalian cells can utilize: mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), homologous recombination (HR) and non-homologous end joining repair (NHEJ).

1.2.1 Mismatch repair (MMR)

The MMR system recognizes and corrects misincorporated bases, erroneous insertion and deletion made by DNA replication polymerases. Cells lacking MMR have increased number of mutations, organisms with defective MMR genes are characterized by variety of cancers including Lynch syndrome or known also as hereditary non-polyposis colon cancer. The MMR is a strand specific pathway that remained quite conservative from bacteria to primates. The process consists of three main steps: recognition, excision and repair. In the first step mispaired bases are recognized, in the second one the error containing strand is degraded, leaving a gap, and in the third one DNA is synthesized to fill the gap.

1.2.2 Nucleotide excision repair (NER)

NER machinery recognizes the bulky distortions of the double helix. Such DNA distorting lesions are cisplatin-DNA intrastrand crosslinks, pyrimidine dimers and 6-4 photoproducts caused by UV light. The process consists of the same biochemical steps both in prokaryotes and in eukaryotes: damage recognition, verification, dual incisions,

excision, repair synthesis and ligation. While the NER in prokaryotes takes only six proteins, in eukaryotes more than thirty proteins are involved. The process mediated by the sequential assembly of repair proteins and the correct positioning at the site of the DNA lesion. Defects in NER cause severe diseases including xeroderma pigmetosum, Cockayne syndrome and trichothiodystrophy, caused by genetic mutations of NER proteins. All of them characterized by extreme sun sensitivity and predisposition to cancer, neurodegeneration, immunological defects and premature aging. The NER system contain two subpathways: global genome repair (GG-NER) and transcription-coupled repair (TC-NER). The two differ in the damage recognition step and while GG-NER eliminates lesions from the whole genome, TC-NER initiated by the stalling of the RNA polymerase on the coding strand of DNA being transcribed.

1.2.3 Double-Strand Break Repair

DNA double strand breaks (DSBs) are amongst the threats that endanger genome stability and cell viability. They can be generated naturally during programmed genome rearrangement by nucleases, V(D)J recombination and from damaging agents, including ionizing radiation, UV lights and chemicals. Failure to repair them can cause chromosomal aberrations, deletions leading to genomic instability or development of cancer. Organisms use two pathways to repair DBSs: homologous recombination (HR) and non-homologous end-joining (NHEJ). HR pathway utilizes the undamaged sister chromatid as template and restricted to the late S and G2 phases of the cell cycle. NHEJ repair pathway was named "non-homologous" because the break ends are directly ligated without the need for a homologous template. NHEJ considered being an error-prone repair, which operates in all phases of the cell cycle.

1.2.4 Base Excision Repair (BER)

Base excision repair pathway removes small, non-helix distorting lesions from the DNA. The process initiated by DNA glycosylases which excises mismatched (uracil) or damaged bases derived from alkylation (3-methyladenine), deamination (hypoxanthine) or oxidation (8-oxoguanine). There are at least twelve DNA glycosylases with very narrow substrate specificity. They all use a common “flipping” mechanism by which the

damaged base is flipped to an extrahelical position for excision. DNA glycosylases cleave the N-glycosidic bond between the base and its deoxyribose leaving an abasic (AP) site. These AP sites then processed by apurinic/apyrimidic endonuclease 1 (APE1) which hydrolyzes the phosphodiester backbone 5' to the AP site, generating a single-strand break bordered by 3'-OH and 5'-deoxyribose phosphate (5'-dRP) termini. The resulting single-strand break can be further processed by either short patch repair with a single nucleotide replacement or long patch repair where 2-10 nucleotides are replaced.

1.2.5 OGG1, a versatile DNA repair enzyme

OGG1 is the dedicated enzyme to excise the 8-oxoG during the DNA base excision repair (BER) process. OGG1 is a bifunctional glycosylase: it is able to both cleave the glycosidic bond of the mutagenic lesion and the phosphodiester bonds (3' and 5') causing a strand break in the DNA backbone. OGG1-initiated BER encompasses four key steps, including damaged base recognition and excision, 3'deoxyribose phosphate end-processing by AP endonuclease 1 (APE1), filling in the nucleotide gap by DNA polymerase β, and nick-sealing by DNA ligase. OGG1's repair activity is modulated by post-translational modifications, including phosphorylation, acetylation, and by interactions with canonical repair and non-repair proteins. Depending on the last exon sequence of the C-terminal region of the OGG gene there are two major splice variants of OGG: nuclear (type 1 with 3 isoforms) and mitochondrial (type 2 with 5 isoforms). All variants have the N-terminal region in common. In eukaryotes, the N-terminus of this gene contains a mitochondrial targeting signal, essential for mitochondrial localization.

Accumulation of 8-oxoG in DNA has conventionally been associated with various diseases, accelerated telomere shortening, inflammatory and aging processes. In addition, unrepaired 8-oxoG lesion is potentially one of the most mutagenic lesions among oxidatively modified DNA bases, because its pairing with A will cause a GC→AT mutation. Unexpectedly, OGG1 knock out ($OGG1^{-/-}$) mice have an unaltered lifespan, and show only moderate increases in tumor formation, no organ failure despite the supraphysiological levels of 8-oxoG in their DNA. Furthermore, $OGG1^{-/-}$ mice showed an increased tolerance to chronic oxidative stress (induced by $KBrO_3$ treatment),

while 8-oxoG levels in the DNA increased by 250- to 500-fold compared to the wild type. Mabley and co-workers studied the role of OGG1 in inflammatory processes. They used three models of inflammation: endotoxic shock, diabetes, and contact hypersensitivity. OGG1 knockout mice were extremely resistant to most of the lipopolysaccharide-induced effects: LPS-induced organ dysfunction, neutrophil infiltration and oxidative stress, when compared to wild-type ($OGG1^{+/+}$) controls. In case of multiple low-dose streptozotocin-induced type I diabetes, $OGG1^{-/-}$ mice were found to have significantly lower blood glucose and higher insulin levels followed by fewer incidence of diabetes as compared with wild type mice. In their allergy model of oxazolone-induced contact hypersensitivity, results showed reduced neutrophil accumulation, chemokine (MIP-1, MIP-2), Th1 (IL-1, TNF-α) and Th2 cytokine levels (IL-4) in the ear tissue of $OGG1^{-/-}$ mice. It has been hypothesized that DNA-dependent kinases recognize the single strand gaps made by OGG1 and trigger inflammation. In this point of view, it looks more advantageous to down-regulate OGG1 and leave the 8-oxoG in the DNA. This hypothesis also can explain why $OGG1^{-/-}$ mice with significantly fewer DNA nicks are less prone to inflammation.

1.3 Small GTPases

Small GTPases are a type of G-proteins found in the cytosol that are homologous to the alpha subunit of heterotrimeric G-proteins (large GTPases). They can hydrolyze guanosine triphosphate (GTP) to form guanosine diphosphate (GDP). The GDP-bound form is their inactive while the GTP-bound form is their active state in which they can activate downstream pathways by binding to effectors. Small GTPases work like molecular switches with the help of guanine nucleotide exchange factors (GEFs) that facilitate GDP dissociation and of GTPase activating proteins (GAPs) that stimulate GTP hydrolysis.

1.3.1 Ras

The name “Ras”is an abbreviation of “Rat sarcoma” referring the way the first members of the protein family were discovered. Ras protein family members belong to small GTPases, as they can bind and hydrolyze GTP. Ras is encoded by the ras gene and the prototypical member of the Ras superfamily of proteins, which are all related in 3D structure. They regulate diverse cell behaviors and are involved in transmitting signals within cells.

Activation of Ras GTPases involves the displacement of GDP with GTP, a process mediated by guanine nucleotide-releasing/exchange factors (GEF). GEFs first interact with GTPase and dissociate GDP at an increased rate, and then the bound GTP promotes the release of exchange factor leaving the GTPase in an active form. Well known GEFs are Son of Sevenless (Sos) and cdc25. Ras-GTP binds to the RBD domain of the Raf1 serine/threonine kinase, and its subsequent phosphorylation is essential, but not sufficient, for mediating Raf1’s mitogen-activated protein kinase (MAPK) activity, as phosphorylated Raf1 requires additional protein-protein and -membrane lipid interactions. The MAPK cascade transmits signals downstream and results in the transcription of genes involved in cell growth and division. While RasGEFs catalyze a "push and pull" reaction which releases GDP from Ras, RasGAPs enhance the catalytic machinery of Ras, since the protein intrinsic GTPase activity is very slow. Thus, GAPs accelerate Ras inactivation. Because intracellular concentration of GTP is approximately 10 fold more relative to GDP, GTP predominantly re-enters the nucleotide binding pocket of Ras. The balance between GEF and GAP activity determines the guanine nucleotide status of Ras, thereby regulating Ras activity. Another protein may augment the activity of Ras is GDI (GDP Disassociation Inhibitor). This functions by slowing the exchange of GDP for GTP and thus, prolonging the inactive state of Ras. Ras-regulated signal pathways control cell growth, migration, differentiation, actin cytoskeletal integrity, cell adhesion, apoptosis, proliferation and survival.

The clinically most notable members of the Ras subfamily are H-RAS, K-RAS and N-RAS, mainly for being implicated in many types of cancer. Ras and ras-related proteins are often deregulated in cancers, leading to increased invasion and metastasis, and decreased apoptosis. Mutations in ras genes can result the production of continuously

active Ras proteins. As a result, the signaling pathway(s) remain “switched-on” and the overactive signaling can lead to uncontrolled cell growth and cancer. Ras mutations are found in 20-25% of all human tumors and up to 90% in certain types of cancer (e.g. pancreatic cancer).

1.3.2 Rac1

Rac1, also known as Ras-related C3 botulinum toxin substrate 1 is a protein ubiquitously expressed and involved in signal pathways that regulate mobility and other processes related to membrane trafficking and cell morphology. It is encoded by the rac1. The Rac protein belongs to the Rho GTPase family. The classical members of the four subfamilies of Rho are: Rac, Cdc42, Rho and Rif. Similarly to other GTPases, the classical Rho GTPases cycle between active GTP-bound and inactive GDP-bound forms. Their cycle is also controlled by three types of regulatory proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Rac subfamily has three Rac isoforms: Rac1, Rac2 and Rac3. Rac1 protein is widely expressed in different tissues, whereas as Rac2 expression is restricted to cells of hematopoietic origin. Rac3 is predominantly found in the central nervous system. Rac1 is a well characterized member of the Rac subfamily. Previous studies found that Rac1 regulates a diverse array of cellular events, including the formation of lamellipodia and membrane ruffles, cell cycle, cell adhesion and mobility. In addition, Rac1 regulates endocytic and exocytic trafficking pathways. Rac1 has a characterized role in clathrin-dependent endocytosis. Other physiological functions of the Rac1 GTPase includes: modulation of the cellular redox state, regulation of cell movements, cellular signaling, gene expression, and cell differentiation. In most types of cells, Rac is connected to the production of low levels of ROS which have an implicated role in growth, differentiation, migration, and angiogenesis, as well as in inflammation. Rac1 is ubiquitously expressed in nonphagocytic cells, including lung epithelial cells and fibroblasts. Rac1 is one of the three Rac family molecules that control NADPH oxidase (NOX) activity (NOX1, NOX2, and NOX4 as well as NOX3) both in phagocytes and in non-phagocytic cells.

1.4 DNA repair-independent functions of OGG1

Previous studies have implied roles for OGG1 in multiple cellular processes in addition to that of being a canonical DNA BER protein. It has been shown that OGG1 colocalizes with centrioles (microtubule organizing center), microtubule networks, and mitotic chromosomes implicating that OGG1 takes part in chromatin remodeling and transcriptional initiation. OGG1 was reported to act as a chaperon for aconitase and to prevent mitochondrial dysfunction and apoptosis by interacting with it. OGG1 was found to play a role in innate immunity by enhancing Cxcl2 (a chemokine produced by monocytes and macrophages) expression after TNF-α treatment. OGG1 facilitated the recruitment of transcription initiation factor II-D, NF-κB/RelA, Sp1 (specificity protein 1) and p-RNA pol II (p-RNA polymerase II) by binding to the Cxcl2 promoter region.

As described above, $OGG1^{-/-}$ mice have increased resistance to inflammation. This observation raises the possibility that it may not be the genomic level of 8-oxoG but the free 8-oxoG generated by BER that provides the linkage to disease/aging processes. While several aspects of the involvement of OGG1 in DNA repair-independent cellular functions have already been revealed, the role of OGG1 and free 8-oxoG in the activation of canonical small GTPase-mediated pathways has not been investigated so far.

2. Objectives of the study

Previous studies on OGG1−/− mice suggest that a lack of OGG1 activity is accompanied by dysfunction of signaling pathway(s) linking oxidative stress to cellular responses. These observations raise the possibility that the 8-oxoG base released from the genome by OGG1 could have physiological/pathophysiological relevance. The aim of this study was to reveal whether 8-oxoG and/or OGG1 are able to activate small GTPase-related signaling pathways.

Our hypotheses were the following:

- 8-oxoG has a biological role after being excised and not just a neutral byproduct of DNA repair,
- both exogenously added and endogenously excised 8-oxoG induces cellular responses,
- free 8-oxoG base can bind to OGG1,
- 8-oxoG/OGG1 complex can activate small GTPases, Ras and Rac1,
- 8-oxoG/OGG1 complex can induce ROS production via NADPH oxidases by activating Rac1.

3. Materials and methods

3.1 Cell cultures

Human diploid fibroblast (MRC-5) and human cervix carcinoma (HeLa-S) cells were maintained in Earle's minimum essential and Dulbecco's modified Eagle's low glucose medium, respectively. A549 type II alveolar epithelial cells (ATCC # CCL-185) were cultured in Ham's F12 (GIBCO-BRL), U937, a human monocytic cell line, were grown in and RPMI-1640. The human myelomonocytic KG-1 (ATCC# CCL-246) cells were grown in Iscove's Modified Dulbecco's Medium. All media were supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin; cells were grown at 37 °C in a 5% CO_2. KG1 cells were stimulated with 10 ng/ml PMA (Sigma-Aldrich, Steinheim, Germany) and 100 ng/ml ionomycin (Sigma-Aldrich) for 4 days.

Monocyte-derived DCs were developed from isolated Buffy Coat by Ficoll-Pacque (Amersham Biosciences, Uppsala, Sweden) gradient centrifugation and monocytes were isolated by magnetic cell separation using positive selection with anti-CD14-coated beads (Miltenyi Biotech, Bergish Gladbach, Germany). Purified monocytes were plated at 2×10^6 cells/ml concentration and cultured in serum-free AIMV medium (Gibco) in the presence of 100 ng/ml IL-4 and 75 ng/ml GM-CSF (Peprotech EC, London, UK) given on days 0 and 2. Cells were differentiated for 5 days. Activation of immature DC was induced by an inflammatory cocktail containing 10 ng/ml TNF-α, 5 ng/ml IL-1β, 20 ng/ml IL-6, 75 ng/ml GM-CSF and 1 μg/ml PGE2 (Sigma-Aldrich).

3.2 Animals and treatments

Animal experiments were performed according to the National Institutes of Health Guidelines for Use of Experimental Animals and approved by the University of Texas Animal Care and Use Committee (Protocol number: 0807044A). Eight-week-old female BALB/c mice (The Jackson Laboratory) were challenged intranasally with 60 μl of 8-oxoG (1 μM) in saline (or with control saline) under mild anesthesia. The animals were sacrificed after 15 min, their lungs homogenized in lysis buffer then extracts were prepared for measuring the Rac1/Ras levels.

3.3 Assessment of GTP-bound Ras and Rac levels

Ras- and Rac-GTP levels were quantified with the Active Ras/Rac pull-down and detection kit (Pierce, Thermo Scientific Inc. Waltham, MA) per the manufacturer's instructions with slight modifications. The activated Ras/Rac1 was eluted with Laemmli buffer and quantified by Western blotting and densitometry.

3.4 Protein interaction assays

The interaction of OGG1 with H-, N-, or K-Ras was analyzed on nickel- nitrilotriacetic acid (Ni-NTA)-agarose beads (Qiagen Inc., Valencia, Ca) in interaction buffer and incubated for 30 min at 4 °C. After three washes in interaction buffer, untagged OGG1 ± 8-oxoG was added in the presence or absence of GTP or GDP. The samples were incubated for 30 min at 4 °C and washed twice with interaction buffer, and the proteins eluted with Laemmli buffer were analyzed by Western blotting.

Interactions between OGG1 and Rac1, were determined by enzyme-linked immunosorbent assays (ELISA). Briefly, Rac1 antibody-coated wells were washed with PBS-T, and then guanine nucleotide free (empty) Rac1, GDP-, or GTP-loaded Rac1 protein was added to parallel wells in PBS-T alone or together with OGG1 and 8-oxoG for 1 h at room temperature. Unbound proteins were removed by washing before incubation with anti-OGG1 Ab. HRP-conjugated secondary Ab was added and color was developed using TMB substrate. Absorbance was determined on a Microplate Reader.

3.5 Western blot analysis

Extracts from lung or cell lysates were clarified by centrifugation, and the supernatants were collected. Protein samples were mixed with sample loading buffer, heated for 5 min at 95°C, and separated by 5-20% SDS-PAGE. Proteins were transferred to Hybond-ECL nitrocellulose membrane by electroblotting. The membranes were then blocked with 3% BSA in TBS containing 0.1% Tween (TBS-T) for 3 h and incubated overnight at 4°C with the primary antibody diluted in 3% BSA in TBS-T. The blots were then washed and incubated for 1 h with HRP-conjugated secondary Ab in 5% non-fat dry

milk in TBS-T. After washing, immunoreactive bands on membranes were visualized by chemiluminescence using an ECL substrate.

3.6 Preparation of 8-oxoG solution

8-OxoG is provided as a hydroacetate salt, and was dissolved in 12 mM NaOH (4 mM final concentration). An 8-OxoG stock solution was prepared freshly, diluted in PBS (w/o Ca^{2+}/Mg^{2+}, pH: 7.4), and used within 1 h for experiments. All nucleotide bases and nucleosides (2'-deoxyguanosine, guanine, adenine, guanosine, 8-oxodG, 8-OH-ade and FapyG were solubilized in the same manner.

3.7 Fluorescence spectroscopy

The binding of 8-oxoG to OGG1 was assessed by monitoring the decrease in intrinsic tryptophan fluorescence. Briefly, 0.5 µM OGG1 (100 µl) was incubated with increasing concentrations of 8-oxoG base (or 8-oxodG or FapyG as controls; 0-2.0 µM) for 10 min at 24 °C in Tris-HCl containing 1 mM DTT. The tryptophan fluorescence (λ_{em} = 290–400 nm; λ_{ex} = 280 nm) was analyzed in a SPEX FluoroMax spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ). The binding constant K_d was calculated by plotting ΔF (change in fluorescence emission maximum, 336 nm) versus ligand concentration according to the equation $\Delta F = \Delta F_{max}$ [ligand]/K_d = [ligand].

3.8 Guanine nucleotide exchange assay

Nucleotide-free H-Ras was loaded with an equimolar amount of GDP or GTP in a buffer containing 20 mM Tris (pH 7.5) with 50 µg of bovine serum albumin. Guanine nucleotide exchange assays were initiated by the addition of OGG1±8-oxoG in the presence of a 10-fold excess of GTPγS or GDP. The molecular ratio of Ras and OGG1±8-oxoG was 1:1 or 10:1. After 0, 0.5, 1, 2, 4, 8, 16, or 32 min. Ras-GTP levels were determined using Active Ras pull-down assays. Changes in Ras levels were analyzed by Western blotting.

The GDP-GTP and GTP-GDP exchange on Rac1 were determined by real-time fluorescence spectroscopic analysis. Rac1 was loaded with the nucleotide ^{Mant}GTP or ^{Mant}GDP in exchange buffer containing 20 mM Tris with 50 µg of bovine serum albumin

for 30 min. In the case of GDP-GTP exchange, Rac-1-^{Mant}GDP and OGG1 protein + 8-oxoG base were mixed with untagged GTP. A similar strategy was used to monitor GTP-GDP exchange. Kinetic changes in the fluorescence of Rac1-^{Mant}GDP or Rac1-^{Mant}GTP were determined using a POLARstar Omega reader (BMG: Bio Medical Gurrat; LABTECH). Curves were fitted using MS Excel. The half-life of Rac1-^{Mant}GDP was determined using POLARstar Omega software.

3.9 Gene expression and molecular network analysis

MRC-5 cells were treated with 10 μM 8-oxoG or mock solution and harvested at various time points. RNA was isolated, and after synthesis of double-stranded cDNA and biotin-labeled cRNA, the cRNA was hybridized to Affymetrix GeneChip® Human Genome Focus Arrays. The initial data were produced by Affymetrix Microarray Suite software and further processed for network, pathway, and functional analyses using the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems).

3.10 Flow cytometry

Expression level of cell surface markers was measured after direct or indirect immunofluorescence labeling using FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Ten-thousand cells were counted and analyzed by the CellQuest program. Subcellular particles were gated out on the basis of forward and side scatter, the list-mode data were analysed by the WinMDI software.

3.11 Latex bead uptake

Unstimulated and activated cells (5×10^5/ml) were incubated with $5x10^6$/ml carboxylate modified latex beads of 1 μm diameter (Sigma-Aldrich) for 48 h at 37 ºC, washed three times with staining buffer and analyzed by flow cytometry. Control cells were incubated at 4 ºC under the same conditions.

3.12 Lucifer Yellow uptake

Unstimulated and activated cells (1×10^6/ml) were incubated with 250 μg/ml Lucifer Yellow (Sigma-Aldrich) at 37 ºC for 1 h, washed three times with the staining buffer

and analyzed by flow cytometry. Control cells were incubated at 4 ºC under the same conditions.

3.13 FITC-dextran uptake

FITC-dextran uptake was measured as described previously. Briefly, 1 mg/ml FITC-dextran (Sigma-Aldrich) was added to 1×10^6 unstimulated or activated cells and incubated for 1 h. Cells were washed three times with the staining buffer and analyzed by flow cytometry. Control cells were incubated at 4 ºC under the same conditions.

3.14 Chemotaxis assay

Cell migration was assessed in a multiwell microchemotaxis chamber (Neuroprobe, Gaithersburg, MD, USA). 1×10^6 cells were resuspended in 1 ml RPMI 1640 medium supplemented with 0.5% BSA. 3.5×10^5 cells in 350 µl RPMI medium were placed into the upper well chambers, which were separated by a filter of 5 µm pore size from the lower wells containing 430 µl medium containing various concentrations of the recombinant chemokine MIP-3β (Peprotech) or medium alone. Cells were allowed to migrate for 3 h, 4 h and 5 h at 37 ºC in a CO_2 incubator. Non-migrating cells were removed by washing the filter with PBS containing 2 mM EDTA. Cells, which migrated to the lower wells were centrifuged and resuspended in 50 µl medium. The amount of migratory cells was assessed by MTT assay.

3.15 Down-regulation of gene expression

Cells were transfected with control siRNA (siGENOME nontargeting siRNA) or target-specific siRNA: H-Ras, K-Ras, N-Ras, OGG1, Rac1 and NOX4 siRNAs using INTERFERin™ transfection reagent per the manufacturer's instructions. Briefly, siRNAs (20 nM final concentrations, as determined in preliminary studies) were mixed with INTERFERin™ transfection reagent and added to cells.

After 3 h incubation in serum-free medium, growth medium was added for 72 h. p22phox siRNA and a second control were purchased from Santa Cruz Biotechnology, Inc. OGG1 was depleted via a simultaneous siRNA transfection and plating method.

Depletion of the target genes' mRNA levels was determined by qRT-PCR and Western blot analysis.

3.16 Quantitative real-time PCR

qRT-PCR was done by the SYBRGreen method using an ABI 7000 System equipment and software per the manufacturer's recommended protocol. A dissociation stage was added at the end of the run to verify the primers' specificity. Expression levels (fold change) were determined by the delta-delta Ct method (ΔΔCt).

3.17 Oligonucleotide incision assay.

OGG1' base excision repair activity in nuclear lysates was determined by using a ^{32}P-labeled 31-mer oligonucleotide substrate containing one 8-oxoG. The cleaved product was separated from the intact substrate in a 20% polyacrylamide gel containing 8 M urea in Trisborate-EDTA buffer, pH 8.4. Radioactivity in the separated DNA bands was visualized by using a Storm 860 PhosphorImager and quantified by densitometry using ImageQuant software.

3.18 Assessment of cellular ROS levels

Changes in intracellular ROS levels were determined by using the fluorogenic probe 2'-7'-dihydro-dichlorofluorescein diacetate (H_2DCF-DA). Briefly, cells were grown to 70% confluence and loaded with 50 μM H_2DCF-DA at 37 °C for 30 minutes. Cells were then washed twice with PBS and exposed to nucleic acid bases, nucleosides, or solvent. Changes in DCF fluorescence were recorded in an FLx800 microplate reader at 485 nm excitation and 528 nm emission.

3.19 Microscopic imaging

Cells were transfected with pHyPer-Cyto, pHyPer-dMito, or pHyPer-Nuc and 72 h later challenged with 8-oxoG (10 μM) or H_2O_2 (10 μM). At times indicated, cells were washed in PBS, fixed with formalin (3.7%), dried, and mounted on microscope slides. Images were taken by a NIKON Eclipse Ti System (magnification: x125).

To visualize colocalization, cells were cultured on microscope coverslips then mock-treated or pulsed for 30 min with 10 μM 8-oxoG base and fixed in 4% paraformaldehyde at 4 ºC and then permeabilized with Triton X100 at 37 ºC. The cells were then incubated for overnight at 4 °C with primary antibody to OGG1, Rac1 and NOX4 antibody. After washing cells were incubated for 1 h at RT with Alexa 488-, Alexa-594 and/or Texas Red-conjugated secondary antibodies. Microscopy was performed on a NIKON Eclipse Ti System. Magnification: x125. Co-localization was visualized by superimposition of green and red images using Nikon NIS Elements.

Co-localizations (overlap coefficient) of proteins were calculated according to Manders. $R = \Sigma S1 \times S2 / \sqrt{\Sigma(S1)^2 \times \Sigma(S2_1)^2}$ where S1 represents the signal intensity of pixels in channel 1 and S2 represents signal intensity of pixels in channel 2. The overlap coefficients k_1 and k_2 split the value of co-localization into a pair of separate parameters: $k_1 = \Sigma S1 \times S2 / \Sigma(S1)^2$; $k_2 = \Sigma S1 \times S2 / \Sigma(S2)^2$, where S1 represents the signal intensity of pixels in channel 1 and S2 represents signal intensity of pixels in channel 2.

3.20 Statistical analysis

The data are expressed as the mean ± SD. Results were analyzed for significant differences using ANOVA procedures and Student's t-tests (Sigma Plot 11.0). Differences were considered significant at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****p< or =0.0001).

4. Results and Discussion

4.1 Signaling pathways induced at the transcriptomal level by 8-oxoG

The free 8-oxoG base is generated exclusively during the repair of 8-oxoG in DNA, initiated by OGG1. To mimic a transient increase in its intracellular level, we added 8-oxoG base to OGG1-proficient cells (MRC-5) and analyzed the impact on the transcriptome using Affymetrix GeneChip. Ingenuity Pathways Analysis (IPA) of microarray gene expression data showed that 8 of the top 10 pathways that responded to 8-oxoG involved the small G protein Ras.

4.2 Ras activation in cell culture and lungs

To confirm Ras activation, we showed that the addition of 8-oxoG increased GTP-bound Ras levels in a time- and dose-dependent manner. The time course of Ras activation was in line with rapid cellular uptake of 8-oxoG base. At 1 min after the addition, ~70% of 8-oxoG was taken up by the cells, as shown by LC/IDMS analysis.

The lowest dose of 8-oxoG base that increased Ras-GTP to a detectable level was 100 nM in MRC-5 cells with no concentration-dependent increase in Ras-GTP levels above 10 μM. Free 8-oxoG was unique in increasing Ras-GTP levels as neither 8-oxodG nor other oxidized bases (FapyG or 8-oxoA) nor the original guanine base displayed this activity. To test the in vivo relevance of these findings we challenged mice intranasally with 8-oxoG and found that exposure of mice to 8-oxoG markedly increased Ras-GTP levels in the lungs.

4.3 Ras is not activated when OGG1 silenced

As 8-oxoG is recognized and excised by OGG1 in the genome, we hypothesized that 8-oxoG bound to OGG1 mediates Ras activation. To prove our hypothesis we down-regulated OGG1 in MRC-5 and HeLaS cells and measured the Ras-GTPase levels after 8-oxoG treatment. OGG1-depleted MRC-5 or HeLa-S cells showed reduced OGG1 RNA levels (20% compared to control cells) and adding 8-oxoG did not cause an increase in Ras-GTP levels.

4.4 Ras activation by excised 8-oxoG: the KG-1 model

To investigate the effects of intracellularly generated 8-oxoG on Ras activation we used KG-1 cell line. The KG-1 is a human acute myelogenous leukemia cell line that represents an early stage of hematopoietic differentiation. The cell line composed predominantly of myeloblasts and promyelocytes. KG-1 can spontaneously differentiate to granulocyte and macrophage like cells and can be induced to show DC morphology and phenotype by using a defined cytokine cocktail. Functionally, KG-1-derived DCs were able to induce allogenic T-cell response and phagocyte latex beads.

We previously described some important functional features of KG-1 cells compared to immature dendritic cells (DCs) of monocytic origin. We found that the efficiency of Lucifer Yellow uptake by pinocytosis is similar in monocyte-derived DC and in KG-1 cells. Phagocytosis of latex beads, however, was less efficient by KG-1 cells than by immature DC. Both immature DC and KG-1 cells internalized FITC-dextran and expressed the mannose receptor (MR). The difference in the efficacy of receptor-mediated internalization correlated with MR expression, which was higher in monocyte-derived immature DC than in KG-1 cells. Our results show that unstimulated as well as activated KG-1 cells express high levels of the chemokine receptor CCR7 but in contrast to monocyte-derived DCs, KG-1 cells were not able to migrate to the direction of chemokine (MIP-3β) attraction. Our observations altogether suggest that KG-1 cells resemble professional myeloid antigen presenting cells but also possess special phenotypic and functional characteristics.

The unique feature that makes KG-1 cell line a suitable model for study the 8-oxoG-mediated Ras activation is the expression of a thermolabile OGG1 mutant ($OGG1^{R229Q}$). At physiological temperature (37 ºC), these cells accumulate supraphysiological levels of 8-oxoG in their genome due to the lack of $OGG1^{R229Q}$'s enzymatic activity, but at a lower temperature (e.g., 25 ºC) OGG1 regain its activity. Intriguingly, the viability of KG-1 cells is similar to that of cells expressing wild-type OGG1. Importantly, the KG-1 cell model allowed us to examine the consequences of 8-oxoG repair without exposing cells to ROS.

First we confirmed that the catalytic activity of OGG1 in KG-1 cells is defective under our culture conditions and could be re-established at 25 ºC. The control U937 cells

expressing wild-type OGG1 showed nearly identical 8-oxoG excision activity at both 25 ºC and 37 ºC. To determine Ras-GTP KG-1 cells were transferred from 37 ºC to 25 ºC for 45 min or 90 min and control cells were kept at 37 ºC. The results show that at 25 ºC, GTP-bound Ras levels were elevated at 45 min and further increased at 90 min while at 37 ºC, there was no change. U937 cells showed no change in active Ras levels at 25 ºC. Incubation of KG-1 and U937 cells at 25 ºC resulted in no alteration in total Ras levels. These data support the idea that OGG1-initiated BER is associated with activation of Ras GTPase as we proposed previously.

GEFs' and GAPs' activities can be affected by temperature change, so they could influence the relative levels of Ras-GTP and Ras-GDP in KG-1 cells. To exclude this possibility, OGG1 expression in KG-1 cells was downregulated using siRNA, and the cells then incubated at 25 ºC for 90 min. OGG1-depleted cells showed no increase in Ras-GTP levels. In contrast, Ras-GTP levels were increased in cells transfected with control siRNA.

4.5 OGG1 binds free 8-oxoG at an independent site and not in the DNA lesion-recognition site

In many cases, the binding of the ligand to the protein changes its intrinsic tryptophan (or tyrosine) fluorescence. If the ligand is close enough to the tryptophan(s) or if a conformational change is associated with ligand binding and this brings a quenching group into the vicinity of the fluorophore, then the intensity decreases and the K_d value of protein-ligand binding can be determined. A concentration-dependent decrease in Trp fluorescence indicated the OGG1 conformational change as a result of the interaction.

The binding constant (K_d) 0.56 ± 0.19 nM calculated from the binding isotherms indicates its high affinity for 8-oxoG. In controls, OGG1 did not bind either 8-oxodG or free FapyG base an equally good OGG1 substrate. Thus, the 8-oxoG base is unique in its strong affinity for OGG1.

We observed that 8-oxoG base stimulated OGG1's activity in a concentration-dependent fashion, which indicates that the 8-oxoG base serves as a cofactor to OGG1. This observation also implies that the 8-oxoG base binds tightly at an independent site of OGG1 and not in the DNA lesion-recognition site.

4.6 OGG1/8-oxoG complex functions as a GEF on Ras

We explored a possible interaction between OGG1 and Ras and observed that in the presence of 8-oxoG, OGG1 was able to bind to H-Ras.

However, OGG1 alone did not interact with H-Ras under identical conditions, suggesting that an 8-oxoG-induced conformational change in OGG1 allows its binding to Ras. GTP was more effective than GDP in inhibiting this interaction. These data suggest that the conformation of nucleotide-free Ras allows the most stable interaction with OGG1/8-oxoG, which is consistent with observations showing high affinity binding between nucleotide-free Ras and its GEF.

In the presence of 8-oxoG, OGG1 caused replacement of GDP-bound to Ras with GTP at equimolar or higher molar ratios of H-Ras:OGG1. We subsequently showed that OGG1 also catalyzed the release of H-Ras-bound GTP replacement with GDP.

OGG1 or 8-oxoG alone did not induce guanine nucleotide exchange. We measured similar kinetics between GDP → GTP and GTP → GDP exchange on Ras, suggesting that OGG1 indiscriminately releases the nucleotide in vitro and allows rebinding; thus its activity is similar to that of other Ras-GEFs.

4.7 Ras activation induced by 8-oxoG leads to phosphorylation of MAPKs

Ras-GTP binds to the Ras-binding domain (RBD) of the Raf1 serine/threonine kinase for mediating its mitogen-activated protein kinase (MAPK) activity. Increasing the cellular 8-oxoG level in MRC-5 cells induced rapid phosphorylation of the MAPK kinase (MEK1/2) and extracellular signal-regulated kinase (ERK1/2) and the nuclear translocation of the latter. The MEK1/2 inhibitor PD98059 decreased phosphorylation and the nuclear translocation of ERK1/2 after treatment of cells either with 8-oxoG base or PDGF.

To confirm that ERK1/2 phosphorylation is Ras-dependent in 8-oxoG-exposed cells, we depleted the canonical Ras family members H-, K- and N-Ras with siRNA. The phosphorylation of ERK1/2 decreased after 8-oxoG addition, primarily in N-Ras-ablated MRC-5 cells.

These results so far suggest a complex signaling network activated by OGG1, and triggered by the generation and repair of 8-oxoG in the genome.

4.8 Treatment with 8-oxoG also increases cellular ROS levels

Downregulation of OGG1 decreases the house dust mite (HDM) extract-induced ROS in MLE-12 cells. It is proposed that expression of OGG1 affects the levels of oxidative stress during asthmatic conditions. Based on these previous observations we also tested whether upon addition of free 8-oxoG base to the cells it increases cellular ROS levels via an OGG1 dependent mechanism. We found that the treatment of MRC-5 or A549 cells with 8-oxoG elevated the intracellular ROS levels. Pretreating the cells with N-acetyl-L-cysteine, a precursor of glutathione biosynthesis and a scavenger of oxygen radicals, prevented the 8-oxoG-mediated increase in intracellular ROS levels. Furthermore, 8-oxoG induced significantly lower levels of ROS in OGG1-depleted cells. The 8-oxoG base is unique in increasing ROS levels, because the guanine base, FapyG, 8-OH-Ade, adenine, and guanine do not have this activity.

The kinetics of intracellular ROS increase induced by 8-oxoG indicate that an indirect mechanism is responsible for the observed phenomenon and the involvement of regulator molecules.

4.9 8-OxoG induces ROS via activating NADPH oxidase

We hypothesized that the increase in cellular ROS levels by 8-oxoG base is due to the activation of cellular oxido-reductases, which may require small GTPase(s). One particular protein that has a direct role in ROS production is Rac1, a multifunctional GTPase, that is involved in the modulation of cellular redox state via regulating NADPH oxidase(s) activities. To prove our hypothesis we pretreated cells with DPI, a widely used inhibitor of NADPH oxidases, prior exposure to 8-oxoG. The pretreatment decreased cellular ROS levels by 62±3 and 70±7% in MRC-5 and A549 cells, respectively. We used siRNA-mediated depletion of p22phox, Rac1, and NOX4, expressed in both MRC-5 and A549 cells to get further evidence of the association of NADPH oxidase activity with cellular ROS increase.

The siRNA mediated depletion of p22phox lowered ROS levels by 64±8% (MRC-5 cells) and 69±6% (A549 cells), NOX4 specific siRNA decreased it by 67±9.5% for MRC-5, and 76±4% for A549, while depletion of Rac1 decreased ROS levels by 72±3% in MRC-5 and 68±7% in A549 cells after 8-oxoG exposure. These data show the significance of Rac1 in the 8-oxoG mediated ROS production via NADPH oxidase so next we investigated the role of OGG1 in the activation of this small GTPase.

4.10 Rac1 activation in cultured cells

First we explored whether exposure of the cells to the 8-oxoG base changes cellular Rac1-GTP levels. At time 0, the percentage of GTP-bound Rac1 was 0.78 ± 0.2 and 0.74 ± 0.1% in MRC-5 and A459 cells, respectively, whereas after a 5-min exposure to 8-oxoG, the percentage of GTP-bound Rac1 levels increased to 6.5 ± 2% (MRC-5) and 7.05 ± 1.7% (A459) cells. These data indicate that the kinetics of Rac1 activation in these cells was faster than that we observed in case of Ras-GTPase.

To confirm that the 8-oxoG-induced increase in Rac1-GTP levels required OGG1, was confirmed by siRNA in both MRC-5 and A549 cells. These results indicate that both Ras and Rac1proteins require the presence of OGG1 in order to get activated by free 8-oxoG.

4.11 Rac1 activation in lung

Next, we examined whether 8-oxoG base increases Rac1-GTP levels in a tissue environment. The 8-oxoG base was instilled into the lungs of mice, and Rac1-GTP levels were determined. Compared to saline-challenged lung there was a robust increase in Rac1-GTP levels at 15 min post-challenge, consistent with our cell culture studies. According to our measurements, Rac1 is abundantly expressed in lung tissues, while Rac2 and Rac3 expression was nearly undetectable. These results are in line with those generated with normal diploid lung fibroblasts (MRC-5) and A549 cells (type II alveolar lung epithelial cells). Rac1 has various roles in lung tissues. One of them is taking part in the reorganization of actin cytoskeleton, which leads to endothelial barrier recovery and elimination of interendothelial gaps. Lung endothelial cells (EC) have all components of NOXs and are major contributors of ROS related lung inflammation.

4.12 Changes in ROS levels begin at perinuclear membrane

NOX type 4 (NOX4) have been reported to be localized to cytoplasmic compartments and nuclear membranes, and are involved in multiple cellular processes, including localized redox modulation, and cellular signaling. To determine the cellular localization of NOX4 that generate ROS in response to 8-oxoG exposure, cells were transfected with the biosensor pHy-Pers, expressed in cytoplasm (pHyPer-Cyto) or targeted to nucleus (pHyPer-Nuc), and mitochondria (pHyPer-Mito). The control, H_2O_2-induced pHyPer signal was localized to cytoplasm, mitochondria, and nuclei of cells. When cells were exposed to 8-oxoG pHyPer-Nuc fluorescence appeared first at the perinuclear region (at 10-12 min) and then a nuclear fluorescence was observed (~20 min). pHyPer-Nuc signal decreased to the basal level by 60 min. pHyPer-Cyto-mediated fluorescence was initiated around the nuclei of cells (at 10-12 min) and then spread to the cytoplasm. The pHyPer-Mito fluorescence appeared at 20 min but only few mitochondria showed fluorescence suggesting that pHyPer-Mit oxidation is a secondary event and possibly due to ROS generated by NOX4. Together these data suggest that NOX4 generating ROS is localized to the nuclear membrane and 8-oxoG exposure-induced ROS generation is transient.

4.13 Rac1 and OGG1 co-localize with NOX4 in nuclear membrane

To obtain an insight on the close proximity of OGG1, Rac1 and NOX4 at perinuclear regions, we immunostained A549 cells by using specific antibodies to these proteins. We found that OGG1 was primarily localized to the cell nuclei, but a fraction of it was associated with the nuclear membrane. Microscopic imaging showed that like NOX4, Rac1 is associated with the nuclear membrane. We utilized Manders' formula, a method which allows a reliable estimation of close proximity localization of proteins, to determine the colocalization of OGG1 with Rac1 and NOX4. Our results showed a close proximity localization of OGG1 and Rac1 and NOX4. In controls, OGG1 was localized to the nuclear membrane, like lamin A/C; however, the Manders' equation showed that OGG1 and lamin A/C are not in close proximity. The close proximity of OGG1 with Rac-1 and NOX4 is consistent with an increase in nuclear membrane-associated ROS

generation. Colocalizations in the nuclear membrane of OGG1 with Rac1 and NOX4 are novel observations, while another report has already documented the nuclear membrane localization of NOX4 (and NOX1, 2) and their activators of Rac1, as well as p22phox (and p47phox), and their contribution to localized ROS generation.

4.14 OGG1 physically interacts with small GTPase Rac1

We tested if physical interaction between OGG1 and Rac1 occurs similarly to that we experienced with Ras proteins. According to our measurements OGG1 protein alone interacted poorly with guanine nucleotide-free (empty) Rac1 protein. However, in the presence of 8-oxoG, physical interactions between OGG1 and empty Rac1 were significantly increased, as detected by ELISA and Ni-NTA pull-down assays. OGG1 (with 8-oxoG) bound most extensively to GDP-loaded Rac1, while binding to Rac1-GTP was significantly lower than to either empty Rac1 or GDP-Rac1. Together these data reveal a physical interaction of the OGG1/8-oxoG complex with Rac1, and also suggest that the conformation of GDP-bound Rac1 allows the most stable interaction with OGG1/8-oxoG.

4.15 OGG1/8-oxoG complex acts as a GEF on Rac1

To examine the possibility that OGG1/8-oxoG) may serve as a GEF and increase Rac1-GTP levels, we performed guanine nucleotide exchange assays utilizing fluorescently labeled guanine nucleotides (^{Mant}GDP and ^{Mant}GTP).

When Rac1 protein was loaded with ^{Mant}GDP (1:1 molar ratio), the fluorescence intensity of ^{Mant}GDP increased from $1.36x10^5$ FU to $1.9x10^5$ FU. On addition of OGG1 and 8-oxoG along with unlabeled GTP to Rac1-^{Mant}GDP, the fluorescence rapidly decreased, indicating that Rac1-bound ^{Mant}GDP was replaced by nonfluorescent GTP (50% in 45 s). In controls, OGG1 plus GTP did not change Rac1-^{Mant}GDP fluorescence. Rac1 bound to ^{Mant}GTP increased its fluorescence intensity from $1.38x10^5$ FU to $2.02x10^5$ FU similar to Rac1-^{Mant}GDP. Addition of OGG1 plus 8-oxoG along with non-labeled GDP resulted in a slow guanine nucleotide exchange. These data are in line with the poor interaction of OGG1 with Rac1-GTP and show that OGG1 in complex with 8-oxoG catalyzes the release of GDP efficiently, and may function as a GEF.

These observations resemble those reporting interaction between Rac1 and GEFs such as T lymphoma invasion and metastasis (Tiam) protein or Vav2. It has been shown that GEFs require either posttranslational modification(s) or binding to regulatory molecules for interaction and catalysis of guanine nucleotide exchange on Rac1 GTPases. For example, Tiam1's interaction with Rac1 and its GEF activity are increased by association with phosphoinositides in its N-terminal pleckstrin homology domain. Our data suggest that the OGG1 protein is functionally similar to other Rac family GEFs, as it requires binding of a cofactor (8-oxoG) to gain the proper conformation necessary for its binding to Rac1-GDP.

4.16 The role of ROS-OGG1-Rac1 triangle in cellular responses

It seems that ROS could alter OGG1's repair activity as well as subcellular localization in response to the cell's need to repair oxidatively damaged DNA. It has been shown that OGG1 is redistributed in response to locally generated oxidative stress within the nucleus. It has also been shown that NOX4-derived ROS cause DNA oxidation resulting in 8-oxoG and alterations in gene expression, especially those associated with inflammation. OGG1 considered to be a canonical BER protein, our observations show that in complex with 8-oxoG also functions both as a Ras and Rac1 GEF. We propose that the complex not only activates small GTPase dependent pathways, but initiates signal transduction for transcriptional activation of downstream genes. A recent study reviewed the role of OGG1-BER in the activation of pro-inflammatory chemokines/cytokines while another study showed the involvement of K-Ras and NF-κB in innate immune response initiated by OGG1-BER. Although we only show that activated Rac1 increases ROS levels via NOX4, it could be also involved in regulation processes. Supporting that, a recent study has shown that in the presence of 8-oxoG, OGG1 physically interacts with Rho GTPase and increases Rho-GTP levels in cultured cells and lungs, which mediates α-smooth muscle actin (α-SMA) polymerization into stress fibers. This way OGG1 could be involved in the cytoskeletal changes and organ remodeling observed in various chronic diseases.

5. New scientific results

The new scientific results claimed by the author are the following:

- By binding 8-oxoG, OGG1 goes through sterical changes, which enable its physical interaction with small GTPases.
- These interactions result in GDP to GTP exchange activating both Ras and Rac1.
- Both externally added and intracellularly generated 8-oxoG bases are able to induce Ras activation.
- Administration of 8-oxoG increases both Ras-GTP and Rac1-GTP levels in the lungs of experimental mice.
- OGG1/8-oxoG complex formation can lead to MEK/ERK phosphorylation via Ras, or a NOX4-mediated increase in cellular ROS levels via Rac1.

6. Summary

DNA is a frequent target of oxidative agents from both inner and outer sources. One of the most studied DNA adduct among the oxidized bases is 8-oxoG and determination of its level can be used as a reliable gauge of the oxidative stress load of an organism. The 8-oxoguanine DNA glycosylase 1 is the dedicated enzyme to excise the 8-oxoG during the DNA base excision repair process from nuclear and mitochondrial genomes. Accumulation of 8-oxoG in the DNA connected to various disorders like cancer, aging, Alzheimer disease, although OGG1 knock-out mice have normal life span, show only moderate increases in tumor formation, have an increased tolerance to chronic oxidative stress and are less susceptible to inflammatory processes. In this work we describe that OGG1 can exert non-repair functions after binding its excised product, 8-oxoG. We demonstrated that OGG1 has a non-catalytic binding site for 8-oxoG. In a complex with its product, OGG1 not only becomes more efficient removing oxidized guanine, but also goes through sterical changes, which enable its physical interaction with small GTPases, Ras and Rac1 proteins. These interactions result in GDP to GTP exchange activating these small GTPases. We found that intranasal administration of 8-oxoG increases both Ras-GTP and Rac1-GTP levels in the lungs of experimental mice. Our results also show that both externally added and intracellularly generated 8-oxoG bases are able to form a complex with OGG1 leading to Ras activation. We revealed that OGG1/8-oxoG complex formation can lead to MEK/ERK phosphorylation via Ras or a NOX4-mediated increase in cellular ROS levels via Rac1.

While the biological significance of our findings has yet to be fully elucidated, it appears that activation of the small GTPase Rac1, leading to localized ROS generation could be part of a physiological DNA damage/repair response initiated by OGG1. As the OGG1/8-oxoG not only activates Rac1 but also the canonical Ras family GTPases, it could be considered that Rac1/NOX4/ROS and Ras-mediated signaling are involved in maintaining cellular homeostasis. We also speculate that a failure in the control of OGG1 activity may lead to excessive release of 8-oxoG from DNA, resulting in unscheduled activation of Rac and Ras family GTPases that could lead to pathophysiological cellular responses, contributing to diseases and aging processes.

7. Publications

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Registry number: DEENK/67/2015.PL
Subject: Ph.D. List of Publications

Candidate: György Hajas
Neptun ID: M2SSZY
Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. **Hajas, G.**, Bácsi, A., Aguilera-Aguirre, L., Hegde, M.L., Tapas, K.H., Sur, S., Radák, Z., Ba, X., Boldogh, I.: 8-Oxoguanine DNA glycosylase-1 links DNA repair to cellular signaling via the activation of the small GTPase Rac1.
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IF:5.71

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8. Keywords

8-Oxoguanine, 8-Oxoguanine DNA glycosylase-1 (Ogg1), Base excision repair, Cell signaling, NADPH oxidase 4, Oxidative stress, Rac1 GTPase, Ras GTPase.

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