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PARP-1 protein as target for stress response in human lung cells

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Referat und bibliographische Beschreibung

In der vorliegenden Arbeit verwendeten wir primäre humane Lungen-epithelzellen, um uns auf das Material aus authentischen Fällen zu konzentrieren, in denen Beruf und Lebensgewohnheiten zusammen mit dem genetischen Hintergrund Lungenkrebs verursacht haben. Aus histologisch normalem Lungengewebe aus Resektionsmaterial konnten Kulturen von normalen humanen Bronchialepithelzellen (NHBEZ) und peripheren Lungenzellen gewonnen werden. Nach dem Auswachsen des ersten subkonfluenten Monolayers konnten die Zellen mehrmals gesplittet und dadurch vermehrt werden. Die Farbstoffe Hämatoxylin und Eosin wurden zum Anfärben der Lungenzellen eingesetzt. Die Expression von PARP-1-Protein wurde mittels Western Blot-Technik nachgewiesen. Wir haben untersucht, ob PARP-1 in normalen humanen Lungenzellen in Kultur exprimiert wird und ob Expression und Funktion von PARP-1 durch externe Faktoren, wie die Art des Test- materials (Explantat oder Passagen) moduliert werden. PARP-1 wurde sowohl in NHBEZ als auch in der Tumorzelllinie A549 exprimiert. Bei semi-quantitativen Analysen der Immunoblots war das Signal in A549 Tumorzellen wesentlich höher als in NHBEZ. Die PARP-1 Expression in NHBEZ variierte zwischen verschiedenen Patienten etwa um den Faktor 2,5. Die PARP-1 Proteinexpression in langfristigen Kulturen von NHBEZ wurde ebenfalls untersucht und zeigte von der zweiten bis zur fünften Generation eine Induktion im Vergleich zur ersten Generation. Die basale PARP-1-Aktivität wurde immunozytochemisch nach Induktion von DNA-Schäden bestimmt. Die Intensität des Fluoreszenzsignals korrelierte mit der Konzentration von H_2O_2. In den Proben wurden inter-individuelle Unterschiede in der PARP-1-Aktivität mit einem Faktor von 2,3 zwischen der höchsten und der niedrigsten relativen Aktivität beobachtet. In Langzeitkulturen von NHBEZ wurde eine schrittweise Reduktion der PARP-1-Aktivität nachgewiesen. Vor der Untersuchung der Wirkung verschiedener Substanzen auf Expression und Aktivität von PARP-1 wurden diese zunächst mittels MTT-Assay getestet. Nach kurz- und langfristiger Behandlung mit Kupfer oder Quecksilber kam es zu einem nicht signifikanten Anstieg von PARP-1. Kupfer und Quecksilber reduzierten bei kurzfristiger Behandlung die H_2O_2 induzierte PARP-1 Aktivität. Zigarettenrauchkondensat erhöhte die PARP-1 Expression bei kurz- und langfristiger Exposition entsprechend 1,4 und 1,6-fach im Vergleich zur Kontrolle (basale zelluläre PARP-1 Expression). Zigarettenrauchkondensat induzierte die PARP-1-Aktivität bei kurz- und langfristiger Exposition in Abwesenheit von H_2O_2 und verstärkte die H_2O_2 induzierte Aktivität.

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List of abbreviations

AECG	Airway Epithelial Cell Growth Medium
ANOVA	Analysis of variance
AMD	a central auto modification domain
ATP	Adenosin-5´-triphosphat
BCA	Bicinchoninic acid
BER	base excision repair
BSA	Bovine Serum albumen
Cal/EPA	California Environmental Protection Agency
CHD	chronic hypertensive disease
COPD	chronic obstructive pulmonary disease
CSC	Cigarette smoke condensate
$CuSO_4$	Copper sulphate
DAPI	4;6-diamidino-2-phenylindole
DBD	DNA-binding domain
DMEM	Dulbecco´s modified Eagl´s medium
DSBs	double strand breaks
ECL	enhanced chemiluminescene
FBS	Fetal Bovine Serum
FITC	fluorescien isothiocyanate
$Hg\ Cl_2$	Mercuric chloride
KD	kilo Dalton
MMR	mismatch repair
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
NAD^+	Nicotinamid-adenin-dinucleotid
NLS	nuclear localization signal
NHBEC	normal human bronchial cells
PARP-1	poly(ADP-ribose) polymerase-1
PARG	poly(ADP-ribose) glycohydrolase

PAGE	Polyacrylamide Gel Electrophoresis of Proteins
PAR	poly(ADP-ribose) polymer
PBS	phosphate buffer saline
PLC	peripheral lung cells
RT	room temperature
ROS	reactive oxygen species
SDS	Sodium dodecyl sulfate
SSBs	single-strand breaks
SOD	superoxide dismutase
TCA	trichloroacetic acid
IARC	International Agency for Research on Cancer
USDHHS	U.S. Department of Health and Human Services
CDC	Centers for Disease Control and Prevention
SIDS	sudden infant death syndrome
LEC	Liver endothelial cells
WHO	World Health Organization
ELISA	enzyme-linked immunosorbent assay
CPT	Camptothecin
3-ABA	3-aminobenzamide
CSE	Cigarette Smoke Extract

1. Introduction

Lung is a target organ for the toxicity of inhaled compounds. The respiratory tract is frequently exposed to elevated concentrations of these compounds and become the primary target site for toxicity. The lung has a significant capability of biotransforming such compounds with the aim of reducing its potential toxicity. In some instances, the biotransformation of a given compound can result in the generation of more reactive, and frequently more toxic, metabolites. Indeed, lung tissue is known to activate pro-carcinogens into more reactive intermediates that easily form DNA adducts. Several enzymes are expressed in bronchial, bronchiolar epithelium, Clara cells, type II pneumocytes, and alveolar macrophages involved in the metabolising of xenobiotics (Castell et al., 2005).

The challenges for lung toxicology are to address the cellular response toward abundant risk factors for toxic stressors in air, such as dust, environmentally related exposure of metals or cigarette smoke. In order to clarify these important tasks for human health, in vitro approaches are needed that help to clarify basic mechanisms of cellular responses to stress factors. The strengths and weaknesses of experimental concepts can be elucidated by a profound knowledge of the anatomical and physiological characteristics of human lung.

1.1. Lung architecture and function

The respiratory system includes the lungs, which are the major organs of the respiratory system, and are divided into sections, or lobes. The right lung has three lobes and is slightly larger than the left lung, which has two lobes. A system of tubes link the sites of gas exchange with the external environment , and act as an air pump whose basic function is to supply all of the body cells with a continues supply of oxygen and to get rid of the gaseous metabolic by-product, carbon dioxide. The tube system is divided into two principal regions (a) a conducting portion, consisting of the nasal cavity, nasopharynx, larynx, trachea, bronchi, bronchioles, and terminal bronchioles, and (b) a respiratory portion (where gas exchange take place), consisting of respiratory bronchioles, alveolar ducts, and alveoli **(Fig. 1).**

Each primary bronchus branches dichotomously 9-12 times, with each branch becoming progressively smaller until it reaches a diameter of about 5 mm. Bronchioles, intralobular airways with diameters of 5 mm or less, have neither cartilage nor gland in their mucosa; there are only scattered goblet cells within the epithelium of the initial segments. The epithelium of the terminal bronchioles is ciliated simple columnar or cuboidal, and contains Clara cells. These cells, which are devoid of cilia, present secretory granules in their apex and are known to secrete glycosaminoglycans that probably protect the bronchial lining.

The conducting portion serves two main functions: to provide a conduit through which air can travel to and from the lung, and to condition the inspired air. In order to ensure an uninterrupted supply of air, a combination of cartilage, elastic and collagen fibers, and smooth muscle provides the conducting portion with rigid structural support and the necessary flexibility and extensibility. To condition the inspired air before it enters the lungs, the inspired air is cleansed, moistened, and warmed. To carry out these functions the mucosa of the conducting portion is lined by a specialized respiratory epithelium, and there are numerous mucous and serous glands as well as a rich superficial vascular network in the lamina propria. Most of the conducting portion is lined by ciliated pseudostratified columnar epithelium. Deeper in the bronchial tree, this epithelial cell population is modified in a transition to simple squamous epithelium. As the bronchi subdivide into the bronchioles, the pseudostratified organization gives way to a simple columnar epithelium, which is further reduced to a simple cuboidal layer in the smallest (terminal) bronchioles.

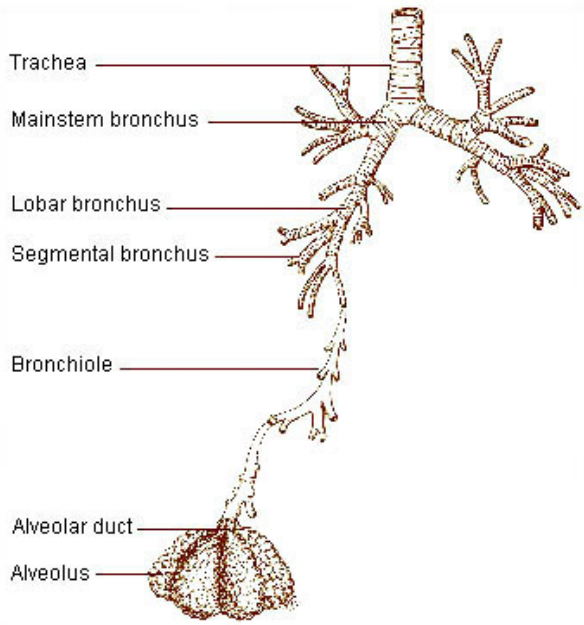


Fig. 1. structure of the conducting portion of the respiratory system

Typical respiratory epithelium consists of five cell types (as seen in the electron microscope). Ciliated columnar cells, constitute the most abundant type, each cell possesses about 300 cilia on its apical surface. The next most abundant cells are the mucous goblet cells. The remaining columnar cells are known as brush cells, because of the numerous microvilli present on their apical surface. These cells have afferent nerve endings on their basal surfaces and are considered to be sensory receptors. Basal (short) cells are small rounded cells that lie on the basal lamina but do not extend to the luminal surface of the epithelium. These cells are believed to be generative cells that undergo mitosis and subsequently differentiate into the other cell types. The remaining cell type is the small granule cell, which resembles a basal cell except that it possesses numerous granules 100-200 nm in diameter with dense core. Histochemical studies reveal that these cells constitute a population of

cells of the diffuse neuro endocrine system. These endocrine like granule cells may act as effectors in the integration of the mucous and serous secretory processes (Breeze and Wheeldon, 1977).

Alveoli are saclike evaginations, about 200 µM in diameter, of the respiratory bronchioles, alveolar ducts, and alveolar sac. Alveoli are responsible for the spongy structure of the lung. Structurally, alveoli resemble small pockets that are open on one side. The structure of the alveolar walls is specialized for enhancing diffusion between the external and internal environments. Generally, each wall lies between two neighbouring alveoli termed an interalveolar septum, or wall. An alveolar septum consists of two thin squamous epithelial layers between which lie capillaries, fibroblasts, elastic and reticular fibers, and macrophages. The capillaries and connective tissue matrix constitute the interstitium, within which is found the richest capillary network in the body. Oxygen from the alveolar air passes into the capillary blood through these layers, CO_2 diffuses in the opposite direction. The approximately 300 million alveoli in the lungs considerably increase their internal exchange surface, which has been calculated to be approximately 140 m^2. Alveoli have two types cell lining, Type I cell, also called squamous alveolar cells, which are extremely attenuated cells that line the alveolar surfaces. Type I cells make up 97% of the alveolar surfaces (Type II cells make up the remaining 3%). The main role of this cell is to provide a barrier of minimal thickness that is readily permeable to gases (Evans, 1975).

Type II cells, or great alveolar cells, are roughly cuboidal cells that are usually found in groups of two or three along the alveolar surface at points where the alveolar walls unite and form angles. The main role of this cell is the production of the pulmonary surfactant, which aids in reducing the surface tension of the alveolar cells (Kikkawa and Smith, 1983).

1.2. Heavy metals as toxic stressors

"Heavy metals" are chemical elements with a specific gravity that is at least 5 times the specific gravity of water. Some well-known toxic metallic elements with a specific gravity of 5 or more are arsenic, 5.7; cadmium, 8.65; iron, 7.9; lead, 11.34; and mercury, 13.546 (Lide, 1992).

Heavy metals are natural constituents of the Earth's crust. Other sources of heavy metals include industrial sources, e.g. mines, foundries and smelters, and diffuse sources such as combustion by-products, traffic, etc. They are stable and cannot be degraded or destroyed, therefore continuous minimal exposure occurs. In some regions emission sources prevail and drinking water comes from lead pipes and thus the exposure exceeds normal levels. Also the intake by the food chain is variable because it depends from personal factors and local food sources.

As trace elements, some heavy metals (e.g. copper, selenium, zinc) are essential to maintain the metabolism of the human body such as Cu/Zn superoxide dismutase, cytochrome *c* oxidase, and tyrosinase. However, at higher concentrations heavy metal poisoning could result. Exposure to heavy metals is a common event due to their environmental distribution. The toxic manifestations

of metals are caused primarily due to imbalance between pro-oxidant and antioxidant homeostasis. Besides, heavy metals have high affinity for thiol groups containing enzymes and proteins, which are responsible for normal cellular defence mechanism.

Long term exposure to these metals could lead to apoptosis. Signaling components affected by metals include growth factor receptors, G-proteins, MAP kinases and transcription factors (Flora et al., 2008).

The assessment of the impact of metals on human health is difficult because metal tend to bio-accumulate which may lead to a lag phase between uptake and effect. Bioaccumulation means an increase in the concentration of a chemical in a biological organism over time, compared to the chemical's concentration in the environment. In our study two elements of heavy metals have been used as representatives for the metal group, copper sulphate and mercuric chloride, to investigate their effect on PARP-1 protein expression and activity in cultivated human lung cells.

Copper

Cu is an essential nutrient. Infancy represents one of the most critical periods in life in terms of Cu requirements because rapid growth increases Cu demands, whereas diets based on milk provide low amounts of the element (Lönnerdal, 1996)**.** Its role as a cofactor for crucial enzymes has been well established. These include cytochrome *c* oxidase (the terminal enzyme in electron transport and respiration), Cu/Zn superoxide dismutase and ceruloplasmin (which deal with superoxide and other potentially damaging radicals), as well as tyrosinase (producing melanin pigment), lysyl oxidase (which cross-links elastin and collagen), dopamine-monooxygenase (necessary for catecholamine production), and peptidyl glycine α-amidating monooxygenase (required for modification of neuropeptide hormones) (Linder et al., 1996).

In living matter, Cu has two oxidation states: cuprous (Cu^{1+}) and cupric (Cu^{2+}). Cu^{2+} is fairly soluble, whereas Cu^{1+} solubility is in the sub-micromolar range. In biological systems, Cu is found mainly in the Cu^{2+}form, since in the presence of oxygen or other electron acceptors Cu^{1+} is readily oxidized to Cu^{2+}. Cu oxidation is reversible since Cu^{2+} can accept an electron from strong reluctant such as ascorbate and reduced glutathione (Galhardi et al., 2004).

In the general population the primary source of copper intake is diet, with an estimated intake of approximately 2 mg/day from food and 0.15 mg/day from drinking water. The estimated average requirement for copper is 0.7 mg/day and the recommended dietary allowance (RDA) is 0.9 mg/day for adults (ATSDR, 2004). Cu is necessary for an efficient immune response (Huang and Failla, 2000). In infection, Cu is essential for the production of interleukin-2 by activated lymphocytic cells (Percival, 1998). Cu deficiency has been shown to decrease superoxide dismutase (SOD) activity and increase superoxide anions in Cu deficient rat embryos (Hawk et al., 2003) as well as in adult Cu deficient rats (Lynch et al., 1997). In addition to decreased CuZn-SOD activity, it is postulated

that the Cu deficiency-induced decreases in cytochrome c oxidase activity, and the oxidative inactivation of complex I (NADH:ubiquinone oxidoreductase) contribute to the increased production of ROS in Cu deficient animals (Johnson and Thomas, 1999).

Similar to copper deficiency, copper overload may also show adverse effects as copper toxicity can result in oxidative stress and subsequent tissue damage. Free copper ions or low molecular copper complexes catalyse Fenton-type reactions, generate reactive oxygen species (ROS) and induce protein and nucleic acid oxidation (Uriu-Adams et al., 2005; Evans and Halliwell, 1999, Koppenol, 1994). In addition, its ability to bind to proteins and nucleic acids enables copper to specifically promote oxidative modification reactions (Cecconi et al., 2002; Bar-Or et al., 2001).

Chronic, excessive copper accumulation in liver, brain, and some other organs, as occurs in human Wilson disease, results in liver cirrhosis as well as degenerative changes in brain and some endocrine organs. As demonstrated histologically, the initial stage of Wilson disease is characterized by an accumulation of copper in hepatocyte mitochondria (Goldfischer et al., 1980).

Another potential consequence is programmed cell death, or apoptosis. Numerous studies have implicated oxidative stress in promotion of apoptosis (Sandstrom et al., 1994). It is important to note that some investigators have suggested that the chronic consumption of drinking water with elevated Cu concentrations may represent a potential health risk for susceptible populations including infants, young children (Eife et al., 1999). Copper-induced apoptosis has been demonstrated in thymocytes against which metallothionein protects (Deng et al., 1999).

Mercury

Mercury is a toxic and hazardous metal that is wide spread distributed. Natural phenomena such as erosion and volcanic eruptions, and is widely distributed in the environment as a result of human activities as anthropogenic activities like metal smelting and industrial production and use may lead to substantial contamination of the environment with mercury. Through consumption of mercury in food, the populations of many areas, particularly in the developing world, have been confronted with catastrophic outbreaks of mercury-induced diseases and mortality (UNEP, 2002; Tchounwou et al., 2003).

Three main forms of mercury are found in the environment: elemental mercury or quicksilver (Hg^0), inorganic mercury (Hg^+ and Hg^{2+}), and organic methyl-, ethyl- and phenylmercury. Each form has a different solubility, reactivity, and toxicity (Clarkson, 1997; Goldman and Shannon, 2001).

The major forms of mercury to which humans are exposed to are mercury vapour, Hg^0 primary emission (natural, coal burring, natural gas, industry poisons) and methyl mercury compounds. Mercury vapour emitted from both natural and anthropogenic sources is globally distributed in the atmosphere, and is returned in a water soluble form via precipitation to fresh and ocean waters.

Inorganic mercury, present in water sediments, can be subjected to bacterial conversion to more toxic methyl mercury compounds, which owing to their lipophilic nature are bioaccumulated in the aquatic food chain. Human exposure to mercury vapour arises from dental amalgam (rarely) and industry (Dopp et al., 2004).

Metallic mercury may cause kidney damage, which is reversible after exposure has stopped. Acute mercury exposure may give rise to lung damage. Chronic poisoning is characterized by neurological and psychological symptoms, such as tremor, changes in personality, restlessness, anxiety, sleep disturbance and depression. The symptoms are reversible after cessation of exposure. Because of the blood–brain barrier there is no central nervous involvement related to inorganic mercury exposure (Järup, 2003).

Metallic mercury is an allergen, which may cause contact eczema, and mercury from amalgam fillings may give rise to oral lichen. It has been feared that mercury in amalgam may cause a variety of symptoms. Several experimental studies have shown that mercury vapour is released from amalgam fillings, and that the release rate may increase by chewing (Sallsten et al., 1996). There is mounting evidence suggesting that chronic exposure to low levels of mercury, such as that experienced with mercury amalgam fillings can result in immune dysfunction (Clarkson, 1997). This so-called ‘amalgam disease’ is, however, controversial, and although some authors claim proof of symptom relief after removal of dental amalgam fillings (Lindh et al., 2002); there is no scientific evidence of this (Langworth et al., 2002). The intake of fish products is a major public health concern due to possible methyl mercury exposure, which is especially toxic to the human nervous system (Cortes and Fortt, 2007; Myers et al., 2009).

The general population is primarily exposed to mercury *via* food, fish being a major source of methyl mercury exposure (WHO, 1990). A high dietary intake of mercury from consumption of fish has been hypothesized to increase the risk of coronary heart disease. Furthermore, Shenker et al., 2000 have shown that both organic ($MeHgCl_2$) and inorganic ($HgCl_2$) mercurial species specifically affect mitochondrial function by initiating the development of a permeability transition within the mitochondrial membrane and the subsequent release of the proapoptotic molecule cytochrome c.

Cell cultures of MO3.13 cells treated with 25 and 50 µM $HgCl_2$ for 24 h exhibited chromatin condensation patterns typical of apoptosis, this occurred in many cells that also displayed extensive membrane blebbing. Apoptosis in MO3.13 cells culture was accompanied by detachment and shrinkage of cells. Exposure to higher concentrations of $HgCl_2$ 250 µm induced a necrotic phenotype including cell swelling and lysis whilst at (100 µmm) both apoptotic and necrotic cells were observed in the same culture. The toxic effects of $HgCl_2$ were observed to be both time and dose dependent (Issa et al., 2003). Heavy metals such as mercury do not usually induce obvious genotoxic effects but interfere with numerous cellular activities such as cellular repair enzymes (Williams et al., 1986) to enhance genotoxicity (Au et al., 1996).

Mercuric ion has also been postulated to use different mechanisms to interact with DNA repair enzymes or DNA proteins essential for repair. One of these mechanisms is the inhibition DNA polymerase. The latter mechanism probably affects proteins with zinc finger structures in their DNA-binding motifs. Within these structures, zinc is complexed to four cysteines and/or histidines. Mercury has a strong affinity for thiol bonds, which are present in cysteines in zinc finger structures. The binding of mercuric ion to these thiol groups can severely distort the structural integrity and activity of these proteins (Cebulska-Wasilewska et al., 2005).

1.3. Cigarette smoke and human health

Smoking of tobacco is practised worldwide by over one thousand million people. However, while smoking prevalence has declined in many developed countries, it remains high in others and is increasing among women in developing countries. Between one-fifth and two-thirds of men in most populations smoke (IARC, 2002). In Germany about 33,9 % of adults are smokers and approximately 140,000 of population die each year from the direct effect of smoking (Bätzing, 2009).

Smoking harms nearly every organ of the body; causing many diseases and reducing the health of smokers in general (USDHHS, 2006). The adverse health effects from cigarette smoking account for an estimated 438,000 deaths, or nearly 1 of every 5 deaths, each year in the United States (CDC, 2006) More deaths are caused each year by tobacco use than by all deaths from human immunodeficiency virus (HIV), illegal drug use, alcohol use, motor vehicle injuries, suicides, and murders combined (McGinnis and Foege, 1993; CDC, 2006).

Cancer is the second leading cause of death and was among the first diseases casually linked to smoking. Smoking causes about 90 % of lung cancer deaths in men and almost 80% of lung cancer deaths in women. The risk of dying from lung cancer is more than 23 times higher among men who smoke cigarettes, and about 13 times higher among women who smoke cigarettes compared with never smokers. Smoking causes cancers of the bladder, oral cavity, pharynx, larynx (voice box), oesophagus, cervix, kidney, lung, pancreas, and stomach, and causes acute myeloid leukaemia (USDHHS, 2006).

Smoking causes coronary heart disease, and approximately doubles a person's risk for stroke (USDHHS, 2006; Ockene and Miller, 2006). Smokers are more than 10 times as likely as non-smokers to develop peripheral vascular disease (Fielding et al., 2006). Smoking causes abdominal aortic aneurysm (USDHHS, 2006). 90% of all deaths from chronic obstructive lung diseases are attributable to cigarette smoking (USDHHS, 2006). Cigarette smoking has many adverse reproductive and early childhood effects, including an increased risk for infertility, preterm delivery, stillbirth, low birth weight, and sudden infant death syndrome (SIDS). Postmenopausal women who smoke have lower bone density than women who never smoked. Women who smoke

have an increased risk for hip fracture than never smokers (USDHHS, 2006). Active smoking by the mother during pregnancy has causal adverse effects on pregnancy outcomes that are well documented (USDHHS, 2001; 2004). Most recently, the report of the California EPA (Cal/EPA) found the evidence to be conclusive for second-hand smoke as a cause of premenopausal breast cancer (Cal/EPA, 2005).

Cigarette smoke contains more than 6,000 components, many of which can lead to DNA damage (Lofroth, 1989). Consistent with this, cigarette smoke exposure has been reported to induce DNA damage in a variety of cell types (Baumgartner et al., 2000). Clinically, one of the major cell types affected by cigarette smoke is the airway epithelium, where damage can lead to cancer and may contribute to the development of chronic obstructive pulmonary disease (Agusti et al ., 2002 ; Tuder et al., 2003 ; Hodge et al., 2003). Cigarette smoke condensate (CSC) refers to the sticky particles comprised of thousands of chemicals created by burning tobacco. It is the particulate component of tobacco smoke without nicotine and water. Cigarette smoke extract (CSE) is prepared by combustion of cigarette without filter and bubbling of the smoke through distilled water. This solution is considered to be 100% CSE

CSC is a chemical mixture containing different compounds, of which 100 or more are known carcinogens, co-carcinogens, mutagens and tumor promoters (Hoffmann et al., 2001), oxidants and aldehydes, all of which have the potential to cause inflammation and damage cells. Oxidants are thought to play a major role in cell injury induced by tobacco smoke since each puff of tobacco smoke contains approximately 10^{17} oxidant molecules (Church and Pryor, 1985).

Exposure to cigarette smoke activates an inflammatory cascade in the airway epithelium resulting in the production of a number of potent cytokines and chemokines, with accompanying damage to the lung epithelium, increased permeability, and recruitment of macrophages and neutrophils to the airway (Adler et al., 1994). Cigarette smoke has been associated with damage to the alveolar epithelium. In particular it can induce suppression of cell proliferation, increase detachment of cells, DNA strand breaks, and reduced surfactant production (Hoshino et al., 2001; Yokohori et al., 2004). When damage occurs to the DNA of a cell, several responses are possible. Often apoptosis or programmed cell death occurs, a response thought to protect the integrity of the genome (Liu et al ., 2005).

Combined list of chemicals in cigarette smoke and their health effects (Fowles and Bates, 2000)

Chemical	Health effect
1,3- butadiene	Cancer, reproductive/ developmental
Acetaldehyde	Cancer, respiratory irritation
Acrolein	Respiratory irritation
Arsenic	Cancer, cardiovascular, reproductive/ developmental
Benzene	Cancer, reproductive, developmental
Cadmium	Cancer
Carbon monoxide	Cardiovascular
Chlorinated Dioxins and Furans	Cancer, Cardiovascular, reproductive, developmental
Chromium	Cancer, respiratory irritation
M+ P+ O Cresol	Cardiovascular
Formaldehyde	Cancer, respiratory irritation
Hydrogen cyanide	Cardiovascular
N-nitrosonomicotine (NNN)	Cancer
N-nitrosodimethylamine (NDMA)	Cancer
N-nitrosopyrrolidine (NP)	Cancer
Ammonia	Nicotine availability

1.4. Cellular responses to stressors

Poly(ADP-ribose)polymerase-1 (PARP-1)

The mammalian cells have homeostatic pathways that regulate both life and death. Exposure of these cells to noxious agents leads to activation of multiple cellular mechanisms in an attempt to survive. One of these mechanisms is the activation of poly (ADP-ribose) polymerase-1 (PARP-1) in response to DNA damage (Bürkle, 2001; Tong et al., 2001). This process is called Poly(ADP-ribosyl) ation which is a posttranslational modification of proteins that regulates many cellular processes and catalysed for the most part by poly (ADP-ribose) polymerase-1 (PARP-1) (Bürkle,

2001b). Poly(ADP-ribosyl) ation is triggered by the presence of DNA strand breaks and represents one of the immediate cellular responses to DNA damage and functionally associated with DNA repair pathways (Flohr et al., 2003; Süsse et al., 2004). Poly(ADP-ribosyl)ation occurs in all nucleated cells of mammals, plants, and lower eukaryotes, but is absent from yeast. Poly (ADP-ribose) can be synthesized only by the members of the PARP family, but the majority is by the most abundant and active member, PARP-1. In the absence of DNA damage, poly(ADP-ribosyl)-ation levels are very low (Sallmann et al., 2000).

PARP-1 is 113-kD protein composed of 1014 amino acids (Kurosaki et al., 1987) and is a well known DNA binding enzyme (Bouchard et al., 2003). The gene coding for this protein is at the q41–q42 position of chromosome 1 (Cherney et al., 1987). It is the main member of the PARP family, which contains as many as 18 distinct proteins in humans (Amé et al., 2004). PARPs are members of a large family of enzymes that use NAD^+ as a substrate to transfer ADP-ribose onto glutamic acid residues of proteins. In addition to PARPs, this family contains the distinct class of mono(ADP-ribosyl)transferases, which include the well-studied bacterial toxins. Whereas mono(ADP-ribosyl)ases transfer a single ADP-ribose unit, PARPs transfer multiple units, resulting in the formation of poly(ADP-ribose) on a protein acceptor (Smith, 2001). Poly(ADP-ribose) polymerases (PARPs enzymes), also known as poly(ADP-ribose) synthetases and poly(ADPribose) transferases, constitute a family of cell signalling enzymes (e.g. PARPs; Vault PARP and Tankyrases) present in eukaryotes, which catalyze poly(ADP-ribosylation) of DNA-binding proteins. These enzymes have emerged as critical regulatory components of the immediate cellular response to DNA damage (Cepeda et al., 2006).

One of the first clues indicating the presence of multiple PARPs was the observation of PAR activity in mouse embryo fibroblasts derived from PARP-1-knockout mice (Shieh et al., 1998). PARP-1 and at least five other PARP family members (i.e., PARP-2, PARP-3, VPARP, tankyrase 1, and tankyrase2) are associated with various components of the mitotic apparatus, which is required for the accurate segregation of chromosomes during cell division (Smith, 2001; Amé et al., 2004). In addition, several PARP enzymes, including PARP-1, PARP-3, and tankyrases, are associated with centrosomes, the cellular microtubule organizing center that functions as the spindle pole during mitosis (Smith and de Lange, 1999; Kaminker et al., 2001; Augustin et al., 2003; Kanai et al., 2003).

PARP-1 has a highly conserved structural and functional organization including (1) an N-terminal double zinc finger DNA-binding domain (DBD) act as a DNA nick sensor, (2) a nuclear localization signal included in the caspase- cleavage site (DEVD), (3) a central automodification domain (AMD) which includes five conserved glutamate residues that function as poly(ADPribose) acceptors. AMD is considered a regulator segment because it contains regions for dimerization, which may modulate the interaction of PARP-1 with DNA and with proteins. This domain also has

the terminus motif of breast cancer susceptibility protein C (BRCT), very common in many DNA repair and cell cycle proteins. It acts in several protein-protein interactions , as well as 15 glutamic acid residues, which act as acceptor sites for the initiation reaction, and (4) a C-terminal catalytic domain, is the most strictly conserved part of the enzyme Catalytic activities of this domain are related to the synthesis of the polymer and its binding to target proteins and consist of NAD^+ hydrolysis, initiation, elongation, branching and termination of the (ADP-ribose) polymer (**Fig. 2**) (D'Amours et al., 1999; Kraus and Lis, 2003; Cepeda et al., 2006). PARP-1 is constitutively expressed at high levels from a promoter with features typically found in housekeeping genes. PARP-1 mRNA is present in all tissues, at varying levels, with highest levels being observed in testis, spleen, brain and thymus (Meyer-Ficca et al., 2005)

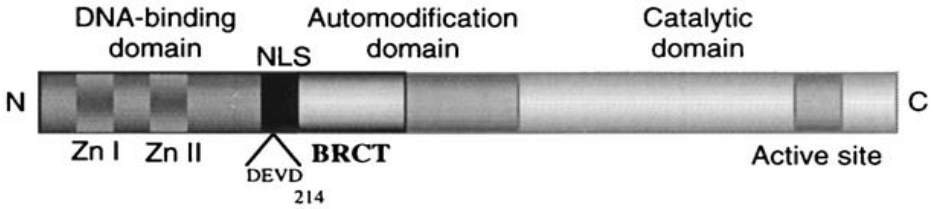


Fig. 2. Structure of PARP-1. PARP-1 can be divided in 3 main domains:a DNA-binding domain (DBD) with 2 zinc fingers (Zn I and Zn II), an automodification domain with a BRCT motif, and a catalytic domain with the NAD+-binding site. The nuclear localization signal (NLS) is located between the DBD and the automodification domain. It comprises the DEVD cleavage site recognized by caspases 3 and 7 (Bouchard et al., 2003).

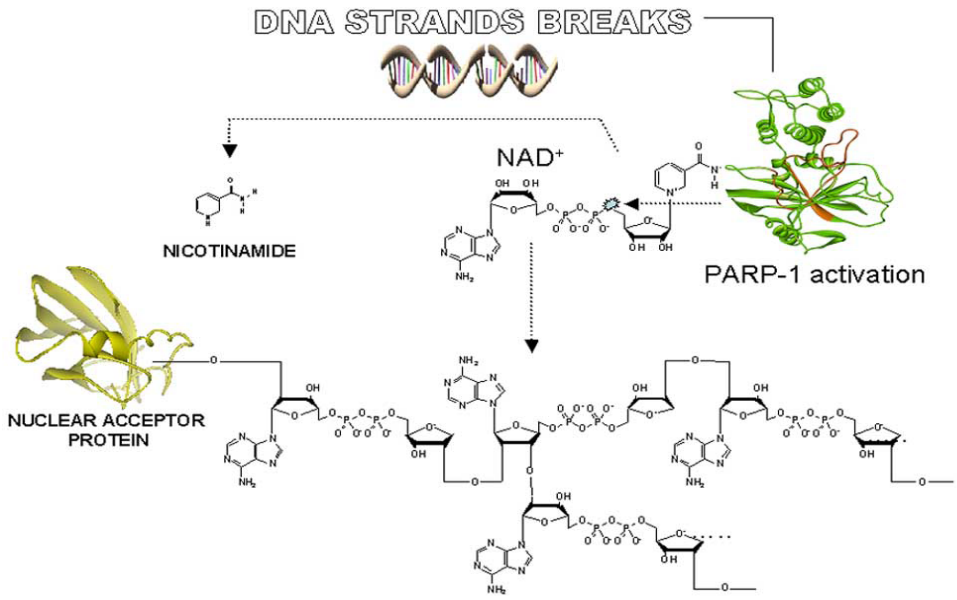


Fig. 3. Function of poly(ADP-ribose) polymerase-1. PARP-1 is activated by DNA strand breaks induced by several events including DNA repair, replication, recombination, oxidative stress and binding of drugs to DNA. Subsequently, PARP-1 catalyzes the cleavage of NAD+ into nicotinamide and ADP-ribose moieties and then uses the latter to synthesize ADP-ribose covalently bound to aminoacid residues of acceptor proteins ('initiation reaction'). ADP-ribose may be also bound to other ADP-ribosyl moieties already transfer to a protein ('elongation reaction') (Cepeda et al ., 2006).

The biological role of PARP-1 includes nine primary functions: 1) DNA repair and maintenance of genomic integrity, 2) regulation of transcription, 3) regulation of replication and differentiation, 4) regulation of telomerase activity, 5) cell elimination pathway, 6) source of energy for BER machinery, 7) regulation of cellular activities by polymer binding proteins, 8) regulation of cytoskeletal organization, and 9) signalling for protein degradation (Virag et al., 2002). PARP-1 is now known to be implicated in several crucial cellular processes: DNA replication, transcription, DNA repair, apoptosis, and genome stability (Bouchard et al., 2003). Genomic stability of cells has long been linked to their poly(ADP-ribosyl)ation capacity (Bürkle, 2001 ; Bürkle et al., 2002). PARP-1's basal enzymatic activity is very low, but is stimulated dramatically in the presence of a variety of allosteric activators, including damaged DNA, some undamaged DNA structures, nucleosomes, and a variety of protein-binding partners (D'Amours et al., 1999; Oei and Shi, 2001; Kun et al., 2002, 2004; Kim et al., 2004). The binding of PARP-1 to damaged DNA, including single-strand breaks (SSBs) and double strand breaks (DSBs), through its double zinc finger DNA-binding domain potently activates PARP-1 enzymatic activity (as much as 500-fold) (D'Amours et al., 1999).

Activation of PARP-1 by DNA strand breaks results in the synthesis of poly(ADP-ribose) at the expense of NAD^+ that is cleaved into ADP-ribose and nicotinamide. PARP-1 catalyzes the binding of ADP-ribose on acceptor proteins, primarily at glutamic residues (Ogata et al., 1980) and subsequently catalyzes the elongation and branching reactions with additional ADP-ribose units (**Fig. 3**). A direct inverse relationship exists between the levels of poly(ADP-ribose) and NAD^+ in a cell. Decreased NAD^+ levels cause an unbalanced NAD/NADH ratio, which, in turn, affects the activation of enzymes involved in glycolysis, the pentose shunt, and the Krebs cycle. Decreased NAD^+ levels also drastically alter the redox state of cells. Because the cell attempts to restore NAD^+ pools by recycling nicotinamide with 2 ATP molecules, excessive activation of PARP-1 depletes pools of intracellular NAD^+ and ATP and, consequently, interferes with most, if not all, energy-dependent cellular processes. Therefore, in the presence of extensive DNA damage, massive synthesis of poly(ADP-ribose) drives the cell to death. When DNA damage levels are limited, poly(ADPribose) polymers are rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG), which possesses endoglycosidase and exoglycosidase activities. PARG removes ADPribose units from the end of the polymer and also cleaves the chain to release free poly(ADP-ribose) (Brochu et al., 1994; Bouchard et al., 2003). As such, PARP-1 can function as a DNA damage sensor. With low levels of DNA damage, PARP-1 acts as a survival factor involved in DNA damage detection and repair. In contrast, with high levels of DNA damage, PARP-1 promotes cell death (Bürkle, 2001a). PARP-1 has been implicated in multiple DNA repair pathways, including the SSB, DSB, and base excision repair (BER) pathways (Bürkle, 2001b; Masutani et al., 2003).

Taken together, the anatomical position of the lung as a primary contact organ for air borne toxicants as well as a secondary contact organ for xenobiotics reaching the blood stream favours the relative frequent incidence of pathological lung insults. Furthermore, exposure to ambient air pollution is associated with pulmonary and cardiovascular diseases and cancer. The mechanisms of xenobiotics-induced health effects are believed to involve inflammation and oxidative stress. The oxidative stress mediated by air pollutants may arise from direct generation of reactive oxygen species from the surface of particles, soluble compounds such as transition metals or organic compounds, altered function of mitochondria or NADPH-oxidase, and activation of inflammatory cells capable of generating ROS and reactive nitrogen species.

Resulting oxidative DNA damage may be implicated in cancer risk and may serve as marker for oxidative stress caused by air pollution. There is overwhelming evidence from animal experimental models, cell culture experiments, and cell free systems that exposure to cigarette smoke and metal particles causes oxidative DNA damage. Similarly, various preparations of metal induce oxidative DNA damage in in-vitro systems.

In addition, the knowledge concerning mechanisms of action of metals and cigarette smoke constituents has prompted the use of markers of oxidative stress and DNA damage for human biomonitoring in relation to ambient air. By means of personal monitoring and biomarkers a few studies have attempted to characterize individual exposure, explore mechanisms and identify significant sources of ambient air pollution with respect to relevant biological effects.

1.5. Aim of the work

Exposure of the cells to noxious agents leads to activation of multiple cellular mechanisms in an attempt to survive. One of these mechanisms is the activation of poly (ADP-ribose) polymerase-1 (PARP-1) in response to DNA damage. Besides its role in DNA repair, at least PARP-1 has been implicated in mammalian longevity and is also considered to be a master switch between apoptosis and necrosis.

Despite its pivotal role for cell survival and tolerance to cope with stressors in all cells types most insights on PARP function and regulation have been obtained from tumor cell lines. Data on normal human epithelial cells are lacking, although these cell types should be much closer to the normal situation and should reflect the basis for risk assessment of chemicals better than cell lines. Tumor cell lines are known to have undergone substantial shifts in apoptosis regulation and DNA repair.

Therefore, an aim of this study is to use normal human lung tissues as a platform to study the expression and activity of PARP-1 as a key element in recognition and regulation of DNA repair. The experimental concept was directed to study whether PARP-1 is expressed in normal human lung cells in culture, and whether PARP-1 expression and function is modulated by external factors. Normal human bronchial cell cultures should be established from explant cultures from human lung tissue. The material is obtained from morphologically normal sections from lob- or pneumectomy material from lung cancer cases.

The following questions should be addressed:

1) Is the level of PARP-1 protein substantially different between individual and may thus reflect the individual tolerance to cope with genotoxic stressors ?
2) What is the adaptive response of PARP-1 expression in culture, because PARP is assumed to play an integral parting the ageing process ?
3) Is PARP-1 a target protein for damage by heavy metals? This problem may have substantial influence of environmentally generated health processes because many toxic metals are abundant in air. Copper and mercury were chosen as examples because they are subject of actual risk assessment in environmental toxicology.
4) What is the role of cigarette smoke constituents on PARP expression and function. This problem is of major significance to understand the individual fate, because tobacco smoking is the most important source for lung toxicity by life style factors. Almost 75 % of the lung cancer cases have a positive smoking history.

2. Materials and Methods

2.1. Materials

Cell culture reagents

AECG-Medium	Promo-Cell, Heidelberg
DMEM	Gibco, Berlin
Fetal Bovine Serum	promo Cell, Heidelberg
Trypsin, PBS, L15-Leibovitz Puffer	Biochrome, Berlin
Penicillin / Streptomycin	Biochrom AG, Berlin
Fibronectine, Collagen R, DMSO	Sigma, Taufkirchen

MTT-Assay reagents

PBS	Biochrome, Berlin
MTT – Reagent	Gibco-Lifescience, Berlin
Isopropanol, Formic acid	Roth, Karlsruhe

Chemicals for Protein Isolation and Determination

TRitidy G	Applichem, Darmstadt
Isopropanol , Ethanol	Roth, Karlsruhe
1-Bromo-3-chloropropane	Merck, Darmstadt
Guanidinhydrochlorid	Roth, Karlsruhe
Protease inhibitor	Sigma, Taufkirchen
BCA, Copper- Sulfate	Sigma, Taufkirchen
Protein Standard (BSA)	Sigma, Taufkirchen
SDS	USB united states Bio

Chemicals for Western Blot

Polyacrylamid	Applichem, Darmstadt
TRIS, sodium chloride, acetic acid	Roth, Karlsruhe
Tween 20, Ponceau S, Bromphenolblau ,	Sigma, Taufkirchen
ß-Mercapto-Ethanol,	
Glycin, Milk powder	Roth, Karlsruhe
Primary antibody PARP-1, Secondary antibody	Santa Cruz Biotechnology
Goat anti-mouse IgG-HR	
Advanced ECL Detections Reagent	Amersham Pharmacia,
Full Range Rainbow Marker	Biotech, Freiburg
Developer, Fixer	Sigma, Taufkirchen

Immunoflourescence reagents

Primary antibody PAR (10H)	Was a generous gift of Professor Alexander Bütkel, University of Konstanz
FITC conjugated secondary antibody (goat anti-mouse IgG-HRP)	Santa Cruz Biotechnology
TCA	Sigma, Taufkirchen
Vectashield mounting medium with DAPI	Vector Laboratores
Hydrogen Peroxide	Roth, Karlsruhe

Chemicals for the treatment of cultured cells

Copper- Sulfate, Mercuric chloride	Sigma, Taufkirchen
Cigarette Smoke Condensate	Was a generous gift of Dr. Schramke and Dr. Haußmann from PhilipMorris Research Laboratories GmbH , Cologne,Germany

Equipment and used materials

Autoclave	Autoclave 3.021	Schütt-Labortechnik, Göttingen
Incubator	BBD 6220	Heraeus-Instrument Hanau
	Function line	Heraeus-Instruments Hanau
	Hera cell	Heraeus-Instrument Hanau
Elektrophoresis	Biometra P25	Schütt-Labortechnik, Göttingen
	Mini-PROTEAN 3 system	Bio-RAD
	Electro Blotting System	EBU- 204
Microscope	Axiovert 25	Zeiss, Jena
	Nikon	Nikon, Japan
Camera	Digital- *Camera*	Nikon
Elisa Reader	Genios	Tecan, USA
Cuvette	Optical Glass- Cuvette 1,5 ml	Hellma
Photometer	DU 7500	Beckman, USA
Water bath	GFL 1083	GFL
Laminar flow	HERA-safe	Heraeus-Instrument
	HERA-safe H12	Heraeus-Instrument
Sterilfiltration	Filter Sartolab-P-plus (0,2-0,45µm)	Sartorius AG, Göttingen
Centrifuge	Biofuge fresco	Heraeus-Instrument

	GS-15 R	Beckman, USA
Weighing machine	analytical balance Bp 211D	Sartorius, Göttingen
Cell culture	24Well and 6 Well- cell culture plate	TPP, Schweiz
	Cell culture plate (6cm∅), Centrifgue tubes (15, 50ml),	
	96Well-Fluorescence plate	Nunc, Wiesbaden
	Cover slip, slides	Schütt-Labortechnik, Göttingen
	Serological pipettes (5ml, 10ml, 25ml)	Eppendorf, Hamburg
	Pipette tip (1000µl, 200µl, 10µl)	Eppendorf, Hamburg, VWR
	Multi-Ecoflex- Tipe(0,5- 200µl), Tubes (1,5ml, 0,5ml, 0,2ml)	Brand, Wertheim
	Disposable cuvette	Brand Wertheim
	disposable steril Glass Pipette: 1,5 und 10ml	Nunc, Wiesbaden
Nitrocellulose- Membrane,		Amersham Pharmacia
ECL- Film		Biotech, Freiburg
Filter paper	Whitman Filter Paper (Chr 3,3)	Biometra, Göttingen

Analyzing Software

Excel	Microsoft, USA
Sigma Stat 8.0	SPSS Inc. USA
Tina 2.09	Raytest, Straubenhardt
AQM Advance 6	

Antibodies

Primary antibody

Mouse monoclonal antibody 10H is directed against Poly(ADP-ribose) [PAR]. PAR is synthesized after activation of the nuclear DNA repair enzyme (PARP)

Secondary antibody

Polyclonal Goat Anti-Mouse Immunoglobulins/FITC. Goat antiserum conjugated with fluorescein isothiocyanate isomer 1. Fluorescein is typically excited by 488 nm line of an argon laser , and emission is collected at 530 nm.

Vectashield mounting medium with DAPI

Mounting Medium contain 4;6-diamidino-2-phenylindole (DAPI) and is intended to be used to counter stain DNA. This product is designed to be used for *in situ* hybridization techniques or for other procedures requiring fluorescent labeling of DNA. DAPI excites at 360 nm and emits at about 460 nm when bound to DNA, producing a blue fluorescence.

Immunodetection of PARP-1 protein by western Blotting

Primary antibody, PARP-1 mouse monoclonal antibody raised against amino acids 764-1014 mapping at the C-terminus of PARP-1 of human origin, used in dilution 1:1000

Secondary antibody, goat Anti-mouse IgG-HRP conjugated, used in dilution 1:20000

Beta-actin Antibody, Mouse monoclonal [AC-15] to beta actin – Loading Control. It is an ideal reagent for use as a cell extract loading control (house keeping gene) on Western blot. Its molecular weight is 42KD.

Cell culture media DMEM and AECG

a) DMEM

The medium used for the tumor cell lines and the basic component of this medium is, Dulbecco´s modified Eagle´s medium, which formed from 3,7 g sodium Bicarbonat in one liter medium. After adjustment to a pH value of 7.4 the DMEM was sterile filtered and stored with 4 °C. Before use 1% Penicillin/Streptomycin (v/v) (10.000 I.E./ml) and 10% FCS (v/v) were added

b) AECG (Airway Epithelial Cell Growth Medium promo cell)

The medium-formulation contains no growth factors or antibiotics. After adding the Supplement Mix the concentrations of growth factors in the complete medium are as follows:

BPE 0.4 %

Epidermal Growth Factor 10 ng/ml

Insulin 5 µg/ml

Hydrocortison 0.5 µg/ml

Epinephrine 0.5 µg/ml

Triiodothyronine 6.7 ng/ml

Transferrin 10 µg/ml

Retinoic Acid 0.1 ng/ml

The medium was stored between 4 °C and 8 °C in the dark, and the Supplement Mix at -20°C. We added Penicillin/ Streptomycin combination (1% of 10.000 I.E./ml) and before use all media were warmed up to 37°C.

Table 1: Description of the study group.

ID	Gender	Age	Diagnosis	Smoker	Chronic disease a)
207	W	49	Adenocarcinoma	yes	
208	W	78	Adenocarcinoma	yes	
209	M	69	Adenocarcinoma	yes	
213	M	77	Bronchogenic carcinoma	Ex-smoker cessation 23 a	CHD
221	M	52	Squamous cell carcinoma	yes 30 pack years	Diabetes CHD
224	M	72	Bronchogenic carcinoma	yes	Diabetes
228	M	65	Adenocarcinoma	yes 40 pack years	
230	M	74	Non small cell carcinoma	yes	CHD
236	M	74	Non small cell carcinoma	Yes 20 pack years	CHD
240	M	57	Non small cell carcinoma	yes	COPD
243	M	62	Non small cell carcinoma	yes 45 pack years	CHD
245	M	54	Non small cell carcinoma	yes 80 pack year	

All patients had primary lung cancer and underwent lob- or pneumectomy due to clinical indication for operation (better prognosis or curative).
All patients underwent symptomatic preoperative treatment: antitussives, analgesics, antiemetics, mucolytics, and bronchodilators

a) Additional chronic disease with medication
b) CHD chronic hypertensive disease; COPD chronic obstructive pulmonary disease.

Sterilization of Materials and solutions

Articles for the cell preparation, as well as cell culture, were sterile from factory. Heatproof glass and plastic materials as well as solutions were sterilized by 25 minutes by autoclave with 120°C. Non heatproof solutions were sterile-filtered. The assigned sterile filters (0.45µm or 0.2µm mesh size) depended on sterility degrees and consistency of the solutions.

2.2. Cell culture of tumor cells

Splitting and cultivation of tumor cell lines

The tumor cells were cultivated in 58 cm^2 culture bottles in the DMEM (Dulbeccos´s minimal essential medium) with 10 % FBS and 1% Penicillin/Streptomycin by 37°C, 5% CO_2 and 95% humidity. The cells were seeded in a density from 20,000- 30.000 cells per cm^2. The culture medium changed three times per week. At 80% confluent growth (within 7-10 days) the cells were replaced and sub cultivated. After sucking the medium and rinsing of the tumor cells two times with PBS , the cells were treated with 10ml Trypsin/EDTA solution (0,05% Trypsin / 0,02% EDTA; in PBS) and incubated 5 min by 37°C. The replaced cells were transferred in a 50ml Falcon centrifugation tubes and centrifuged 5min with 4°C. After removal of the supernatant the cells were suspended in DMEM and the cell number was determined by means of fox Rosenthal chamber. The tumor cells used in the 80% sub confluent growth stage for the experiment.

PBS, NaCl (139 mM); KCL (2,68 mM); Na_2HPO_4(8,2 mM); KH_2PO_4 (1,47 mM); PH 7,4

Tumor cell line

Three human lung cell lines were used in this study: A549, a lung adenocarcinoma cell line with morphological AII cell characteristics; H358, a non small cell lung cancer cell line with AII cell morphology and H322, a non small cell lung cancer cell line with clara cell morphology. The cells were generously provided by Dr. F. Wiebel, Munich. All lung cancer cells were cultured in tissue culture flasks (Techno Plastic Products TPP, Trasadingen, Switzerland) in DMEM medium (Gibco, Berlin), supplemented with 10 % fetal calf serum (c.c. pro, Neustadt/W., Germany) and 1% penicillin/ streptomycine (10 000 IE/ ml). Medium was changed every 2-3 days.

2.3. Explant culture from human lung

Bronchial and peripheral lung tissues were obtained from lung resections of cancer patients. Normal tissue as used in our cell cultures was obtained in proximity of the tumor. Before transport the tissue material was transferred as soon as possible in cold, sterile Leibovitz L15-buffer (Biochrom, Berlin, Germany). The donors were all well documented patients from different hospitals that underwent surgery at the University Hospital of Halle because of a clinical indication. Ages, use of medication or smoking were not exclusion criteria. All patients have given written consent that tissue may be used for cell culture purposes. The medical treatment of patients and diagnosis was not influenced by the study at any time. This study was approved by the ethics committee of the University of Halle-Wittenberg (Tab 1).

Isolation and cultivation of normal human bronchial epithelial cells and parenchymal lung cells

The method of cultivation which was established in our laboratory by Mr. Thomas Stock and modified by Mrs. Dr. Dorothee M. Runge. The modification of the cultivation of normal human bronchial cells (NHBECs) and peripheral lung cells (PLCs) was established according to the method of (Lechner and La Veck, 1985)**.**

Bronchial tissue was obtained from lung resections of cancer patients. Morphologically normal tissue used in our cell cultures was obtained in proximity of the tumor. The diagnosis was done by histopathological examination of the institute of pathology. Before transport the tissue material was transferred as soon as possible in cold, sterile Leibovitz L15-buffer (Biochrom, Berlin, Germany). Bronchi were dissected and cut into pieces of approx. 0.2-0.5 cm². These pieces were transferred onto culture dishes that were pre-coated with 1 % fibronectin/ 2 % collagen/ 0,1% Bovine Serum Albumin in L-15 medium (Seromed, Berlin, Germany). After five minutes serum-free medium (AECG-medium, PromoCell, Heidelberg, Germany) was added. The medium contained 0,4% Bovine hypophyseal extrakt; 0.5 ng/ml epidermal growth factor (EGF).; 5 µg/ml insulin; 0.5 µg/ml hydrocortison; 0.5 µg/ml epinephrin; 6.7 ng/ml triiodothyronin; 10 µg/ml transferrin; 0.1 ng/ml retinoic acid. The tissue cultures were cultivated for 2-3 weeks, cells grew out of the bronchial pieces and adhered to the coated dish surface, until they were 80-90 % confluent (= first generation, 10-14 days). The pieces were then transferred onto new-coated dishes for new and further generations and culturing continued until 80-90 % confluence was reached again (= second generation). Medium was changed every 72 hours.

Primary culture of normal peripheral lung cells (PLC)

Peripheral lung tissue was obtained distal to the bronchial tree, which was microscopically free of bronchial epithelium. Immediately after resection the tissue was maintained in cold L15 Leibovitz buffer (Biochrom, Berlin, Germany). The material was cut into pieces of approximately 1 mm thickness by hand. The pieces were placed onto uncoated 57 mm culture dish (Techno Plastic Products TPP). The cultures were maintained in serum-free AECG medium (AECG, PromoCell, Heidelberg, Germany) containing 0.4% bovine hypophyseal extract; 0.5 ng/ml epidermal growth factor (EGF).; 5 µg/ml insulin; 0.5 µg/ml hydrocortison; 0.5 µg/ml epinephrin; 6.7 ng/ml triiodothyronin; 10 µg/ml transferrin; 0.1 ng/ml retinoic acid. The first subconfluent monolayer was obtained after 4- 5 weeks. Then the pieces could be transferred to new culture dish for new and many generations and the monolayer could be splitted and seeded into new culture dish ($10x\ 10^3$ cells/ cm^2) for new and several passages.

Seeding of cells in passages

After obtaining the first subconfluent monolayer, cells could be splitted into several passages as fellow; Cells in plates were washed 2 times with HBSS and incubated with Trypsin for approx. 5 min by 37°C. The cells were resuspended in TNS (4ml/Plate) and centrifuged with 900/rpm for 5 min; finally the cell pellet was loosened in suspended AECG medium. And the cell number was determined by means of counting slide. Cell pellet (40,000 cells/ml) solved in AECG medium and the cell suspension was used on coated plates for (NHBEC) and on non coated plate for PLC. Cells need approx. 1 week to reach (80 % -90 %) confluence.

2.4. Staining procedure

Staining of cultivated cells with Haematoxylin and Eosin

For this purpose the cells were grown as monolayers on cover glasses placed in cell culture dishes.The medium was removed and the cover glasses were washed with distilled water and fixed by 10% formalin for 10 min and washed with distilled water to remove excessive formalin. Subsequently, cover glasses incubated with haematoxylin for 1 min to stain the nuclei with blue colour and washed with Tape water and subsequently incubated with eosin 5 min to stain the cytoplasm with pink colour. Subsequently, followed by successive 5 min washing in 70%, 90% and absolute ethanol to remove the remnant of water. Cover glasses were cleaned with xylol for 5 min and were mounted on micro slides with mounting medium.

2.5. Vitality test

Cell viability is a determination of the fraction of vital metabolically active cells. There are numerous tests and methods for measuring cell viability e.g. measurement of lactate dehydrogenase (LDH) and ATP which are used as indicators for membrane integrity. One parameter used as an indicator for the metabolic activity of viable cells is the colorimetric assays. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann in 1983, which based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product. The colour can then be quantified using a simple colorimetric assay. All used substances were tested for toxic effects in the (MTT) assay on lung cells in order to check subtoxic concentration ranges.

Tumor lung cells (H322, H358, A549), NHBECs, and PLC were cultivated on 1.9 cm^2 growth area plastic gamma sterilized tissue culture test plates 24 (TPP-Switzerland-Europa). When adherent, cells were incubated with the substance to be tested at 37°C. Afterwards the old medium containing

the test-substance was removed and the cells were washed with PBS. Subsequently, the cells were incubated with the MTT-reagent (3-(4-5dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide) 0.7 mg/ml in PBS for 30 min (37°C) and then washed with PBS. Subsequently a mixture of formic acid (98%) and isopropanol (5: 95 v/v) was added to the MTT-reagent treated cells for 2 min. Formazan was determined by photometric measurement at 570nm.

All assigned substances (H_2O_2 ,$CuSO_4$, Hg Cl_2 , CSC) were used in the following concentration 100µM, 50µM, 20µM, 0.5 mg/ml respectively which were proved by MTT test to be non toxic, and more than 80 % of cells were alive after incubation with these substances up to 24h.

2.6. Immunoblotting techniques

Protein isolation

For this purpose the cells were cultivated on culture dishes with 6 cm diameters, and incubated by tested substance. Cells were washed with PBS 2 times, 1 ml Tritidy G was added for 5 min thoroughly mixed and harvested. This mixture could be kept in tubes with -80°C up to the processing of total protein fraction. For phase separation the tubes were incubated with 100µl 1-Chlor-3-Brom-Propan by room temperature (RT) for 5 min and centrifuged by 11000/rpm for 15 min by 4°C. The upper aqueous phase, which used for the RNA isolation was removed, the remaining aqueous phase and interphase were used for protein isolation.

For the DNA precipitation 300µl ethanol 100% admitted for 5 min by RT and centrifuged by 4500/rpm for 5 min by 4°C. The solution was removed carefully and divided on 2 tubes (2x 400ml). For the precipitation of the proteins 750µl isopropanol were added to the samples in each tube. The precipitation took place within 10 min at RT. The protein pellets formed by centrifugation 11000/ rpm for15 min with 4°C were washed 3 times with a 300 mM Guanidinhydrochlorid solution which composed of 1ml Guanidinhydrochlorid +19ml ethanol 100% (20 min by RT, and centrifugation: 5min, 8000/rpm, 4°C). Protein pellets were washed with 100% ethanol 2 times, incubated for 10 min by RT and centrifugation by 8000/ rpm for 5 min with 4°C. The protein pellets were united and dried 5 min by RT, and 2 min by 55°. Subsequently, the proteins were taken up to 1% SDS solution to get better solubility 5µl of 1N NaOH solution are used for 100µl protein solution.

Protein determination (protein assay)

The protein determination took place by means of Bicinchoninic acid (BCA) method (Smith et al., 1985), the principle of the (BCA) assay is similar to the Lowry procedure, in that both rely on the formation of a Cu^2 protein complex under alkaline condition followed by reduction of the Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the protein present. In the second step, BCA form a complex with Cu^{+1}, which is purple colored and is detectable by ELISA-Reader at 562 nm. Protein + $Cu2^{+}$ => Cu^1+ + BCA => Cu^1+ BCA complex.

For protein determination 5µl of the 1% SDS protein solution were taken and diluted 1:10 with 0.9% NaCl solution. BSA as calibration substance used in the following concentrations: 0.1; 0.2; 0.4; 0.6; 0.8; 1.0µg/µl, as fellow:

1-	100µl	0.9% NaCl		0µg/µl
2-	90µl	0.9% NaCl	10µl BSA	1µg/µl
3-	80µl	0.9% NaCl	20µl BSA	2µg/µl
4-	60µl	0.9% NaCl	40µl BSA	4µg/µl
5-	40µl	0.9% NaCl	60µl BSA	6µg/µl
6-	20µl	0.9% NaCl	80µl BSA	8µg/µl
7-	0µl		100µl BSA	10µg/µl

Subsequently, 10µl from the diluted protein solution and the standard protein (BSA) were used in two repeats of 96 microwell plate. The proof reagent consisted of 1part copper (II)-sulfate (4% w/v) and 49 parts of BCA. 200µl from proof reagent were laid to each well and the microwell plate was incubated for 30min with 37°C in the hybridizing incubator. ELISA-Reader measures the absorbance at 562 nm. From the BSA- calibration row the protein concentrations of the samples were determined.

Western Blot

For the Western Blot 30-50 µg protein were used. The samples were adjusted with distilled water to a uniform sample volume. Used sample buffer concentration is (33mM Tris, 0.6 % ß-Mercapto-Ethanol, 23mM SDS, 6.7 % Glycerin, $6x10^{-3}$ % Bromphenolblau). The samples were denatured for 5min with 95°C and cooled down afterwards on ice. The proteins were laid on a 10% SDS- PAGE gel (7 % collecting gel). Isolation of the proteins took place with 200V, 25mA/Gel for approx. 1h (run buffer: 26mM Tris, 190mM glycine, 1 % SDS). Subsequently; the proteins were transferred by means of wet transfer to a Nitrocellulose membrane. The membrane, the PAGE gel as well as 2 filter papers (Whatman chromatography paper) were moistened by transfer puffer (50 mM Tris, 0,5 mM Glycin, 0,05 % SDS). This sandwiche (filter paper, gel, membrane and filter paper) was placed in a transfer chamber and the protein bands were transferred from the gel into the membrane by overnight with 100 mA. The membrane was stained by Ponceau-red stain (0.1 % [w/v] in 5 % acetic acid[v/v]) to detect the presence of transferred proteins on the membrane. The Ponceau stain could be removed from the membrane with TBS-T (20mM Tris, 137mM NaCl, 0.1 % Tween [v/v], pH 7.3). The membrane was incubated with blocking solution (5 % Blocking agent, milk powder [w/v] in TBS-T) for one hour. Then the membrane was cut into 2 parts, upper one for PARP-1 protein(116 KD) which incubated with primary (PARP-1) antibody (Santa Cruz Biotechnology) in dilution 1:1000 for one hour by RT. The other one was stained for the Beta-actin (42KD), that was used as a house keeping gene which incubated with Beta-actin antibody (Novus Biologicals) in

dilution 1:5000 for one hour. The membranes were washed with TBS-T for 15 min and 2 times for 5min and were incubated with the secondary antibody goat-anti mouse HRP (Santa Cruz Biotechnology) with dilution 1: 20000 for one hour by RT. All these antibodies were diluted in the blocking solution. Again membrane was washed with TBS-T for 15 min and 2 times for 5min. Protein bands were visualized by Chemiluminescence's (ECL-Advanced- detection kit) (Amersham). This solution A and B of the kit which used 1:1, and about 3,5 ml of this mixture was used for one membrane for 5 min. The membrane were exposed to ECL films in dark room. The films were developed by solution from Kodak. The bands were quantitatively evaluated by Chemiluminescence densitometry in a scanner and analyzed by TINA 2.09 software.

2.7. Analysis of PARP-1 activity

The product of PARP-1 activity poly(ADP-ribose) (PAR) polymers binds specific to primary mouse monoclonal antibody (10H) raised against (PAR). This complex could be quantified using secondary antibody labelled with Fluorescein isothiocyanate (FITC) (goat anti-mouse IgG-HRP from Santa Cruz, Biotechnology) in situ immunoflurescence assay. PARP activity could be provocated through induction of DNA damage. This was achieved by treating the cells with H_2O_2. Degree of activity was related to the concentration of H_2O_2.

Cells were grown as monolayers on cover glasses placed in cell culture dishes and treated as indicated for the required experiments. Cover glasses were washed with PBS before addition of diluted H_2O_2 which is taken in tubes (according to the desired final concentration). Medium is added to the tubes with sterile pipette and poured back in the cell culture dishes which kept at 37°C for exactly 5 min. Subsequently, cover glasses were removed, rinsed with phosphate buffer saline (PBS) and fixed in ice-cold 10% trichloroacetic acid (TCA) for at least 10 min. After that they were rinsed with (PBS), followed by successive 5 min washing in 70%, 90% and absolute ethanol (-20 °C). Cover glasses were air-dried, rehydrated in PBS and incubated with 5 ug/ml purified monoclonal antibody 10H directed against (PAR) in blocking reagent (PBS,PH 7.4, 5% skim milk powder and 0.05%Tween 20) (dilution 1:300 in blocking reagent). This step was carried out in a humid chamber at 37°C for 30 min, followed by repeated washing of the cover glasses in PBS. The secondary, FITC conjugated goat anti-mouse antibody (dilution 1:50 in blocking reagent) was applied accordingly.

Finally Vectashield mounting medium containg 1 ug/ml Diamidine phenyl indol (DAP I) was used, and the Cover glasses were mounted onto micro slides. Fluorescence intensity was detected by using Zeiss microscope, X 40, and X100. Pictures of fluorescence signals and nuclear DAPI staining were captured with a digital camera. At least 50 cells per slide were selected. DAPI filter was used to see the stained DNA inside the nuclei. Fluorescence intensity was quantified using AQM Advance 6 imaging systems software by comparing the fluorescence intensities of individual

cells measured after the respective treatments as compared to H_2O_2-treated cells which were positive controls for PARP activity.

2.8. Statistical methods

Data are expressed as mean ± SE of the independently reproduced experiments. Statistical analysis were carried out using paired samples T test, independent samples T test, and ANOVA test by using SPSS program version 9.0.

3. Results

3.1. Test system

3.1.1. Lung cell culture

The culture of lung tumor cells

The tumor lung cells, A549, H358 and H322 (**Fig. 4**), have been used as model cultures and have been cultivated under the culture conditions mentioned in materials and methods.

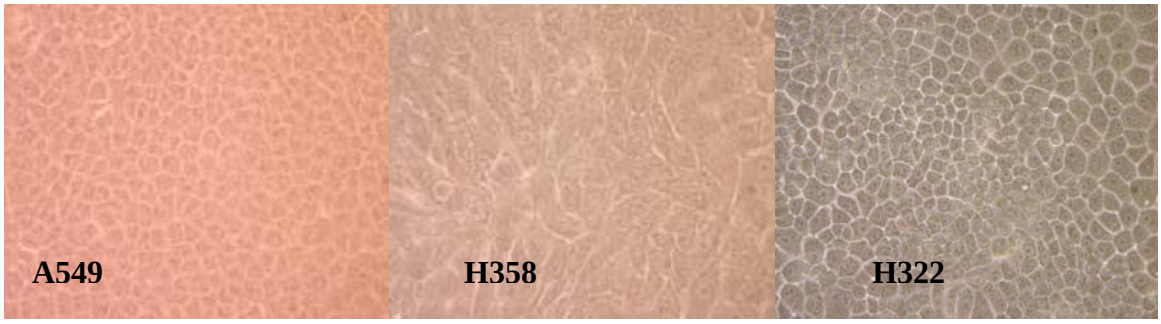


Fig. 4. Microscopic image of human lung tumor cells. A549, a lung adenocarcinoma cell line with morphological AII cell characteristics; H358, a non small cell lung cancer cell line with AII cell morphology and H322, a non small cell lung cancer cell line with Clara cell morphology.

The culture of normal human lung cells

For investigation of the cellular expression and activity of PARP-1 protein served cultivated human lung cells, which differentiated on suitable culture conditions. Safety margin tissue specimens obtained from patients that underwent lob- or total pneumonectomy as a result of primary or lung metastases insults, which were proven to be free of tumor or pathological signs by histopathological examination during operation, were used to obtain serum-free explant cultures of normal bronchial and peripheral lung cells. These cells were cultivated under serum free conditions in order to expand the cultures of NHBECs and PLCs (**Fig. 5**). The cultures consisted of cells with nearly 95 % epithelial characteristics. This was indicated by positive immunohistochemical staining for cytokeratins (CK7 and CK8/18) which are present in epithelial cells, and negative counterstaining for anti-human vimentin, smooth muscle actin and desmin which present in mesenchymal cells and muscle cells as stated by (Lehmann et al.,2001; Torky et al., 2005).

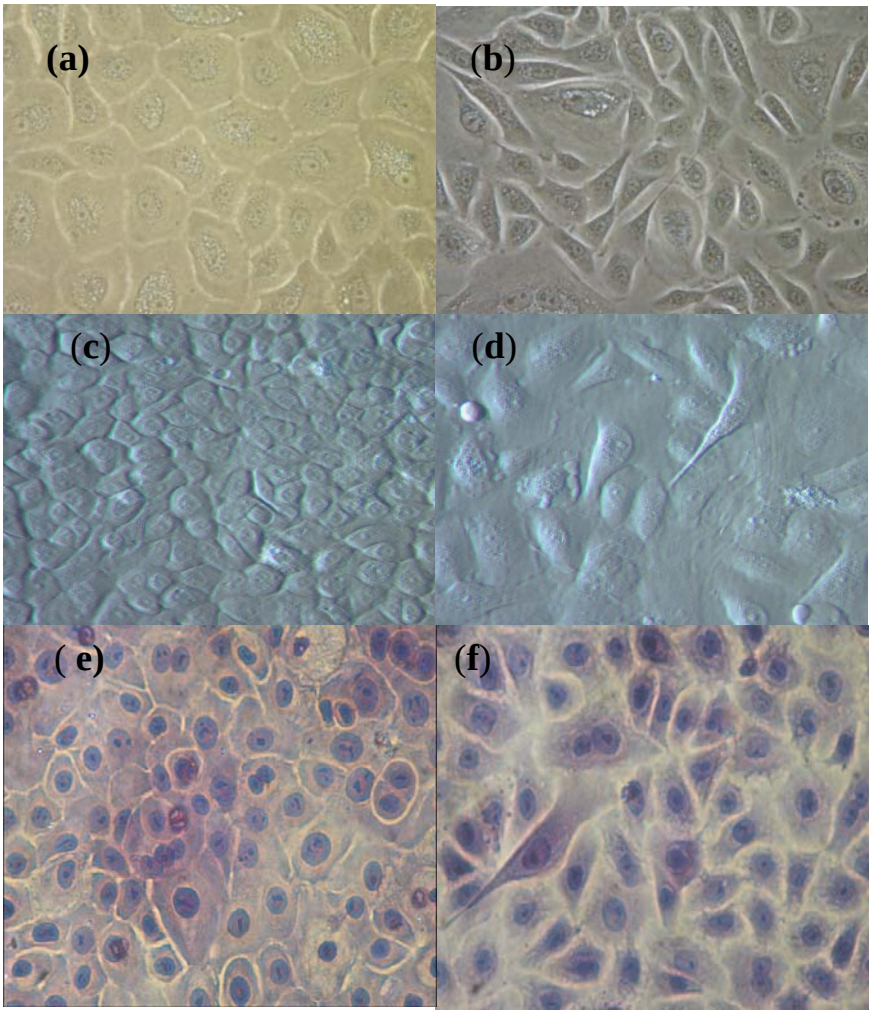
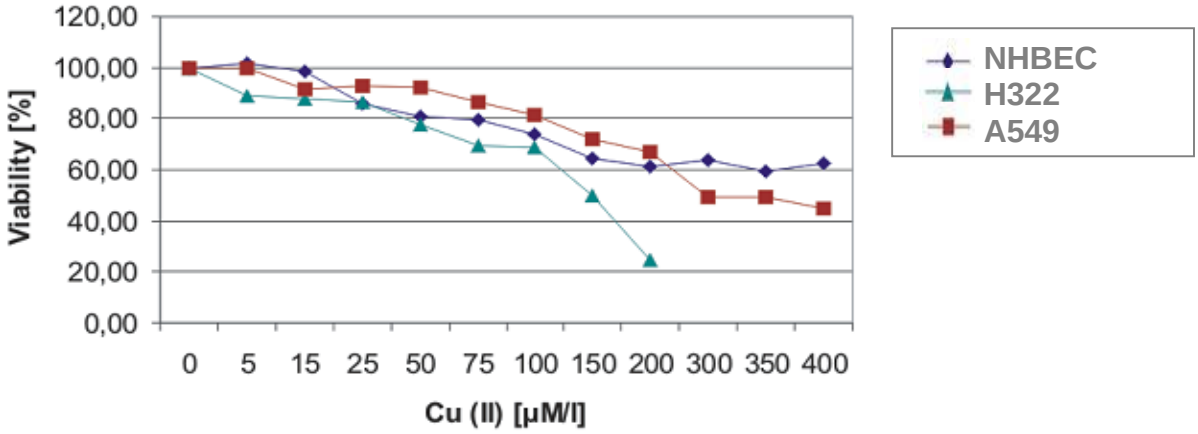


Fig. 5. Microscopic image of normal human lung cells. Monolayer of normal human bronchial epithelial cells (NHBECs) and peripheral lung cells (PLCs), in light microscopy (a-b), Hofmann contrast microscopy (c-d) and stained with Haematoxylin and Eosin (H&E) showing dividing cells (e-f)

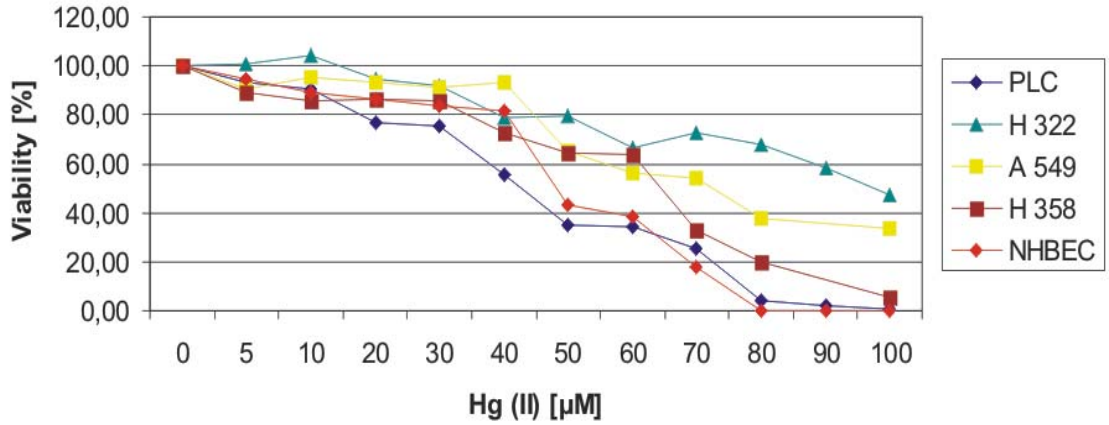
3.1.2. Viability test

All used test-substances were examined for toxic effects using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromids (MTT) assay on lung cells in order to check subtoxic concentration ranges. Basically this test depends on the conversion of the yellow MTT-reagent into the blue formazan under the influence of cellular dehydrogenases which takes place only in vital cells. Hydrogen peroxide (H_2O_2), copper sulphate ($CuSO_4$), mercuric chloride ($Hg\ Cl_2$) and Cigarette smoke condensate (CSC) were tested for duration up to 24h (**Fig. 6**).

(A)



(B)



(C)

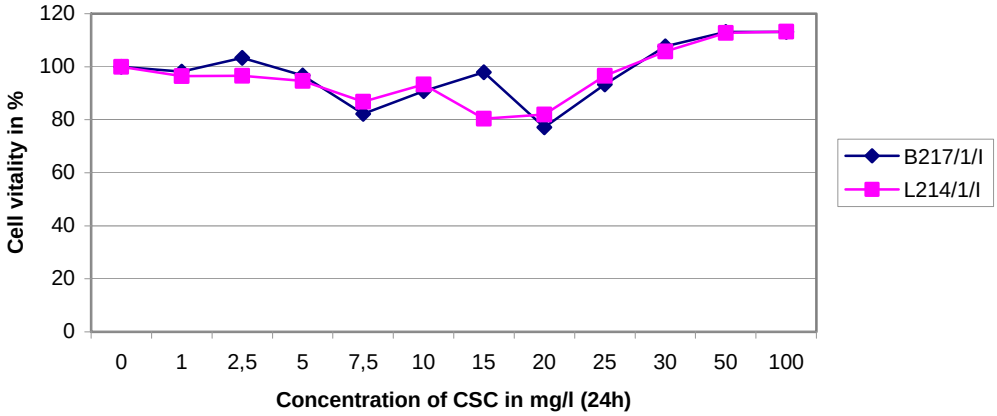


Fig. 6. Cytotoxicity of Copper, Mercury and Cigarette Smoke Condensate. Cu SO_4 (A), $HgCl_2$ (B), and CSC (C) in tumor cell line (A549, H358 and H322), normal human bronchial epithelial cells. (HBECs), and peripheral lung cells (PLC).The cultures were treated with test compound for 24h.Cytotoxicity was monitored by the MTT assay. Untreated cultures were used as controls and their values were set as 100 %.

3.2. Basic PARP-1 protein expression

3.2.1. Normal bronchial cells versus A549 lung tumor cells

Most studies on PARP-1 expression have been performed on either animal cells or human tumor cells. In this work we have studied whether PARP-1 is expressed in normal human lung cells in culture, and whether these cultures are suitable for studying the modulation of PARP-1 expression and function by external factors. The focus of interest was whether A549 and NHBECs are different with respect to basal expression of PARP-1 because many control mechanisms in cellular homeostasis are modified in tumor cells compared with normal cells. The cells were cultivated on culture dishes with 6 cm diameters and proceeded for immunoblotting. PARP-1 protein is expressed in both lung cell types. The amount of PARP-1 protein was measured by Western blotting and peroxidase stain and visualized by enhanced chemiluminescence ECL with advanced ECL reagent. The signal in semi-quantitative analysis of the immunoblots is substantially higher in A549 tumor cells than in NHBECs (**Fig. 7**).

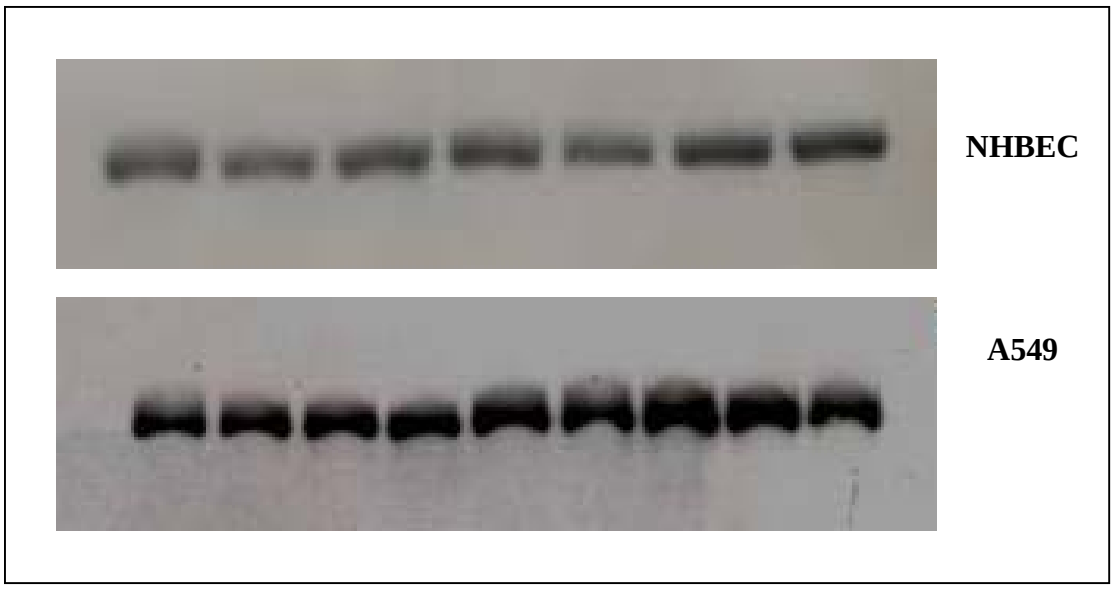


Fig. 7. Basic expression of PARP-1 protein in NHBEC and A549 human lung cell line
NHBEC lanes: total protein (30 μg) from ID 207
A 549 lanes: total protein (30 μg) from different passages
Protein expression was measured by Western blot analysis with a primary (parp-1) antibody (Santa Cruz Biotechnology) and a secondary antibody goat-anti mouse HRP (Santa Cruz Biotechnology). The signal was recorded by chemiluminescence on ECL film and densitometrically measured in a photo imager by TINA2.09 software.

3.2.2. Inter-individual differences

The human lung is exposed to and affected by many airborne and bloodborne foreign compounds, and this exposure is variable between individuals. A different level of PARP-1 protein may contribute to this variation. The expression of PARP-1 protein in NHBECs was compared between six donors in order to get an impression about inter-individual differences. Total protein of first explants from bronchial epithelium was assayed by immunoblotting for PARP-1 expression in six patients (all patients were male and had a positive smoking history). The levels for the highest and lowest expression of PARP-1 protein differed by a factor of 2.3 in between NHBECs. The difference in the mean expression of PARP-1 between these donors was statistically significant (P value <0.05, ANOVA test) (**Fig. 8**).

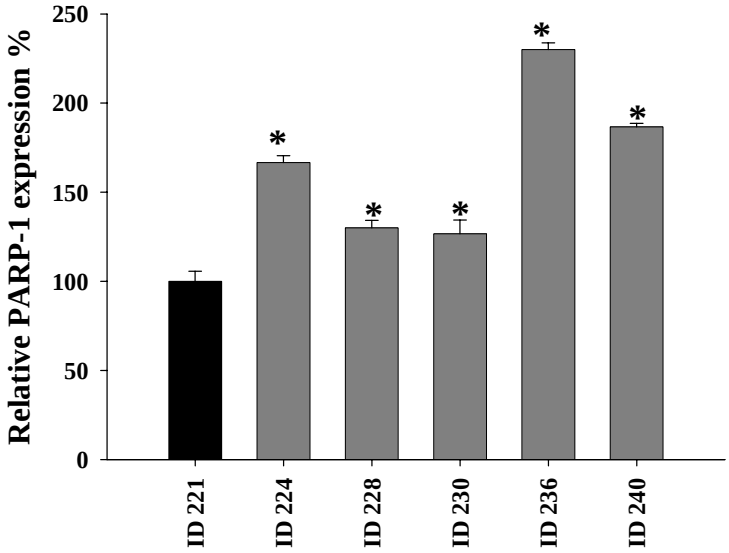


Fig. 8. Inter-individual variations of PARP-1 protein expression in NHBEC. Total protein was obtained from the first explant of resection material from lung cancer cases, separated by PAGE and immunochemically detected by Western blotting. Immunoreactive bands were visualized using the peroxidase reaction plus enhanced chemiluminescene (ECL). The individual values of expression levels are correlated to ID 221 which is represented as 100 %. The horizontal axis refers to the patient ID. * = P value <0.05.

3.2.3. Adaptation to culture

For three patients, ID 207 (female, age 49 y), ID 208 (female, age 78 y) and ID 230 (male, age 74 y), four and five consecutive bronchial explant cultures (generations) were followed in order to investigate the expression of PARP-1 protein in long-term culture of NHBECs. The level of PARP-1 protein expression was measured by immunoblotting and densitometric quantification of the signals. PARP-1 expression was increased from the second to the fifth generation in comparison to the first one. This increase in PARP-1 expression was statistically not significant in each generation compared to the first one (P value > 0.05 independent T test). The values for the highest and lowest protein expression differed among generations by factor 1.3. Generally the difference between expression in different generations was statistically not significant (P value > 0.05, ANOVA test) (**Fig. 9**). Although this experimental group is small, the expression of PARP-1 protein in vitro is relatively constant.

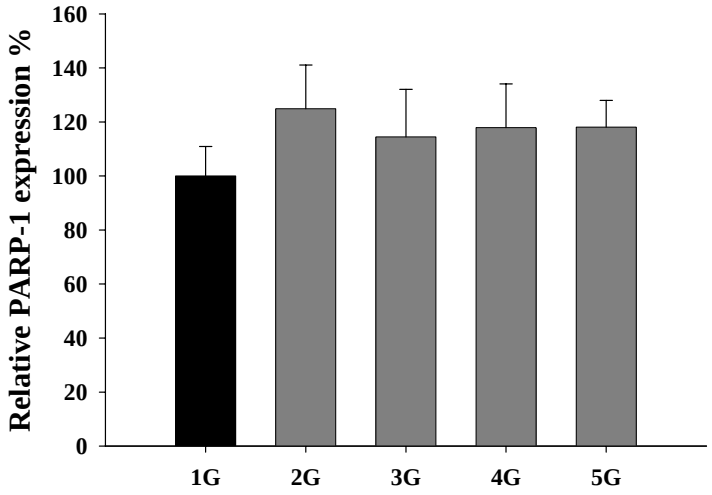


Fig. 9. Follow up of PARP-1 protein expression in progressive generations of NHBECs. The level in the first explant culture (generation) was taken as the reference (100 %) for correlation. The culture duration for monolayers was 97 days for ID 207 and 108 days for ID 208 until 5th generation and 85 days for ID 230 until 4th generation.

3.3. Effect of metals on PARP-1 protein expression

Exposure to heavy metals is a common phenomenon due to their environmental persistence. Some heavy metals are known to be toxic for humans. A common toxic mechanism is that metals and / or their oxides enhance the formation of reactive species. These induce conformational changes of biomolecules or oxidative DNA damage. Copper has a role to maintain the metabolism of the human body. However, at higher concentrations can lead to poisoning. Mercury has toxicological relevance to human being because of its abundant presence as methylmercury in the food chain. In our study two elements of heavy metals copper sulphate ($CuSO_4$) and mercuric chloride ($HgCl_2$) have been used as representatives for the metal group. Furthermore, the exposure to metals may be either single or repeated, it was essential to verify the effect of these metals in both short and long term exposure.

3.3.1. Short term exposure to copper

Cultivated NHBECs from 3 different patients (ID 227, 228, 243) were incubated with Cu SO_4 (50µM) for 24h. After that, the cells have been harvested and tested by western blotting for the amount of PARP-1 protein expression. In the overall population, the expression of PARP-1 correlated with treatment with $CuSO_4$. Accordingly, the expression of PARP-1 in response to CuSO4 was statistically non significant higher by factor 1.23 in cells with than without, (P value >0.05, paired samples T test) (**Fig. 10**).

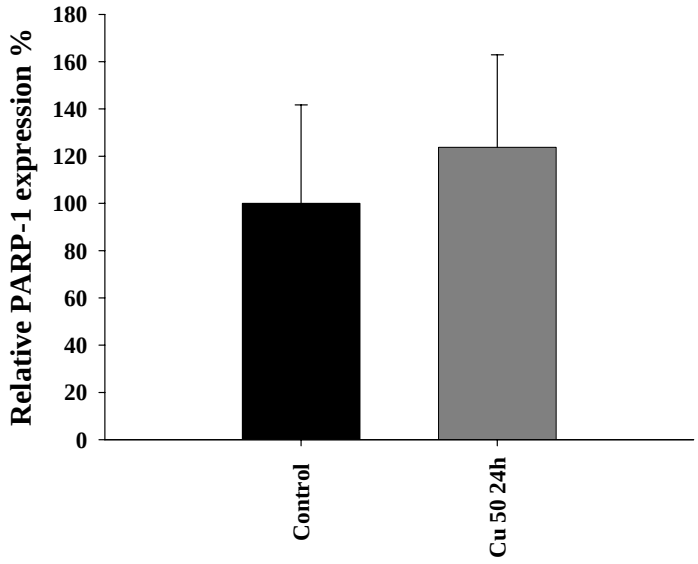


Fig. 10. Expression of PARP-1 protein under the effect of copper in short term. NHBEC were incubated with $CuSO_4$ 50µM for 24h. The total protein was laid on a 10% SDS- PAGE gel (7 % collecting gel) for electrophoresis and separated proteins were transferred by means of wet transfer to a Nitrocellulose membrane. The membrane was incubated with primary (PARP-1) antibody in dilution 1:1000 for one hour by RT and with the secondary antibody goat-anti mouse HRP at dilution 1: 20000 for one hour by RT. Detection took place by means of Chemiluminescence's on ECL films in dark room. The quantitative evaluation of the attempts densitometries was done by means of TINA2.09 software. The control was taken as the reference for correlation 100 %.

3.3. 2. Long term exposure to copper

NHBECs were treated with $CuSO_4$ over three weeks in order to clarify whether long term exposure will lead to an adaptive response which is different from short term exposure. The level of PARP-1 protein expression was statistically non significant between $CuSO_4$ treated cells versus non treated cells. The protein level was quantified by Western Blotting. This finding is identical to the effect of short term exposure (**Fig. 11**).

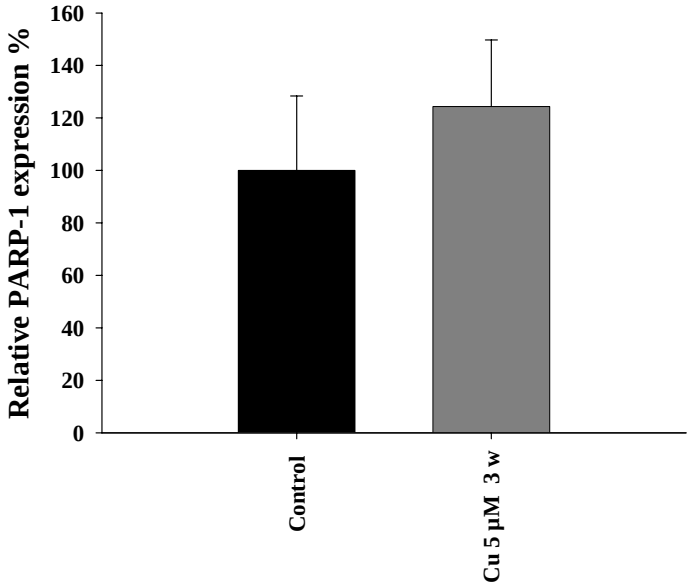


Fig. 11. Expression of PARP-1 protein under the effect of copper in long term. NHBECs were incubated with $CuSO_4$ 5µM for 3 weeks. The proteins were laid on a 10 % SDS- PAGE gel for electrophoresis and transferred by wet transfer to a Nitrocellulose membrane. The membrane was treated with primary (PARP-1) antibody and then with the secondary antibody goat-anti mouse HRP. Detection took place by means of Chemiluminescence's on ECL films. The quantitative evaluation of the attempts densitometries took place by means of TINA2.09 software. The horizontal axis refers to the control and used $CuSO_4$ and the vertical axis refers to the relative PARP-1 expression.

3.3. 3. Short term exposure to mercury

The NHBECs were cultivated on culture dishes with 6 cm diameters, and then incubated with non lethal concentration of ($HgCl_2$ 20µM) for 24h as indicated by vitality test. After that, the cells have been harvested and protein expression was detected immunologically by western blotting. $HgCl_2$ has increased the expression of PARP-1 protein by factor 1.3 in comparison to the control in cells derived from 3 different patients (ID 227, 228, 243) in 3 independent experiments. This increase in expression could not be proved statistically to be significant (P value >0.05, paired samples T test) (**Fig. 12**).

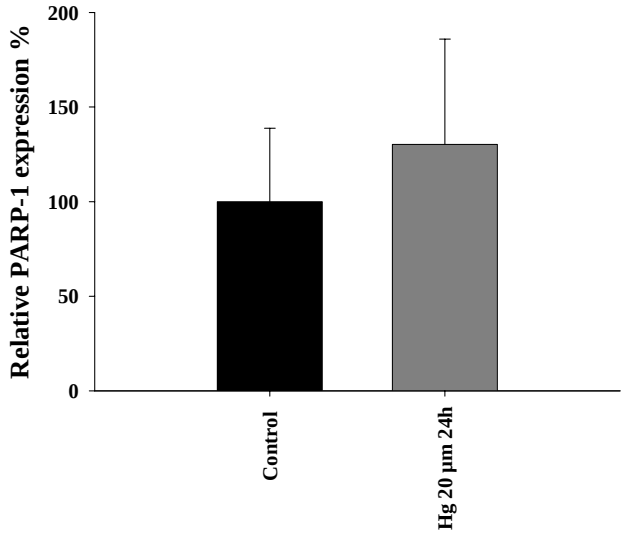


Fig. 12. Expression of PARP-1 protein under the effect of Mercury in short term.
NHBECs were incubated with $HgCl_2$ 20 µM for 24h. The proteins were laid on a 10 % SDS- PAGE gel for electrophoresis and were transferred to a Nitrocellulose membrane. The membrane was incubated with primary (PARP-1) antibody and then with the secondary antibody goat-anti mouse HRP. Detection took place by means of Chemiluminescence's on ECL films. The quantitative evaluation of the attempts densitometries took place by means of TINA2.09. The control was taken as the reference for correlation 100 %.

3.3.4. Long term exposure to mercury

To compare short and long term exposure to mercury. NHBECs derived from 3 patients (ID 227, 228, 243) were incubated with Hg Cl_2 2.5µM for 3 weeks. The expression was increased by factor 1.27 compared to the control. Again this increase was statistically not significant (P value >0.05, paired samples T test) (**Fig. 13**).

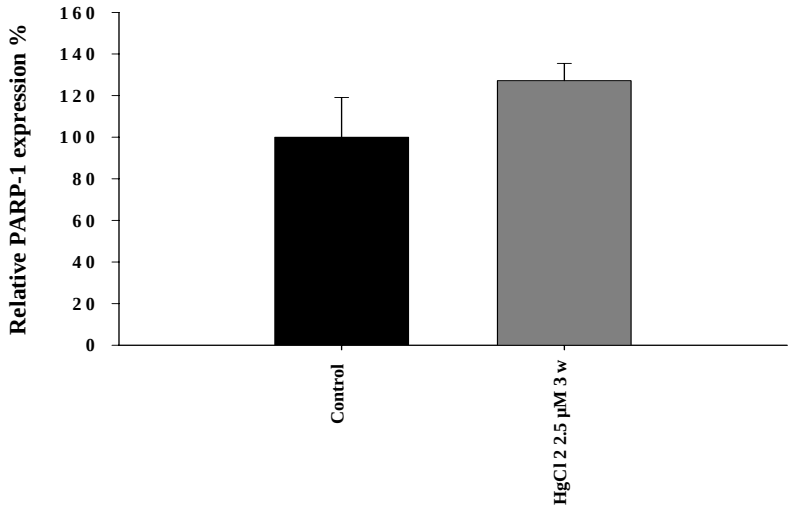


Fig. 13. Expression of PARP-1 protein under the effect of Mercury in long term.
NHBECs were incubated with $HgCl_2$ 2.5µM for 3weeks, and the expression of PARP-1 was tested by Western blot. The control was taken as the reference for correlation. The horizontal axis refers to the control 100 % and used $HgCl_2$ and the vertical axis refers to the level of PARP-1 expression.

3. 4. Effect of cigarette smoke condensate on PARP-1 protein expression

After detection of the basal expression of PARP-1 protein in NHBECs and the investigation of its modulation by $CuSO_4$ and $HgCl_2$, the effect of Cigarette Smoke Condensate in PARP-1 protein has been tested, not only because of its relevance as lung inflammation inducing agents but also as metal containing complex mixture. CSC was used in non lethal concentration as proved by MTT test.

3.4.1. Short term exposure

The NHBECs cells derived from 3 individual (ID 230, 240, 244) were cultivated then incubated with CSC (0.5 mg/l) for 24h. Subsequently, the cells were harvested and subjected to analysis of protein by western blotting. CSC increased parp-1 expression by 1.4 fold compared to the control (basal cellular PARP-1 expression) but this increase was statistically not significant (P value >0.05, paired samples T test) (**Fig. 14**).

CSC (0.5 mg/l) for 24h

ID	PARP-1 expression in control	PARP-1 expression with CSC
230	100	159
240	100	117
244	100	114

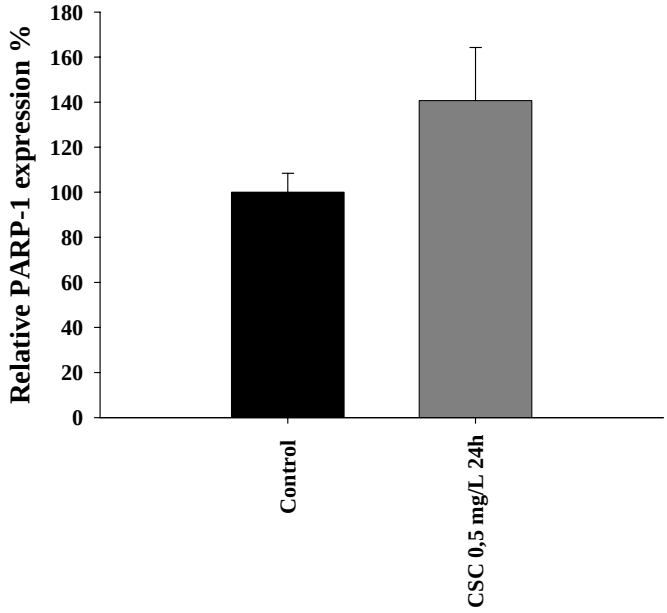


Fig. 14. Expression of PARP-1 protein under the effect of CSC exposure in short term.
Cultivated NHBECs were incubated by CSC 0.5mg/L for 24h. After that the cells harvested and tested by western blotting for the amount of PARP-1 protein expression. Detection took place by means of Chemiluminescence's on ECL films in dark room. The quantitative evaluation of the attempts densitometries took place by means of TINA2.09. The horizontal axis refers to the control (100 %) and used CSC and the vertical axis refers to the level of PARP-1 expression.

3.4.2. Long term exposure

Hence the chronic exposure of human-being to cigarette smoke is not uncommon, it was pivotal step to investigate the effect of prolonged exposure to CSC on PARP-1 expression. PARP-1 expression was assessed by western blotting in NHBECs obtained from 3 different patients (ID 230, 240, 244). Semiquantification of PARP-1 showed significant increase of PARP-1 expression in CSC (0.5 mg/l for 3 weeks) treated cells compared to the non treated control (P value < 0.05 paired samples T test) (**Fig. 15**).

CSC (0.5 mg/l for 3 weeks)

ID	PARP-1 expression in control	PARP-1 expression with CSC
230	100	171
240	100	162
244	100	148

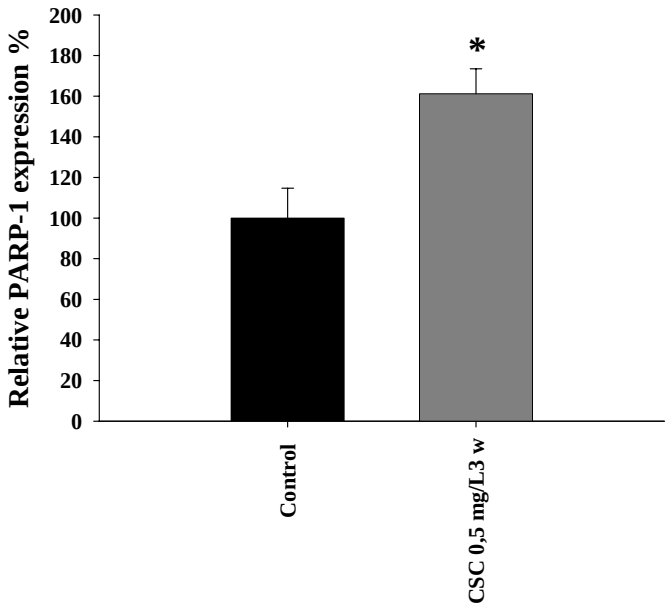


Fig. 15. Expression of PARP-1 protein under the effect of CSC exposure in long term.

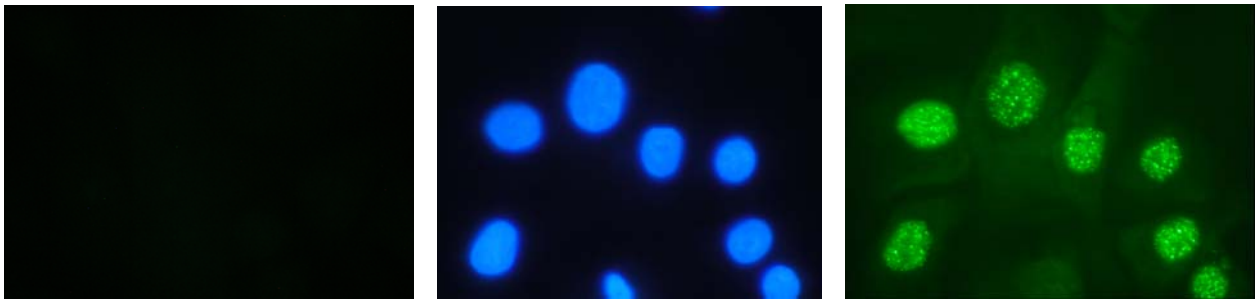
Cultivated NHBECs were incubated with CSC 0,5 mg/l for 3 weeks. After that the cells harvested and tested by western blotting for the amount of protein expression. The protein level was detected by Chemiluminescence's on ECL films in dark room. The quantitative evaluation of the attempts densitometries took place by means of TINA2.09. The horizontal axis refers to the control (100 %) and used CSC and the vertical axis refers to the level of PARP-1 expression. ***** = P value <0.05.

3.5. Activity of PARP-1 in primary lung cells

3.5.1. Basal activity

The functional activity of PARP-1 protein was detected by its early product which is the poly(ADP-ribosyl)ation of damaged sites of DNA in intact cells. The binding of poly(ADP-ribose) (PAR) to DNA strand breaks can be induced by H_2O_2 stress. However, the PAR-DNA complex can be detected only for a short while because it is cleaved very rapidly. The assay was performed by an immunocytochemical method with a highly specific monoclonal antibody against PAR visualized by a secondary FITC-conjugated antibody (Buerkle et al., 2001a). NHBEC were grown on cover glasses and were exposed with H_2O_2, at concentrations of 0.05 – 0.3 mM for 5 minutes to induce DNA damage and as a consequence thereof induce poly(ADP-ribosyl)ation. Untreated control cells showed no nuclear fluorescence signals. H_2O_2-treated NHBECs showed strong nuclear fluorescence signals indicating high PARP activity. Counter staining of the nuclei with DAPI proves that the PAR-related fluorescence signal is localised in the nuclei of NHBEC (**Fig. 16**).

The intensity of the fluorescence signal correlated with the concentration of H_2O_2 (Figure 18 a, b). Some nuclei of NHBEC showed chromatin condensation after exposure to 0.2 mM H_2O_2. Chromatin condensation was found abundant in NHBEC treated with 0.3 mM H_2O_2. NHBECs are relatively sensitive for cytotoxicity by H_2O_2. However, the concentration of 0.1 mM was well tolerated. So, 0.1 mM H_2O_2 was chosen as the trigger to induce poly(ADP-ribosyl)ation of DNA as a marker for PARP-1 activity. Treatment with H_2O_2 for 5 min were sufficient to induce detectable activity (**Fig.17 a, b**).



Control **DAPI stain** **PAR Immunostain**

Fig. 16. Basal activity of PARP-1 protein in normal human bronchial epithelial lung cells.
NHBEC grown on cover glasses were stressed with H_2O_2 (100 µM, 5 min). Immunostaining was performed with 5 ug/ml highly specific monoclonal antibody directed against (PAR) and a FITC conjugated goat anti-mouse antibody. Nuclei of cells were stained with diamidine phenyl indol (DAPI, 1 ug/ml). Fluorescence intensity was imaged with a Zeiss microscope (X 40 and X100) with appropriate filters.

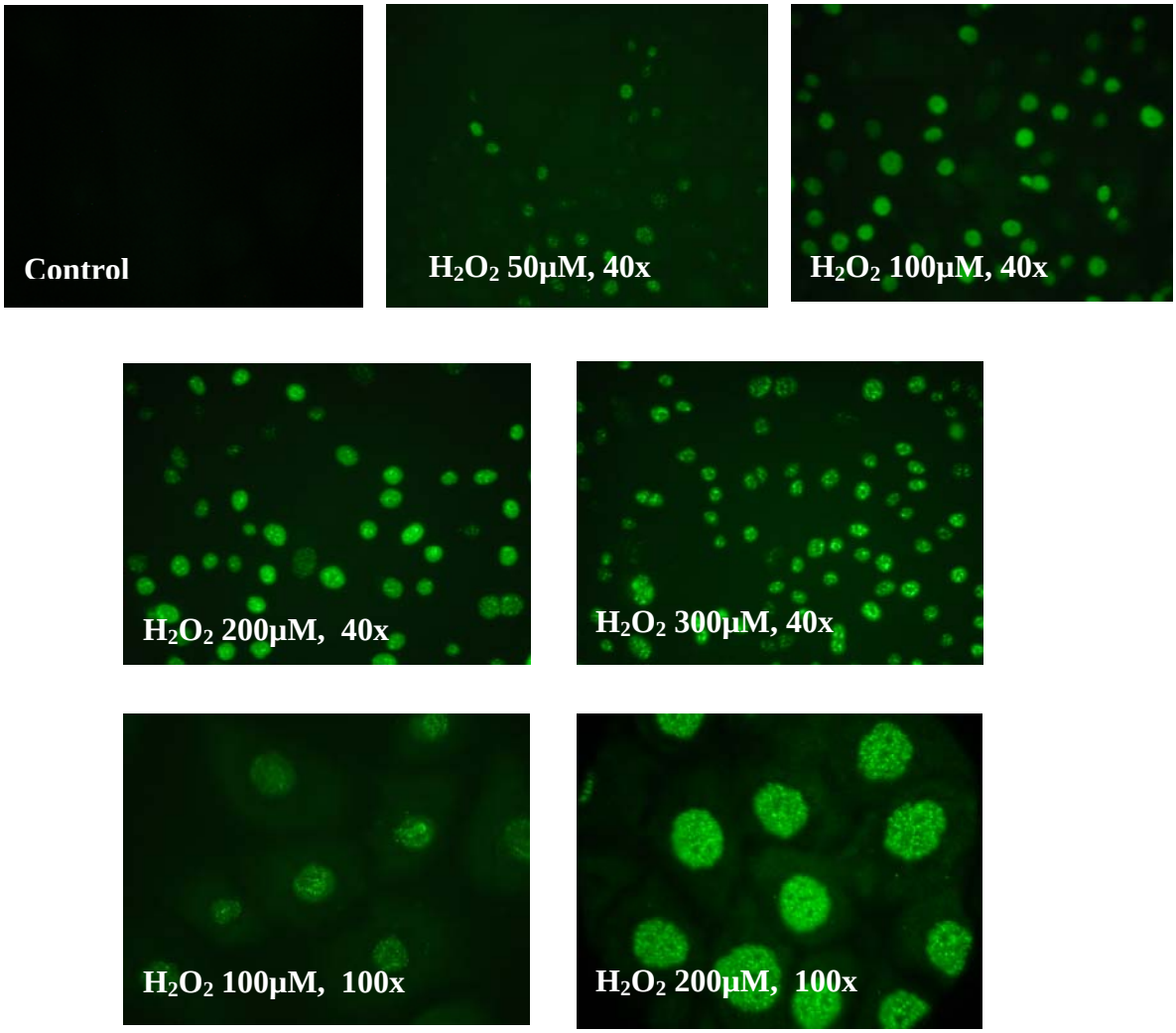


Fig. 17 a. PARP-1 activity in NHBECs with different H_2O_2 concentrations.
Immunofluorescence detection of basal activity of PARP-1 protein in cultivated human lung cells after induction of poly(ADP-ribosyl)ation by H_2O_2 with different doses for 5 min. Cells were washed, fixed with trichloro acetic acid (TCA), and further processed as in **Fig. 14** material and methods. 40 and 100 magnifications were used by fluorescence microscope.

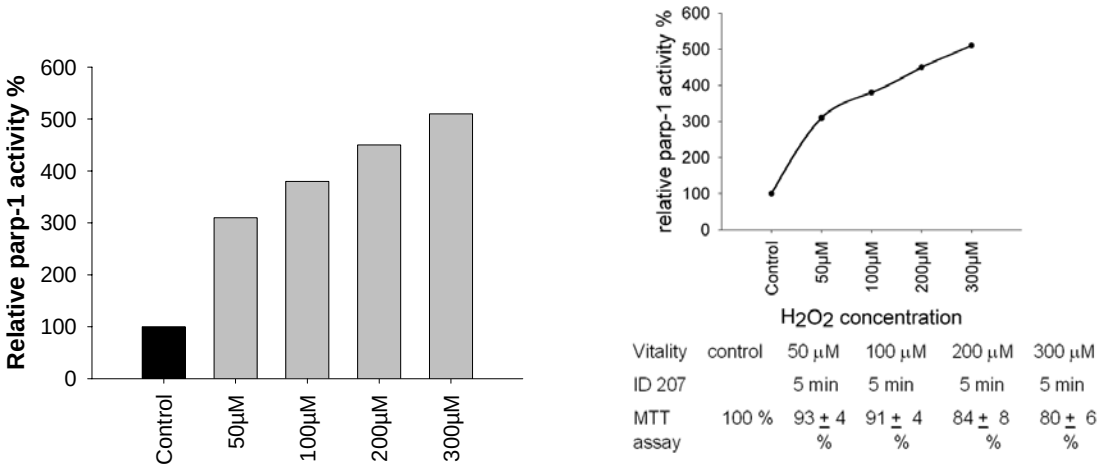


Fig. 17 b. Concentration- effect relation of Poly(ADP-ribosyl)ation in NHBEC after H_2O_2 stress. H_2O_2 was applied for 5 min and PARP-1 activity was monitored by quantitative immunofluorescence imaging as described in methods. Sensitivity of NHBEC against cytotoxicity of H_2O_2 (5 min). Cytotoxicity was quantified by the MTT assay.

3.5.2. Inter-individual differences

Poly(ADP-ribosyl)ation of damaged sites of DNA is a rapid and transient modification of chromatin protein which may be affected by many factors, such as oxidative stress, diseases and medical treatment. The PARP-1 activity in NHBECs from 10 different patients was analyzed in cells from the first explant culture. PARP-1 activity was detected in all samples with an observed inter-individual difference by a factor of 2.3 between the highest and lowest relative activity. ID 207 was taken as reference value. This variation in activity between patients was statistically significant (P value < 0.05, ANOVA test) (**Fig. 18**).

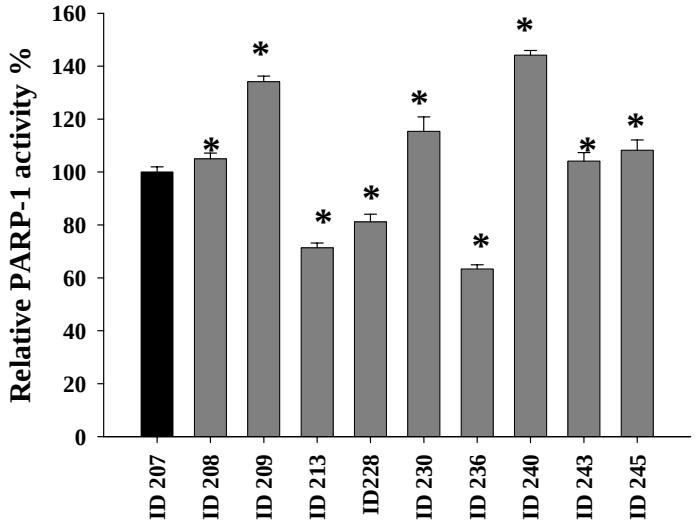


Fig. 18. Inter-individual differences of basal PARP-1 activity in NHBEC. Monolayers from the first explant cultures (generation) were studied for all IDs. The PARP-1 activity is expressed as ratio to the value of activity for ID 207. PARP-1 activity was induced by H_2O_2 (100 µM, 5 min) and measured by quantitative immunofluorescence imaging as described in Methods. * = P value < 0.05.

3.5.3. Influence of culture duration

The effect of prolonged cultivation of NHBECs and PLCs on PARP-1 activity was examined as cultivation itself may contain stress factors and this may affect PARP-1 activity. The intensity of fluorescence signals for poly(ADP-ribosyl)ation decreased gradually from the first to the fifth generation by 10-40 % during long term culture of NHBEC and PLC when compared to the first generation in NHBEC from 3 patients (ID 207, ID 208, ID 230). This decrease in PARP-1 activity was statistically significant in each generation compared to the first one, (P value < 0.05 independent samples T test). Generally, the decrease in the activity from the first generation till the fifth is statistically significant (P value < 0.05, ANOVA test). This may reflect the adaptation of these cells to the cultural condition and attenuation of stress related to culture. Fluorescence images are shown for NHBECs and PLCs obtained from ID 207 (**Table 2**, **Fig. 20**). The monolayers are from explant generations 1 to 5 for NHBEC (4, 6, 8, 11 and 13 weeks after start) and 1 to 4 for PLC (3, 6, 8, 11 weeks after start). A decrease of PARP-1 activity was also observed for subpassages 1 to 5 expanded from the first generation of NHBEC and PLC of ID 207. The time in culture was 5, 6, 7, 8, 9 weeks after start for NHBEC subpassages and 4, 6, 8, 9, 12, 15 weeks after start for PLC (**Table 2**, **Fig. 21**). However, PARP-1 is functioning in normal human lung cells in culture for at least 6- 8 weeks in NHBECs and 9 -12 weeks in PLCs.

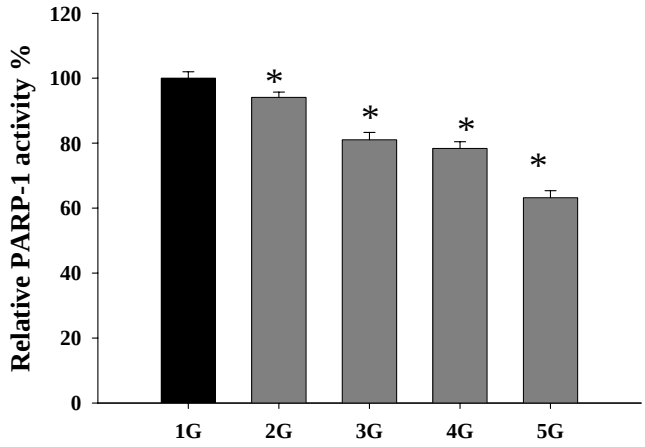


Fig. 19. Follow up of PARP-1 protein activity in progressive generations of NHBEC. Monolayers from the different generations were studied for all patients. PARP-1 activity was induced by H_2O_2 (100 μM, 5 min) and measured by quantitative immunofluorescence imaging as described in Methods. The activity in the first generation was taken as the reference for correlation. ***** = P value < 0.05.

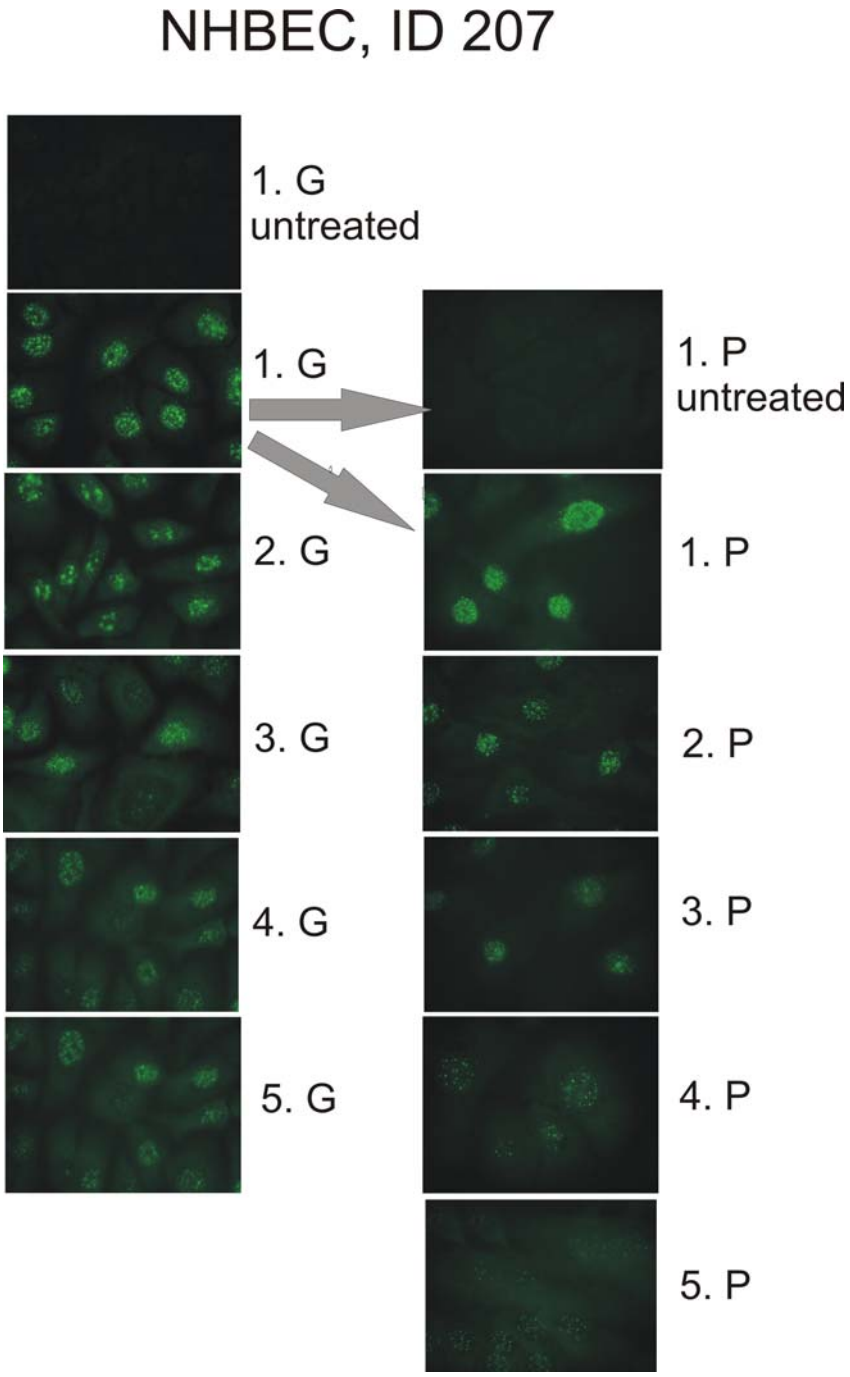


Fig. 20. Immunohistochemical image of PARP-1 activity in NHBEC in cell culture.
G = generations of explant cultures (see **Table 2**) **P** = subpassages from the monolayer of the first explant (see **Table 2**)

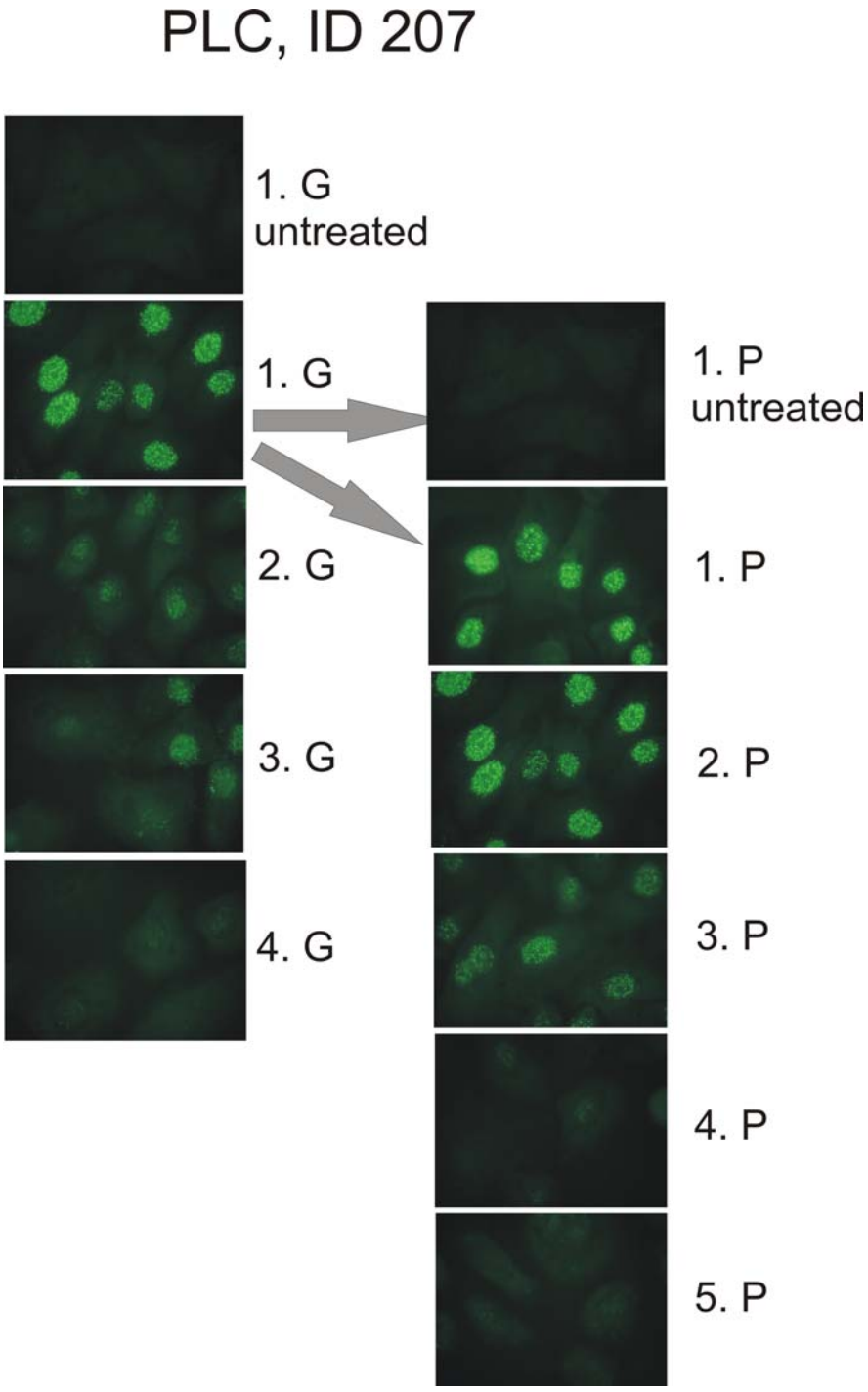


Fig. 21. Immunohistochemical image of PARP-1 activity in PLC in cell culture.
G = generations of explant cultures (see **Table 2**)
P = subpassages from the monolayer of the first explant (see **Table 2**)

Table 2a: Follow up of PARP-1 activity in explant cultures of human lung cells

Generation / Explant		G1	G2	G3	G4	G5
NHBEC	d	26	40	58	76	97
	%	100	96	86	65	60
PLC	d	20	41	58	76	--
	%	100	73	75	51	--

-- = no materials from this generation were available.

Table 2b: Follow up of PARP-1 activity in passages from the first generation of human lung cells

Passage / Explant		P1	P2	P3	P4	P5
NHBEC	days	33	40	47	54	64
	%	100	71	71	63	60
PLC	days	30	41	53	66	90
	%	100	84	60	40	40

PARP-1 activity was followed in NHBECs and PLCs generated from ID 207
(G+nr) indicate the series of explants (generations)
(P+nr) indicate the number of passages from the monolayer of the first generation
d = days in culture from start (= day of lung resection)
% the activity referenced to the first generation or to the first passage.
PARP-1 activity was measured by quantitative immunofluorescence imaging as described in methods .

3.6. Influence of copper on PARP-1 activity

3.6.1. Short term response

The extent of Poly (ADP-ribosyl)ation in intact cells was determined by immunological detection of (PAR). After detection of basal PARP-1 activity we investigated the effect of $CuSO_4$ on PARP-1 activity. Cells derived from 3 different patients (ID 227, 228, 243) were grown as monolayer on cover glasses placed in cell culture plates and treated with $CuSO_4$ (50µM) for 24h. Cover glasses were washed with PBS before addition of H_2O_2 in new medium and kept at 37°C for 5 min. After fixation and antibody treatment the activity was detected. With respect to poly(ADP-ribosyl)ation, no nuclear fluorescence signal was detected in cells exposed to copper sulphate alone at concentration 50µM. Nevertheless, copper significantly decreased the extent of H_2O_2 - induced activity, (P value < 0.05 paired samples T test) (**Fig. 22 a, b**).

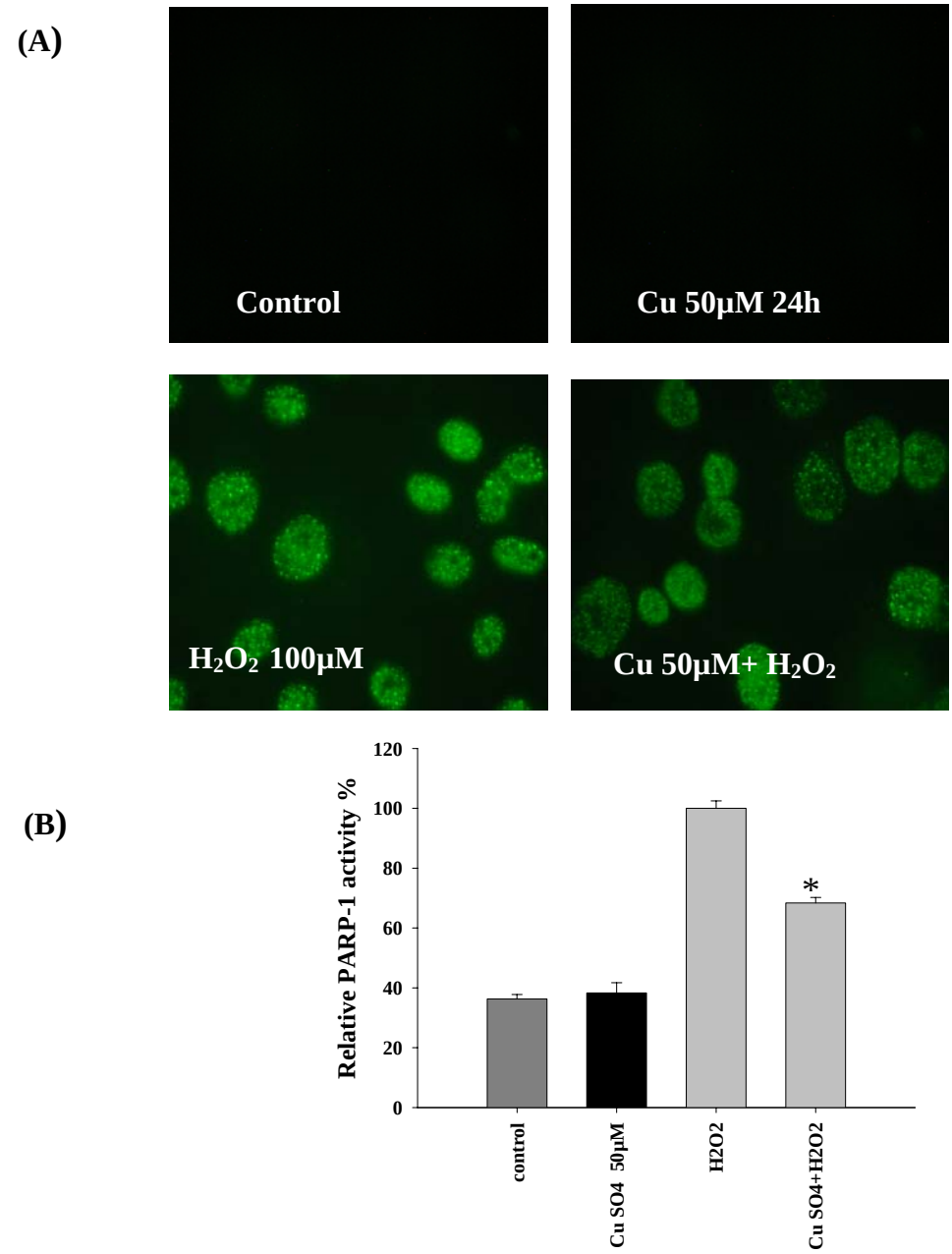


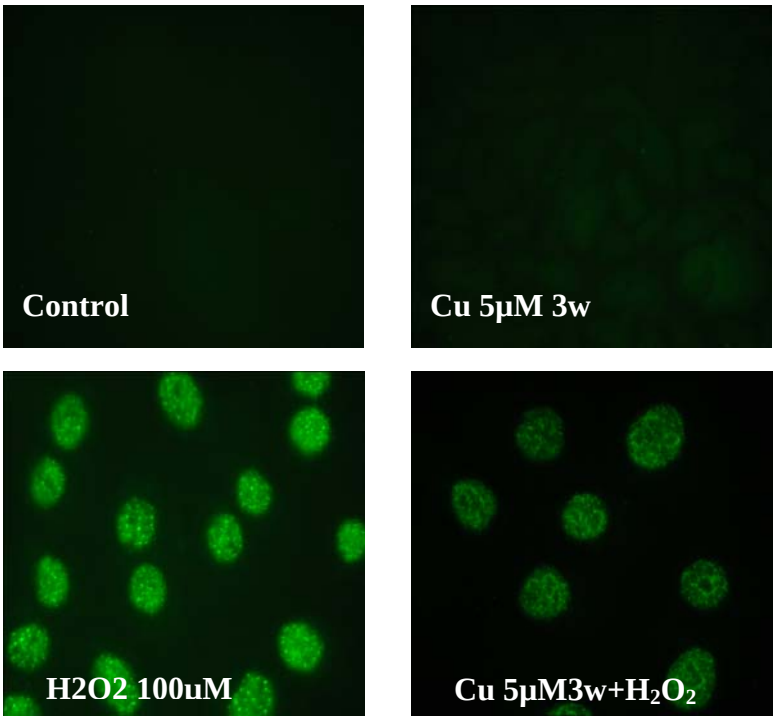
Fig. (22 a, b). Induction of poly(ADP-ribosy)lation by H_2O_2 and the inhibitory effect of Copper. NHBECs first explant, seeded on Cover glasses were pretreated with $CuSO_4$ 50µM for 24h, washed with PBS before addition of H_2O_2 , followed by fixation and immuno-detection with a monoclonal antibody against (PAR), and FITC conjugated secondary antibody. After fixation and antibody treatment the activity was detected. Fluorescence intensity was imaged with a Zeiss microscope (X100) with appropriate filters. In **(Fig. 22 b)** shown values are mean values based on evaluation of at least 150 cells derived from 3 independent experiments. The horizontal axis refers to the negative, positive controls and used $CuSO_{4;}$ and the vertical axis refers to the relative PARP-1 activity. * = P value.

3.6.2. Long term response

Environmental exposure is often long term and repeated, so it was appropriate to investigate the effect of long duration exposure, because the reaction of cells may differ from short term treatment. Cells from 3 different patients (ID 227, 228, 243) were grown as monolayers on cover glasses placed in cell culture plates and treated with $CuSO_4$ (5µM) for 3 weeks. No nuclear fluorescence

signal was detected in cells exposed to copper sulphate alone at concentration (5 µM). Nevertheless, copper significantly decreased the extent of H_2O_2 - induced activity also in this experimental set up, (P value < 0.05 paired samples T test) (**Fig. 23 a, b**).

(**A**)



(**B**)

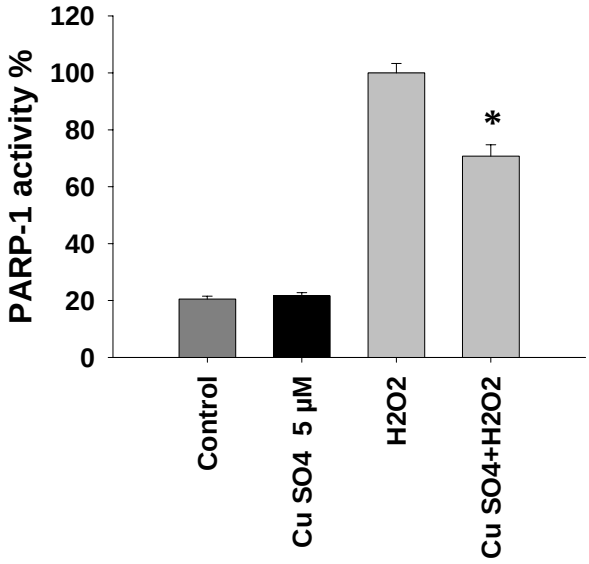


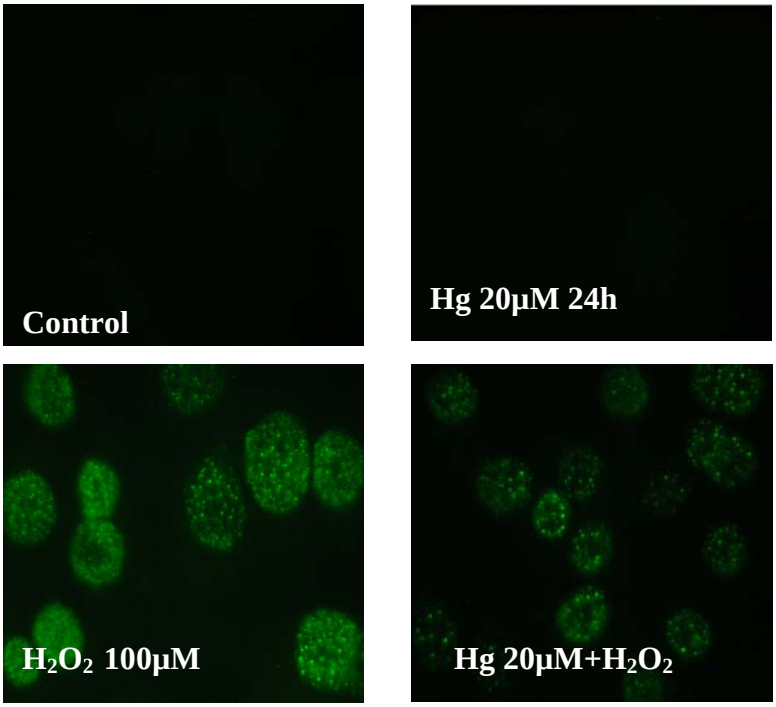
Fig. (23 a, b). Inhibitory effect of Copper on PARP-1 activity in long term exposure. Grown NHBECs on Cover glasses were pretreated with $CuSO_4$ 5 µM for 3 weeks, washed, fixed and further processed as in figure 22a, and as described in material & methods. Fluorescence intensity was imaged with a Zeiss microscope (X100) with appropriate filters. Immunofluorescence signals represent poly(ADP-ribose). In (**Fig. 23 b**) mean values are shown, which calculated from at least 150 cells and the independent repeats. The horizontal axis refers to the negative, positive controls and used $CuSO_4$; and the vertical axis refers to the relative PARP-1 activity. ***** = P value.

3.7. Influence of mercury on PARP-1 activity

3.7.1. Short term response

The effect $HgCl_2$ (20µM) for 24h on cellular poly(ADP-ribosyl)ation capacity are shown in figure 25. The amount of poly(ADP-ribosyl)ation i.e. PARP-1 activation, was negatively and significantly correlated with the exposure to $HgCl_2$ (P value < 0.05 paired samples T test). Although $HgCl_2$ alone did not initiate PARP-1 activation, it was able to decrease significantly the extent of H_2O_2 induced activity (**Fig. 24 a, b**).

(A)



(B)

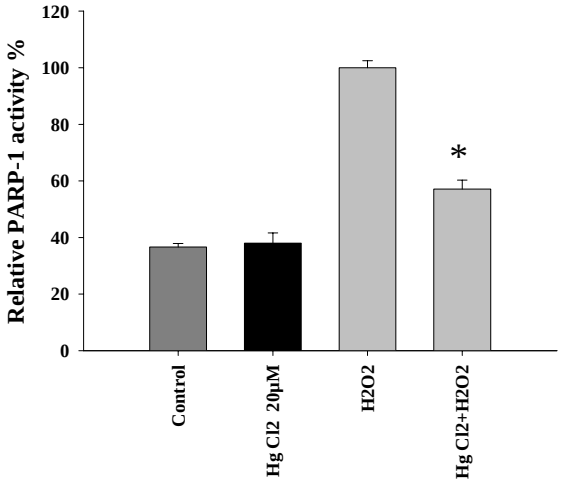
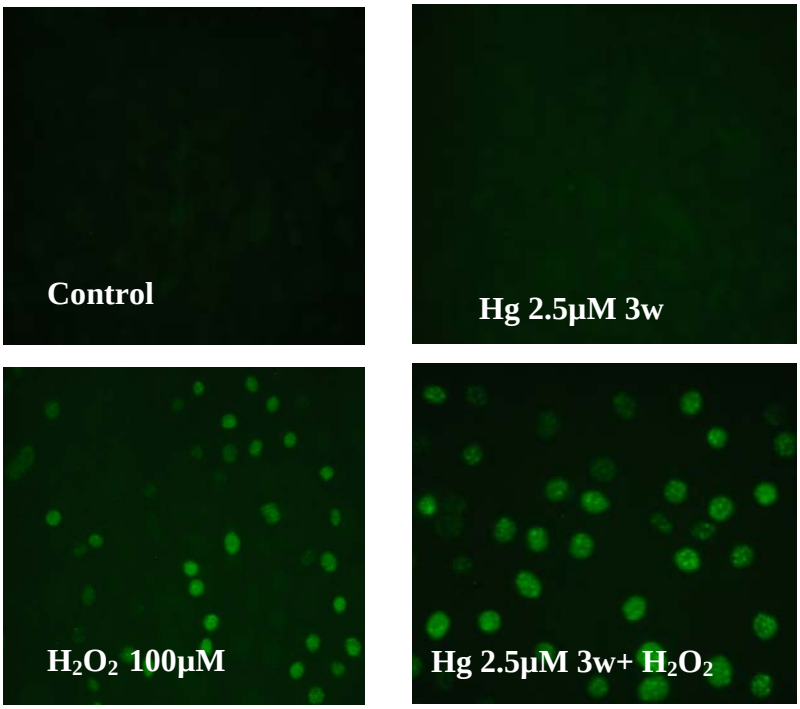


Fig. (24 a, b). Inhibitory effect of Mercury on PARP-1 activity in short term exposure.
Grown NHBECs on cover glasses were pretreated with Hg Cl_2 20 µM for 24h, washed, fixed and further processed as in figure 22a, and as described in material & methods. Fluorescence intensity was imaged with a Zeiss microscope (X100) with appropriate filters. Immunofluorescence signals represent poly(ADP-ribose). In (**Fig. 24 b**) mean values are shown, which calculated from at least 150 cells and the independent repeats from 3 independent experiments (ID 227, 228, 243). The horizontal axis refers to the negative, positive controls and used Hg Cl_2, and the vertical axis refers to the relative PARP-1 activity %. ***** = P value < 0.05.

3.7.2. Long term response

Following the detection of decreased PARP-1 activity with $HgCl_2$ in acute exposure, it was important to investigate the effect of chronic exposure, which is mostly present in our life; therefore we tested $HgCl_2$ in (2.5 µM) for 3 weeks to determine the effect of prolonged exposure. In order to highlight the effect of prolonged increase of $HgCl_2$ (2.5 µM for 3 weeks) in culture medium on the poly(ADP-ribosyla)tion capacity in individual donors (ID 227, 228, 243) the changes in % in PARP-1 activity of mercury, H_2O_2, H_2O_2 and treated cells are plotted against each other. Interpretation of the obtained records indicated that the inhibitory effect of $HgCl_2$ on PARP-1 activity in short term cultures disappeared in the long term one (**Fig. 25 a, b**).

(A)



(B)

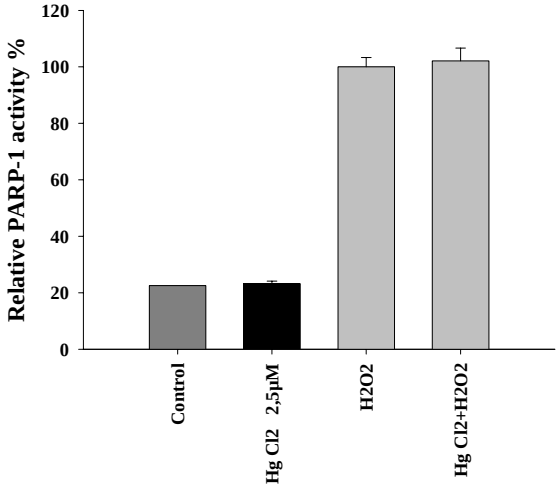


Fig. (25 a, b). PARP-1 activity in NHBECs exposed to Mercury in long term set up.
NHBECs were Grown on cover glasses and treated with Hg Cl_2 2,5 µM for 3 weeks, washed, fixed and further processed as described in material & methods. Fluorescence intensity was imaged with a Zeiss microscope (X40) with appropriate filters. Immunofluorescence signals represent poly(ADP-ribose). In (22b) shown values are mean values based on evaluation of at least 150 cells derived from 3 independent experiments (ID 227, 228, 243). The horizontal axis refers to the negative, positive controls and used Hg Cl_2; and the vertical axis refers to the relative PARP-1 activity %.

3.8. Influence of cigarette smoke condensate on PARP-1 activity

3.8.1. Short term exposure

CSC increased PARP-1 expression in cultivated NHBECs in short term exposure, therefore it was essential to examine the effect of CSC on PARP-1 activity to correlate the expression and activity of PARP-1. Cells derived from 3 different patients (ID 230, 240, 244) were treated with CSC 0.5 mg/l for 24h before addition of H_2O_2 for 5 min. After fixation and immuno-detection with antibodies the activity was measured by quantitative immunofluorescence imaging. With respect to poly(ADP-ribosyl)ation, nuclear fluorescence signal was detected in cells exposed to CSC alone without H_2O_2, and the activity induced by H_2O_2 has been significantly increased by CSC (P value < 0.05 paired samples T test) (**Fig. 26**).

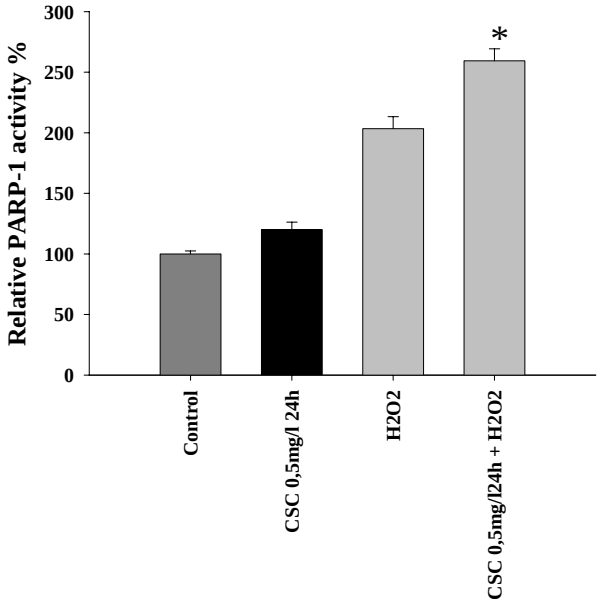


Fig. 26. PARP-1 activity in NHBECs under the effect of CSC in short term exposure.
This plot shows PARP-1 activity induced by H_2O_2 in NHBECs and the effect of CSC 0,5 mg/L for 24 h, alone and with H_2O_2. The designation 100 % refers to the response obtained after treatment with H_2O_2 for 5 min and taken as the reference for correlation. Shown are mean values based on evaluation of at least 150 cells derived from 3 independent experiments. The horizontal axis refers to the negative, positive control and used CSC; and the vertical axis refers to the relative PARP-1 activity %. * = P value < 0.05.

3.8.2. Long term exposure

The effect of prolonged exposure to CSC has been investigated, as in most cases smoking is a chronic habit, so it was important to investigate the response of long term exposure to CSC. Cultivated NHBECs derived from 3 different patients (ID 230, 240, 244) were incubated with CSC 0.5 mg/L for 3 weeks as mentioned under 3.4.6.1. The results obtained by the functional assay revealed increased poly(ADP-ribosyl)ation capacity with CSC treatment. Accordingly, nuclear fluorescence signal was detected in cells exposed to CSC alone without H_2O_2, and the activity induced by H_2O_2 has been significantly increased by CSC (P value < 0.05 paired samples T test) (**Fig. 27**).

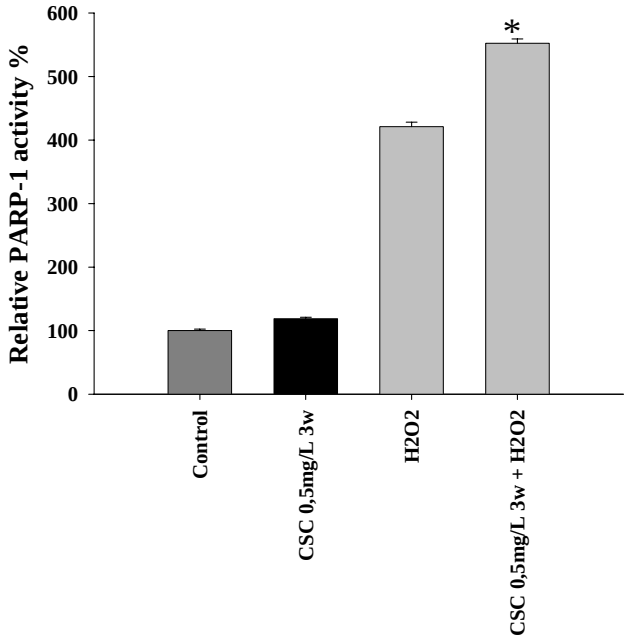


Fig. 27. PARP-1 activity in NHBECs under the effect of CSC in long term exposure.
This curve shows PARP-1 activity induced by H_2O_2 in NHBECs and the effect of CSC 0,5mg/L for 3 weeks alone and with H_2O_2. The designation 100 % refers to the response obtained after treatment with H_2O_2 for 5 min and taken as the reference for correlation. Shown are mean values based on evaluation of at least 150 cells derived from 3 independent experiments. The horizontal axis refers to the negative, positive control and used CSC; and the vertical axis refers to the relative PARP-1 activity%. * = P value < 0.05.

4. Discussion

Multiple chemical substances present in the air are known to elicit a direct toxic damage of lung. The consequences therefore, such as transient inflammatory reaction, a permanent lesion or tumor may not only depend on parameters of exposure but also on cell specific ability to cope with stressors. One of these mechanisms to cope with stressors is the activation of poly(ADP-ribose) polymerase-1 (PARP-1) in response to DNA damage (Bürkle, 2001b; Tong et al., 2001; Meyer-Ficca et al., 2005). At low levels of DNA damage, PARP-1 induces DNA repair and acts as a survival factor (Bürkle, 2001a). In contrast, with high levels of DNA damage, PARP-1 activity induces severe NAD^+ consumption as well as ATP depletion and ultimately failure of energy metabolism promotes cell death (Nicoletti & Stella, 2003; Virag & Szabo, 2002). Yu et al., 2002 showed that PARP-1 could play a role in caspase-independent apoptotic cell death through apoptosis inducing factor (AIF).

4.1. Role of PARP-1 within DNA repair and cellular homeostasis

Deoxyribonucleic acid (DNA) is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms and some viruses. The main role of DNA molecules is the long-term storage of information. Chemically, DNA consists of two long polymers of simple units called nucleotides, with backbones made of sugars and phosphate groups joined by ester bonds. Within cells, DNA is organized into structures called chromosomes. These chromosomes are duplicated before cells divide, in a process called DNA replication. Integrity of genome is a prerequisite for cell viability. Therefore, targeted damage of DNA structure is considered the key approach to the induction of cell death (Glazunova, 2008).

The human genome, comprising three billion base pairs coding for 30000-40000 genes, is constantly attacked by endogenous reactive metabolites, therapeutic drugs and a plethora of environmental mutagens that impact its integrity. Thus it is obvious that the stability of the genome must be under continuous surveillance. This is accomplished by DNA repair mechanisms, which have evolved to remove or to tolerate pre-cytotoxic, pre-mutagenic and pre-clastogenic DNA lesions in an error-free, or in some cases, error-prone way (Christmann et al., 2003).

DNA damage is a relatively common event in the life of a cell and may lead to mutation, cancer, and cellular or organismic death. Damage to DNA induces several cellular responses that enable the cell either to eliminate or cope with the damage or to activate a programmed cell death process, presumably to eliminate cells with potentially catastrophic mutations. These DNA damage response reactions include: (a) removal of DNA damage and restoration of the continuity of the DNA duplex; (b) activation of a DNA damage checkpoint, which arrests cell cycle progression so as to allow for repair and prevention of the transmission of damaged or incompletely replicated chromosomes; (c) transcriptional response, which causes changes in the transcription profile that may be beneficial to

the cell; and (d) apoptosis, which eliminates heavily damaged or seriously deregulated cells (Sancar et al., 2004).

The cell has developed a network of complementary DNA-repair mechanisms, and in the human genome, >130 genes have been found to be involved. Knowledge about the basic mechanisms for DNA repair has revealed an unexpected complexity, with overlapping specificity within the same pathway, as well as extensive functional interactions between proteins involved in repair pathways. Unrepaired or improperly repaired DNA lesions have serious potential consequences for the cell, leading to genomic instability and deregulation of cellular functions (Altieri et al., 2008).

Several DNA-repair pathways have been developed by organisms to cope with the frequent challenge of endogenous and exogenous DNA insults. Endogenous sources of DNA damage include hydrolysis, oxidation, alkylation, and mismatch of DNA bases. Sources for exogenous DNA damage include ionizing radiation (IR), ultraviolet (UV) radiation, and various chemicals agents (Hakem, 2008).

Different DNA-repair pathways exist and perform major roles at both cellular and organismic levels. These pathways include (1) the direct reversal pathway, (2) the mismatch repair (MMR) pathway, (3) the nucleotide excision repair (NER) pathway, (4) the base excision repair (BER) pathway, (5) the homologous recombination (HR) pathway, and (6) the non-homologous end joining (NHEJ) pathway (Hakem, 2008) (**fig. 28).**

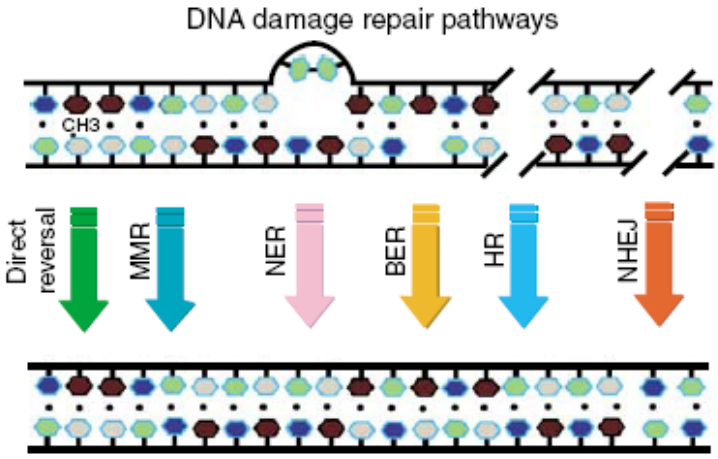


Fig. 28. DNA-repair pathways. Several DNA-repair pathways exist and deal with various types of DNA insults. These pathways include (1) the direct reversal pathway, (2) the mismatch repair (MMR) pathway, (3) the nucleotide excision repair (NER) pathway, (4) the base excision repair (BER) pathway, (5) the homologous recombination (HR) pathway, and (6) the non-homologous end joining (NHEJ) pathway (Hakem, 2008).

The base excision repair (BER) pathway deals with base damage, the most common insult to cellular DNA (Wilson and Bohr, 2007). Two sub-pathways, short-patch BER and long-patch BER, are involved in BER. The short-patch BER sub-pathway typically replaces a single nucleotide, whereas the long-patch sub-pathway results in the incorporation of 2–13 nucleotides. poly (ADP-ribose)

polymerase-1 (PARP-1) as well as the related protein PARP-2 play important roles in DNA repair and maintenance of genomic integrity through a crucial role in the BER pathway (Bürkle, 2005).

4.2. Function of PARP-1 in cultured human lung cells

PARP-1 mRNA is present in all tissues but at varying levels. The highest levels were observed in testis, spleen, brain and thymus. Expression and function of PARP-1 has not been intensively investigated in normal human lung cells, although the expression may be affected not only by substances from air but also by endogenous compounds, that enter the lung through pulmonary circulation. This shortage in the information about PARP in normal human lung may be attributed not only to the severe difficulty to obtain normal human lung tissue but also to the special precautions taken to get monolayer cell culture of human lung. We have clarified that PARP-1 protein is expressed in all cultures from bronchial epithelium but the levels were smaller compared to A549 human lung tumor cells. This observation is in agreement with the general experience that permanent tumor cell lines have undergone several crucial modifications in pathways that regulate cellular homeostasis and cell cycle control.

The level of PARP-1 protein expressed in bronchial cells in vitro varies between individual donors. The ratio between the highest and the lowest level was about 2.3 fold in our study group. The difference in the mean expression of PARP-1 between these donors was statistically significant (P value <0.05) ANOVA test. Several factors are candidates to modify the expression of proteins, such as living habits, diseases, medical treatment, and genetic background. PARP-1-mRNA expression was increased in mononuclear cells (MNCs) from diabetic patients versus controls (Tempera et al., 2005). According to our data diabetes is not likely to be a factor to increase PARP-1 expression in cultivated (NHBECs). However, the number of diabetic patients was too small within our group to draw definitive conclusions about that.

In follow up experiments PARP-1 protein was expressed in NHBECs with relatively constant levels over a time period of 10 weeks (**Fig. 9**). This underpins that PARP-1 protein exerts physiologically important functions in normal human lung cells, such as gate keeper function between DNA repair and necrosis or apoptosis as well as the transcription of some genes (Rosenthal et al., 2000).

Protein expression does not necessarily correlate to protein function. In order to elucidate this point the activity of PARP-1 protein was monitored in cultivated human lung cells by an immunocytochemical assay, where PARP-1 protein activity is triggered by H_2O_2 induced DNA insult. PARP-1 binds to damaged DNA, including single strand break (SSBs) and double strand breaks (DSBs), by its double Zinc finger DNA-binding domain. This binding potently activates PARP-1 enzymatic activity (as much as 500 fold) (D'Amours et al., 1999). Due to the enzymatic activity of PARP-1 poly(ADP-ribose) (PAR) complexes are formed with the histones of the

damaged DNA site and subsequently chromatin aggregates. This nuclear event is a prerequisite for initiation of repair processes. The PAR-histone complex can be marked by specific antibodies and this is visualized by secondary FITC conjugated antibodies and fluorescence imaging.

Owing to the transient nature of PAR histone complexes this process has to be triggered in vitro under controlled conditions. We used H_2O_2 as an initiator for PARP-1 activity in normal human lung cells. Control samples from untreated NHBECs and PLCs cultures had no fluorescence signals. In H_2O_2 treated cells the PARP-1 activity related signal was visible in a dose dependent manner. We applied test concentrations between 0.05 mM and 0.3 mM. At the concentrations of 0.2 and 0.3 mM we observed heterogeneous, spot-like distributions of the signal in the nuclei, which indicates that nuclear condensation has occurred as a side event of toxicity. Nuclear condensation was also reported for a study on the human hepatoma cell line SMMC-7721 after 0.2 mM H_2O_2 (Huang et al., 2000). For these cells the typical morphology of apoptotic nuclei was reported after 48 h, indicated by bright fluorescence at the nuclear membrane due to condensed and aggregated chromatin. Our test concentration of 0.1 mM H_2O_2 was well tolerated by NHBECs and PLCs in the MTT assay for toxicity. Also the fluorescence signal for PARP-1 activity was robust after a treatment time of 5 min.

Basal PARP-1 activity was monitored in NHBECs obtained from 10 lung cancer cases. The highest level of PARP-1 activity was 2.3 fold of the lowest one within this group. This is in good agreement with the inter-individual differences found for PARP-1 protein expression. Only for four cases (IDs 228, 230, 236 and 240) NHBECs could be analysed for protein expression and also functional activity. The data did not correlate well but this should not be overestimated, because the study group is still small. Secondly, for western blotting total protein was prepared directly from the monolayer of the first generation and for immunocytochemistry the detached cells were reseeded onto uncoated cover glasses. So, the material cannot be prepared from identical samples for western blot analysis and functional activity test.

In long term culture of NHBECs and PLCs PARP-1 activity declines latest in the fourth or fifth generation as well as in the fourth or fifth subpassage. However, PARP-1 is active in normal human lung cells from bronchial epithelium as well as from peripheral lung tissue over a time period of 8 to 12 weeks in culture. This time period is pretty long and will enables repeated dosing of test compounds in future studies. Primary NHBECs and PLCs expanded from explant cultures will be a suitable experimental model to study the effect of external factors on regulation of PARP-1 expression and function.

A study conducted by Grube and Bürkel, 1992 showed a large decline of PARP-1 activity with advancing age, along with a changing pattern of modified acceptor proteins. Some of these studies are in account with our study, which showed that PARP-1 activity decreased with progressive

generations, and this may be explained by the adaptation response of cultivated human lung cells to culture conditions. On one hand, long term cell culture may act as a stress per se. On the other hand, some stress factors from living conditions, disease and medication will fade out over time.

Recently, the link between PARP-1 and aging was additionally strengthened by a set of reports from different laboratories showing that PARP-1 physically interacts and functionally cooperates with WRN, the protein deficient in Werner syndrome which is characterized by premature aging (Adelfalk et al., 2003; Lebel et al., 2003; Von Kobbe et al., 2003). In cells derived from Werner syndrome patients, poly(ADP-ribosyl)ation after DNA damage by hydrogen peroxide and methyl methane sulfonate is impaired, pointing to a pathway-specific loss of DNA repair function (Von Kobbe et al., 2003).

Numerous studies have clearly demonstrated that PARP-1 becomes rapidly activated in various pathophysiological conditions, and its activation is prolonged and sustained. For example, direct detection of poly (ADP-ribose) polymer accumulation has demonstrated the activation of PARP in stroke induced by middle cerebral artery occlusion and reperfusion (Endres et al., 1998), and in the heart after myocardial infarction and heart transplantation (Faro et al., 2002; Fiorillo et al., 2002). Similarly, PARP activation has been demonstrated in the gut, heart, and lung in hemorrhagic and septic shock (Watts et al., 2001; Goldfarb et al., 2002), in the lung of mice subjected to a model of acute respiratory distress syndrome as well as in the heart and blood vessels of diabetic animals.

4.3. Metals as stressors for PARP-1

The lung is not only the organ of gas exchange, but shares also the network of metabolism and detoxification in the body and represents a main gate of various inhaled substances including the metals and other elements of pollution such as smoking. Several studies were done to investigate the effect of exposure to heavy metals and cigarette smoking as components of environmental pollution on living organisms especially on human being. These studies were done at different levels, such as histological, histochemical, and molecular levels.

The effects of cigarette smoking are numerous, such as cytotoxicity, impaired mucociliary clearance, cancer at many sites, atherosclerosis and addiction. Cigarette smoking contributes to exposure to substantially toxic metals such as cadmium, arsine, nickel, lead and mercury. In general, environmentally related metal exposure is a powerful risk factor for neurotoxicity, and in some cases carcinogenesity.

DNA repair is a system of defences designed to protect the integrity of the genome; it has been suggested that deficiencies in this system probably lead to carcinogenesis. Current evidence suggests that DNA repair systems are very sensitive targets for many metallic ions. This may be associated with disturbed PARP function resulting in fixation of metal induced DNA changes i.e.

mutation. In addition, the individual may have inherited susceptibility DNA repair genes (Au et al., 2003) that would have caused him/her to have unusual sensitivity.

Studies in the past few decades have shown metals like iron, copper, cadmium, mercury, lead and arsenic possess the ability to generate reactive radicals, resulting in cellular damage like depletion of enzyme activities, damage to lipid bilayer and DNA (Stohs and Baghe, 1995). In this work we have studied the expression and activity of PARP-1 protein as a DNA repair protein under the effect of heavy metals ($CuSO_4$ and $HgCl_2$) and cigarette smoke condensate. All used substances were tested for toxic effects with (MTT) assay in cultivated lung cells in order to check subtoxic concentration ranges. Since the exposure to noxious agents may be single or repeated, it was essential to apply these metals in both short and long term exposures in order to elucidate their effects.

Cultivated NHBECs have been exposed to both short (50µM, 24h) and long term (5 µM, 3 weeks) treatment with $CuSO_4$. In both cases PARP-1 expression has shown slight statically non significant increase (P value >0.05). On the other hand no nuclear fluorescence signal was detected in cells exposed to copper sulphate alone at concentration (50µM, 24h; 5 µM, 3w). Nevertheless, copper could significantly decreased the extent of H_2O_2 - induced activity in all experiments, (P value < 0.05).

This study is in agreement with Schwerdtle et al., 2007 studies who showed that treatment of Hela S3 cells with $CuSO_4$ for 24h significantly decreased the extent of poly(ADP-ribosyl)ation induced by H_2O_2 100µM for 5 Min concentration-dependently at non cytotoxic concentrations, starting at 100µM, reaching 40% residual activity at 300µM. $CuSO_4$ alone only significantly induced strand breaks at 300 µM, however in combination experiments with H_2O_2, $CuSO_4$ increased DNA strand breaks in a concentration dependent manner as compared to H_2O_2 alone. Furthermore, after 10 min preincubation of isolated PARP-1, $CuSO_4$ decreased poly(ADPribosyl) ation concentration-dependently starting at10 µM and reaching 80% inhibition at 250 µM. Further experiments demonstrated a strong inhibition of the activity of isolated PARP-1 by copper, indicating that the observed decrease in cellular poly(ADP-ribosyl)ation might be due to changes in the activity of PARP-1. In the same study of Schwerdtle et al., 2007 copper induced a substantial level of oxidative DNA damage at concentrations already within the range of cytotoxic events. At lower noncytotoxic concentrations of copper the repair of oxidative DNA damage induced by visible light immersion is impaired.

In V79 cells copper nitrate has been reported to induce DNA strand breaks (Sideris et al., 1988) and for copper sulphate an induction of DNA strand breaks was observed in rat hepatocytes (Sina et al., 1983) and leucocytes of mice (Saleha Banu et al., 2004). Copper-induced apoptosis has been demonstrated in thymocytes (Deng et al., 1999), and in CHO cells and some other cells lines at very

high concentrations (500 uM) (Linder, 2001). Copper ions can also enhance apoptosis when given with some other agents. Copper ions have also been reported to reduce protection against apoptosis afforded by nerve growth factor (NGF) to pheochromocytoma cells (Wang et al., 1999). In this case the mechanism of action was by altering the activity and binding of NGF at concentrations of 100uM in the medium. A stronger induction of DNA strand breaks as compared to base lesions was also reported for isolated DNA by copper(II)/H_2O_2 (Kennedy et al., 1997). Furthermore, another study on isolated DNA applying copper (II)/ ascorbate/H_2O_2 assumed that DNA-bound copper(I) primarily mediates DNA base modifications and nonbound copper(I) primarily DNA strand breaks (Drouin et al., 1996). Oikawa et al., 1995 demonstrated that Cu_{12}-MT caused cleavage of isolated DNA fragments, which was increased by piperidine treatment, suggesting both a breakage of the deoxyribose phosphate backbone as well as the induction of base damage and/or a basic site.

Exposure to excess copper ions in water has been demonstrated to enhance apoptosis and cell proliferation in salmon parr (Lundebye et al., 1999). With much lower concentrations and longer exposure (3.2uM for 28 days), copper has been shown to induce apoptosis and necrosis in the gills of fish (Li et al., 1998).

Nevertheless, copper ions do not necessarily promote apoptosis in all circumstances. In dendritic cells, for example, copper ions (100–300 uM) failed to induce changes in gene expression characteristic of apoptosis, although Ni and Co ions did (Manome et al., 1999). Moreover, copper and some of its chelates sometimes prevent apoptosis induced by other factors. Recent examples include Cu(II) prevention of apoptosis due to an Fe-bleomycin analog (Suginaka et al., 1998) or metal removal (Adler et al., 1999), Thus, depending upon the cell type and the presence of other factors such as xenobiotics, copper ions may enhance or hinder apoptosis (Linder, 2001).

A recent study proposed that acute hepatitis in LEC rats impairs the expression and function of two DNA glycosylases, responsible for the repair of oxidative DNA damage (Choudhury et al., 2003).

The results of Schwerdtle et al., 2007 indicated that copper can exert defined genotoxic effects at high exposure levels. One study of Turnlund et al., 2005 provided evidence that, in healthy humans after a high intake of copper for several months the homeostatic mechanisms controlling copper retention may be overcome and accumulation of copper occurs. Turnlund et al., 2004 studies showed that long-term high copper intake resulted in increase in some indexes of copper status, alters an index of oxidant stress, as high level of ceruloplasmin activity, benzylamine oxidase, and superoxide dismutase. Also affection of the immune function which indicated by significantly lower level of antibody titer for the Beijing strain of influenza virus in supplemented subjects with copper after immunization than in unsupplemented control subjects.

Regarding potential mechanisms, it is known that copper shows high affinity to thiols (Witkiewicz-Kucharczyk and Bal, 2006). Binding of PARP-1 to DNA lesions is mediated via two zinc finger

motifs, where zinc is coordinated by three cysteine and one histidine residue, forming a finger-like structure. If $CuSO_4$ binds to the zinc finger domain is displaced zinc and/or the cysteine residue in the zinc finger structure will be oxidized. As a consequence DNA binding affinity of PARP-1 is lost and poly(ADP-ribosyl)ation is decrease.

In our experiments the increased expression of PARP-1 protein may be the result of a positive feed back mechanism. The inhibition of PARP-1 activity by copper stimulated the cells to compensate for the decreased activity. Taken together this result indicates that exposure of cells to copper can hinder DNA repair mechanism, but at the same time induce DNA repair protein (PARP-1) expression to overcome this inhibition of activity.

Mercury is a toxic and hazardous metal that occurs naturally in the earth's crust. Mercury is found in nature in several chemical and physical forms. The general population is primarily exposed to mercury *via* food, fish being a major source of methyl mercury exposure.

The evaluation of metal toxicity is complex, which is especially the case for mechanisms of genotoxicity (Beyersmann et al., 2008; Nampoothiri et al., 2007; Lee et al., 2006; Horiguchi et al., 2006; Barbosa et al., 2006b; Saravana Devi et al., 2008). Even for single metal species several molecular mechanisms contribute to their carcinogenic action. Mercury is a reactive metal that has high affinity for macromolecules and binds to DNA both in vitro and in vivo (Robison et al., 1984; Ariza and Williams, 1996) leading to alterations in DNA structure.

Heavy metals such as mercury do not usually induce obvious genotoxic effects but either interfere with numerous cellular activities such as cellular repair enzymes (Williams et al., 1986) or induce oxidative stress by depletion of naturally occurring thiol, especially GSH which leads to enhanced genotoxicity (Au et al., 1996). In addition to DNA repair processes, one other response to DNA damage is the regulation of cell cycle progression by activation of DNA damage checkpoints. In general, these checkpoints control the ability of cells to arrest the cell cycle in response to DNA damage. Furthermore, they control the activation of DNA repair pathways, the movement of DNA repair proteins to sites of DNA damage, the activation of transcriptional programs. In some cell types, cell death by apoptosis was induced when DNA damage was severe (Zhou and Elledge, 2000).

The potential health effects of mercuric chloride have been a matter of concern because of potential wide human exposure consequent to its wide spread use. Hence, one aim of this work was to determine the susceptibility of cultivated human lung cells to $HgCl_2$ administration and to clarify the effect of $HgCl_2$ on PARP-1 expression and activity to elucidate the mechanism of action of mercury in induction of genotoxic effect and DNA damage.

Cultivated NHBECs have been used as a test model that has been incubated with $HgCl_2$. The experiments were designed to use subtoxic $HgCl_2$ 20µM for 24h and to investigate the level of

PARP-1 expression in cultures from 3 different patients. Hg Cl_2 has increased the expression of PARP-1 protein by factor 1.3 in comparison with the control but this increase in expression was statistically not significant (P value >0.5).

This may be attributed to the compensatory mechanism for the initial inhibitory effect of Hg on PARP-1 activity, as Hg Cl_2 significantly decreased the extent of H_2O_2 - induced activity in short term experiment (P value < 0.05). This is in line with the study of Hartwig et al., 2002 where at low, noncytotoxic concentrations of metal compounds in particular DNA repair was inhibited, as well as cell cycle progression was affected if not even diminished. This was explained by the high affinity of these metal ions toward the sulfhydryl groups. Numerous studies reported on the suppressor effect of metals on PARP activity. PARP activity was decreased in a human T-cell lymphoma derived cell line by As(III) (Yager and Wiencke, 1997), and recent results demonstrated an inhibition of hydrogen peroxide–induced PARP activity in intact cells by Ni(II), Co(II), Cd(II), Cu(II) and concentrations as low as 10 nM As(III) in HeLa cells Hartwig et al., 2002. Hartwig et al., 2003 demonstrated that very low concentrations of arsenite significantly reduce the extent of poly(ADPribosyl) ation in intact mammalian cells.

Many studies showed that Hg induced apoptosis by different mechanisms. One study of Shenker et al., 1997 showed nuclear fragmentation, a typical sign of apoptosis, in mercurial compounds-treated lymphocytes. Araragi et al., 2003 and Kim et al., 2004 confirmed that $HgCl_2$ might induce apoptosis partly via a mitochondria-dependent pathway dependent on ROS production in HL-60 cells, and the release of cytochrome C, proteolytic products of PARP and DNA fragmentation in Hg Cl_2 treated HL-60 cells. Mercurial compounds increase mitochondrial hydrogen peroxide production and lipid peroxidation in LLC-PK cell (Lund et al., 1993). Such findings confirm the conclusion that intracellular mechanism of mercury genotoxicity is related to the increase in reactive oxygen species. Elevated oxidative stress is assumed to be the reason for mutagenicity and not the direct interaction of mercury with DNA (Schurz et al., 2000).

Indeed, several studies indicate the existence of different apoptosis pathways induced by the same stimulation dependent on the cells (Perkins et al., 2000; Hishita et al., 2001). There is the difference between levels of Bcl-2 protein, which is a potent inhibitor of Cyt c release from mitochondria into the cytosol via the voltage- dependent anion channel in the mitochondrial outer membrane, in human T-cells and HL- 60 cells. Guo et al., 1998 reported an elevation of intracellular Bcl-2 protein level in $HgCl_2$-treated T-cells. The intracellular Bcl-2 level is known to differ between several cell types (Sirzén et al., 1998). Mercuric ion can interact with DNA repair enzymes or DNA proteins essential for repair through interference with zinc finger structures in DNA-binding motifs. Moreover mercury has a strong affinity for thiol bonds, which are present in cysteines in zinc finger structures, resulting in severe distortion of the structural integrity and activity of these proteins

(Hartwig et al., 2002; Sekowski et al., 1997). Theoretical explanation for decreased levels of poly(ADP-ribose) could be a reduction in cellular NAD^+ pools by Cu and Hg thus leading to substrate depletion for PARP reaction.

This study demonstrated also that the duration of exposure to mercury is not decisive for increase in PARP-1 expression. 3 weeks application of mercury (2.5µM) to NHBECs has had the same effect as shorter exposure to the same metal regarding PARP-1 expression. Corresponding absence of the inhibitory effect on PARP-1 activity upon long term exposure to mercury has been noticed and registered indicating direct relationship between PARP expression and function as a part of cellular response to increased mercury concentration in culture medium.

This relative increase in PARP-1 activity in long term exposure to Hg may be due to the direct interaction between mercury and DNA molecule which seems to need longer time of exposure to be manifested as increased PARP activity which would stimulate DNA repair. One study from Cebulska-Wasilewska et al., 2005 showed that occupational exposure to low concentrations of mercury did not cause extensive genotoxicity but caused base-excision repair deficiency. The induced DNA repair defects combined with continuous exposure to mercury and possible exposure to other mutagenic agents may significantly increase the health risk of workers. Evidence from a number of sources indicates that chronic exposure to low concentrations of heavy metals, such as mercury, results in immune dysfunction (Clarkson, 1997; Pollard and Hultman, 1997).

Mercury is also discussed to trigger immunologically mediated disease (such as autoimmunity) or to promote chronic infection by immunotoxicity (Silbergeld et al., 1998). Moreover, immune dysfunction is suspected to influence the development and progression of cancer. In vivo studies in male rats have shown that $HgCl_2$ induced dose dependent increase in percentage of chromosomal aberration in male rats following oral administration (Das et al., 1983). It has been reported that methyl mercuric chloride showed a statistically significant increase in micronuclei compared to controls in human lymphocytes (Migliore et al., 1999). The significant DNA damage observed after treatment with Hg Cl_2 agrees with the results obtained for other metals like lead and cadmium in mice with comet assay (Dana Devi et al., 2000;Valverde et al., 2000 respectively) and with chromium in human lymphocytes and gastric mucose cells (Blasiak et al., 1999).

In addition, the inhibition of DNA repair processes may be an important mechanism in metal-induced genotoxicity. In contrast with mechanisms of direct DNA damage, these effects are observed at completely non cytotoxic concentrations of the metal. The inhibition of DNA repair processes may be due to structural changes of the DNA or modifications of repair proteins, or through out the competition with essential metal ions serving as cofactors (Hartwig, 1995). Schmid et al., 2007 study showed that mercuric dichloride induced significant DNA migration in human parotid gland tissue cells and lymphocytes as representative human target cells of carcinogenesis.

Dose-dependent DNA migration could be demonstrated by the Comet assay after incubation with mercuric dichloride and there was no difference between tissue cells and lymphocytes with respect to DNA damage sensitivity. Au et al., 2003 reported that individuals may have inherited susceptibility DNA repair genes that would have caused him/her to have unusual high sensitivity.

In an in vivo experiment with rats Grover et al., 2001 showed a dose-dependent increase in DNA migration in the Comet assay after orally administered $HgCl_2$ doses. Shenker et al., 2000 investigations have clearly demonstrated that mercuric compounds represent a potent class of immunotoxins that induce human lymphoid cell death (T and B lymphocytes as well as monocytes). Sixteen h after exposure to MeHgCl, there was progressive condensation of chromatin and loss of organelle structure. Other physical alterations observed in MeHgCl-treated cells include a decrease in cell size and DNA fragmentation. These changes, however, developed late in the apoptotic process. Another critical hallmark of apoptosis was cleavage of PARP from a 116-kDa protein to an 85-kDa fragment. MeHgCl induced PARP cleavage; this was both time and dose dependent. Minimum cleavage was observed in control cells, and exposure of lymphocytes to MeHgCl resulted in significant PARP cleavage.

Collectively, the exposure to mercury is correlated with DNA damage in the form of stimulated subcellular signal pathway to induce either DNA repair or programmed cell death.

4.4. Cigarette smoke condensate as stressor for PARP-1

Epidemiological data indicate that the risk of cancer formation by tobacco smoke cannot be explained only by data on exposure (dose-effect relationship). Lung tissue from smokers should contain markers which help to understand why only a fraction of 25 – 30 % within smokers develop cancer. There is still a considerable lack of information on the importance of DNA repair in human lung within mechanisms of toxicity by tobacco smoke constituents. Other cooperating factors which are suggested to be included must exist and are decisive.

Clinically, one of the major cell types affected by cigarette smoke is the airway epithelium, where damage leads to cancer and also contributes to the development of chronic obstructive pulmonary disease (Agusti et al., 2002; Tuder et al., 2003; Hodge et al., 2003). Cellular DNA damage usually followed by apoptosis or programmed cell death, a response thought to protect the integrity of the genome. This may activate common effectors mechanisms involving a cascade of intracellular proteases, the caspases, and disrupt DNA repair mechanisms by cleaving repair enzymes such as poly (ADP-ribose) polymerase (PARP) (Vaughan et al., 2002). PARP has been suggested to play a key role in determining whether DNA injury leads to repair or to apoptosis (Oliver et al., 1998). Stressors in cigarette smoke initiate aberrant expression of cancer related genes in lung cancer patients. In response to cigarette smoke extract (CSE)-induced DNA damage, cells may initiate a series of repair processes by activating DNA repair enzymes, such as PARP.

The putative role of triggers for impaired DNA repair should be clarified in in-vitro models of lung tissue. To evaluate whether the DNA repair enzyme PARP is substantially involved in cellular responses toward smoke-induced cell stress human lung cells were exposed to subtoxic concentrations of cigarette smoke condensate. Smoke-induced activation of PARP-1 was assessed by immunofluorescence detection of PARP activity [poly(ADP-ribosy)lation] and the level of PARP-1 expression was assessed by Western blotting.

Several studies have reported that cigarette smoke can induce necrosis or apoptosis in a variety of cells, including an alveolar epithelial cell line (A549) and human fetal lung fibroblasts (Baumgartner et al., 2000; Ishii et al., 2001). It also has been reported that cigarette smoke causes DNA damage in A549 cells by activating endonuclease (Leanderson and Tagesson, 1992), and that cigarette smoke augmented asbestos-induced alveolar epithelial cell injury through a free radical–dependent mechanism (Kamp et al., 1998).

DNA repair largely depends on enzymes such as DNA dependent protein kinase (DNA-PK) and PARP (Bernstein et al., 2002; Virag and Szabo, 2002). PARP is activated in response to DNA damage or inactivated through cleavage by proteases such as caspase 3, resulting in failure of DNA repair (Virag and Szabo, 2002).

Liu et al., 2005 studies using TUNEL and Comet assays, demonstrated that cigarette smoke induced DNA damage in NHBECs. This effect was dependent on the duration of smoke exposure and the concentration of smoke. When the smoke was removed, however, cells did not undergo apoptosis, as assessed by flow cytometry of DNA content and Comet assay, or necrotic death, as assessed by MTT assay. In contrast, Camptothecin (CPT) a cytotoxic alkaloid with strong antitumor activity inhibits DNA topoisomerase, induced not only DNA damage but also apoptosis in these cells. CPT also increased caspase 3 activity. Cigarette smoke did not activate caspase 3 in functional assays and immunoblot, although smoke increased synthesis of the caspase 3 precursor in a concentration-dependent manner. In addition, cigarette smoke–induced DNA damage was reversible as evidenced by the TUNEL assay and clonogenic survival of the cells. Smoke stimulated intact PARP protein expression in a concentration-dependent manner. The PARP inhibitor, 3-aminobenzamide (3-ABA) significantly blocked DNA repair, as revealed by unchanged TUNEL positivity after removal of CSE, but apoptosis was not initiated.

The complex changes in lung function, morphology, and gene expression caused by compounds in cigarette smoke involve a combination of direct and indirect effects on cells, but principally centre around an increase in airway inflammation as a result of cigarette smoking. Exposure to cigarette smoke activates an inflammatory cascade in the airway epithelium resulting in the production of a number of potent cytokines and chemokines, with accompanying damage to the lung epithelium,

increased permeability, and recruitment of macrophages and neutrophils to the airway (Adler et al., 1994).

Hellermann et al., 2002 study showed increased expression of several cytokines at the mRNA level by CSC on cultivated NHBECs , such as IL-1β, which was significantly up regulated in NHBEs following CSC exposure, and has been shown to be important for activation of IL-8. Another major finding is that CSC induces activation of NFκB, a central mediator of proinflammatory responses, which is activated under oxidative stress and upon injury to cells. Six studies have shown that CSE resulted in apoptosis within 3– 24 hours in different cell types (Carnevali et al., 2003; Tuder et al., 2000; Aoshiba et al., 2001). However, Wickenden et al., 2003 showed that CSE exposure only induced necrosis. This might partly be explained by the fact that different cell types and CSE concentrations were used.

Interestingly, two studies showed that exposing cells to low concentrations of CSE induced apoptosis while high concentrations resulted in necrosis Vayssier-Taussat et al., 2001. Suppression of cell proliferation, increased detachment of cells, DNA strand breaks, and reduced surfactant production by alveolar epithelial cells have been associated with cigarette smoke (Hoshino et al., 2001; Yokohori et al., 2004).

Ramage et al., 2006 study indicated that apoptosis can occur in A549 cells following exposure to tobacco smoke. Following treatment with tobacco smoke and hydrogen peroxide expression of early apoptotic markers was seen (Bax-α, and cytochrome C) 4 hours after exposure. However, when the morphology was analysed 48 hours after exposure a much lesser degree of apoptosis was detectable than would be suggested by the level of early marker expression. This may suggest that there are tight control mechanisms that prevent completion of the apoptotic pathway for many cells or that as the pathway progresses the cells convert to a necrotic type of cell death. Bax-α is a member of the Bcl-2 protein family, which are involved in controlling apoptotic events. Thus, high levels of Bcl-2 inhibit apoptosis by preventing cytochrome C release while high levels of Bax-α induce apoptosis by binding to the mitochondrial membrane and increase the permeability allowing the release of cytochrome C (Parone et al., 2002).

In this study the exposure to CSC was designed as short and long term study. The expression and activity of PARP-1 have been investigated. Incubation of NHBECs with CSC 0.5mg/l for 24h increased PARP-1 expression by 1,4 fold compared to the control. Hence the chronic exposure of human being to cigarette smoke is not uncommon, it was pivotal step to investigate the effect of prolonged exposure to CSC on PARP-1 expression. Monolayers of NHBECs were obtained from explants then incubated with CSC 0,5 mg/l for 3 weeks. CSC significantly increased PARP-1 expression by 1.6 fold compared to the control, but the increase in short term exposure was statistically not significant. On the other hand the activity of PARP-1 was induced by CSC.

Moreover, the response to H_2O_2 trigger was significantly strengthened by CSC (P value < 0.05) both in short and long term exposure. Our study is concomitant with Baumgartner et al., 2000 study who has reported that Cigarette smoke exposure has induced DNA damage in a variety of cell types and therefore the increase in PARP functionality is plausible.

Increased PARP-1 expression in our study indicates that CSC in low cellulary subtoxic dose stimulated the cells to form more DNA repair protein to protect the cells from necrosis or inherited mutation, and this in consistent with Liu et al., 2005 studies which showed that the effect of CS was dependent on the duration of smoke exposure and the concentration of smoke.

In general, the above discussion provides an insight into one of the immediate cellular response to DNA damage represented in PARP-1 synthesis and activation in lung cells. In our studies, we have used cultures from normal human lung in order to over come the limitation of animal experiments or permanent lung cell lines. Normal cells are more relevant to assess the crucial effects on DNA repair after exposure to external stressors and to get insight into adaptive responses. An important evidence that PARP-1 is expressed in NHBECs has been obtained.

Taking into account that each individual patient can be recruited only for one occasion and that the parameters of interest, PARP-1 expression and activity and their modulation, are perhaps the results of several competing or supporting factors, we have to consider additional extrinsic and intrinsic factors as aetiological back ground for the inter-individual variability seen in this study regarding PARP expression. In addition, we have pointed to the metal induced DNA toxicity, as metals aggravate cellular stress by cigarette smoke constituents because they inhibit DNA repair by PARP. This mechanism adds on the oxidative stress mechanisms of metals.

Lastly CSC has parallely stimulated PARP-1 expression and activity as a response to DNA damage, which may represent a completion of the available data about the correlation between smoke exposure on one side and histological type and geography- related differences in DNA-lesion-profiles on the other side in lung cancer.

Summary and conclusions

The respiratory system is an important route of exposure to various toxicants and it is also a target for damage. The mechanisms of lung xenobiotics-induced health effects involve inflammation and oxidative stress. A key event with respect to the final outcome by toxic stressors is whether the initiated DNA damage is properly managed by repair systems. One of these mechanisms is the activation of poly (ADP-ribose) polymerase-1 (PARP-1) in response to DNA damage. Poly(ADP-ribosyl) ation is triggered by the occurrence of DNA strand breaks and represents one of the immediate cellular responses to DNA damage and is functionally associated with DNA repair pathways.

There is overwhelming evidence from animal experimental models, cell culture experiments, and cell free systems that exposure to cigarette smoke and metal particles causes oxidative DNA damage. Similarly, various preparations of metal induce oxidative DNA damage in in-vitro systems. In this study we used primary human epithelial lung cells (NHBEC) in order to focus on human lung tissue obtained from authentic clinical cases where occupation and life style together with the genetic background have caused lung cancer. We have studied whether PARP-1 is expressed in normal human lung cells in culture, and whether PARP-1 expression and function are modulated by external factors, such as culture duration, type of material (explant or passages) and response to toxic triggers.

1) PARP-1 protein was expressed in both normal human bronchial epithelial cells and the tumor cell line A549, but the signal in semi-quantitative analysis of the immunoblots was substantially higher in A549 tumor cells than in normal human bronchial epithelial cells. Its expression in NHBECs varies by a factor of about 2.5 between different patients, which may be due to extrinsic and intrinsic factors related to the patient or his environment.

2) The basal activity of PARP-1 protein was quantified immunocytochemically after induction of DNA damage by H_2O_2. The intensity of the fluorescence signal correlated with the concentration of H_2O_2. The basal PARP-1 activity was monitored in NHBECs obtained from 10 lung cancer cases. The highest level of PARP-1 activity was 2.3 fold of the lowest one within this group. This is in agreement with the inter-individual differences found for PARP protein expression.

3) PARP-1 protein was expressed in long term cultures of NHBECs and the level of expressed even raised from the second to the fifth generation in comparison to the first one by (1.24 ± 0.10, 1.14 ± 0.16, 1.17 ± 0.16, 1.18 ± 0.99 fold respectively). The values for the highest and lowest protein expression differed among generations by a of factor 1.3. This increase in PARP-1 expression was not statistically significant in each generation compared to the first one.

4) On the one hand, the expression of PARP-1 protein in vitro is relatively constant within our study group of three cases. On the other hand during long term culture of NHBECs from 3 patients

(ID 207, ID 208, ID 230) the intensity of fluorescence signals for poly(ADP-ribosyl)ation decreased gradually by 10-40% from the first to the fifth generation. This decrease in PARP-1 activity was statistically significant in each generation when compared to the first one. This may reflect the adaptation of these cells to the culture conditions with reduced stress.

5) Both, short and long term treatment with $CuSO_4$, increased the expression of PARP-1 slightly but not significant (1.23 ± 0.39, and 1.24 ± 0.25 fold respectively) in NHBECs..

6) No nuclear fluorescence signals of PARP-1 activation were detected in cells exposed to copper sulphate alone in short and long term exposure, but the response to H_2O_2 trigger was reduced. These results could indicate that exposure of cells to copper can interfere with DNA repair mechanism, but at the same time enhance more formation of DNA repair protein (PARP-1) to overcome this inhibition of activity.

7) $HgCl_2$ has slightly increased the expression of PARP-1 protein in short and long term treatment by (1.30 ± 0.55, and 1.27 ± 0.83 respectively) in NHBECs derived from 3 different patients (ID 227, 228, 243), but this effect was not significant.

8) Although $HgCl_2$ alone did not initiate PARP-1 activation, it was able to decrease significantly the extent of H_2O_2 induced activity in short term exposure. On the other hand there is no statistically significant effect on PARP activity in long term cultures. This indicates that the inhibitory effect of $HgCl_2$ on PARP-1 activity in short term cultures disappeared in the long term exposure system.

9) Incubation of NHBECs with cigarette smoke condensate (CSC 0.5mg/l for 24h) induced PARP-1 expression by 1.4 fold compared to the controls. Hence the chronic exposure of humans to cigarette smoke is not uncommon, it was pivotal step to investigate the effect of prolonged exposure to CSC on PARP-1 expression.

10) Monolayers of NHBECs were obtained from explants then incubated with CSC 0.5 mg/l for 3 weeks. CSC significantly increased PARP-1 expression by 1.6 fold compared to the control, but the increase in short term exposure was statistically not significant. On the other hand the activity of PARP-1 was induced by CSC. Moreover, the response to H_2O_2 trigger was significantly increased by CSC both in short and long term exposure.

In summary, PARP-1 protein is expressed and is active in primary bronchial epithelial cells and varies by a factor of 2.5 in protein levels and by a factor of 2.3 in activity between individuals. It may be a significant factor for the individual sensitivity to DNA damage by abundant stressors in air, such as genotoxic metals, cigarette smoke, dust or particulate matter in general. PARP-1 protein is expressed and is functionally active in primary lung cells in vitro over a period of at least 12 weeks. During that time the cells respond to stressors applied in vitro. The success to follow up the stability and function of PARP-1 over several weeks in primary lung cells in culture proofs the applicability of these non permanent cells as test model to clarify the long term regulatory response

mechanism of PARP protein. Heavy metals such as copper and mercury inhibit DNA repair by PARP and will aggravate cellular stress by other genotoxic agents as well. This underlines that further research should focus on combined effects of toxic agents relevant for human environmental exposure. Finally, cigarette smoke condensate (CSC) stimulated PARP-1 expression and activity in primary human lung cell in vitro. This may be a direct stimulation of protein expression or the result of an indirect effect such as an adaptive cellular response to DNA damage in the first step.

Lastly CSC has simultaneously stimulated PARP-1 expression and activity as a response to DNA damage. The data obtained represents a completion of the available information on the correlation between smoke exposure on the one side and histological type and geography- related differences in DNA-lesion-profiles on the other side in lung cancer.

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Theses

Normal human bronchial epithelial cells and peripheral lung cell cultures can be obtained from resections material of human lung. Explant cultures can be generated over a time period of up to five generations, which equals 12 weeks.

1) Cell monolayers of explant culture can be passaged over at least 5 passages and keep normal morphology of epithelial cells over that time determined by general histological techniques. PARP-1 protein is expressed in normal human bronchial epithelial cells, but the signal in semi-quantitative analysis of the immunoblots was substantially lower for normal human bronchial cells than for A549 human lung tumor cells.
2) The level of PARP protein expression is statistically significant different between individual donors. Thus PARP may be a target within cellular factors for individual sensitivity toward genotoxic stressors.
3) PARP-1 protein is expressed with raising levels in long term culture of normal human bronchial epithelial cells from the second to the fifth generation in comparison to the first one. It seems that PARP activity is essential also for primary cell in culture.
4) The basal activity of PARP-1 protein was detected immunocytochemicaly after induction of DNA damage by H_2O_2. The intensity of the fluorescence signal correlated with the concentration of H_2O_2.
5) PARP-1 activity is different between individual samples by a factor of 2.3 between the highest and lowest relative activity. This variation in activity between patients was statistically significant.
6) Over time of 12 weeks PARP-1 activity gradually decreases in normal human bronchial epithelial cells in long term culture. This decrease in PARP-1 activity was statistically significant in each generation compared to the first one.
7) Under cellular stress with copper sulphate or mercury chloride in subtoxic concentrations PARP-1 protein expression seems to be maintained or even a bit increased.
8) Copper sulphate and mercury chloride do not trigger PARP-1 activity, but it significantly decreased the response toward H_2O_2 -induced DNA damage.
9) In long term treatment of epithelial cells with mercuric chloride the cells adapt to the metal stress, because the cells regain their competence to cope with by H_2O_2 -induced genotoxic stress. The activity profile is even a bit increased, but not significantly.
10) Cigarette smoke condensate (CSC) stabilizes (short term exposure) or even increases (long term exposure) PARP-1 protein expression in vitro.

Cigarette smoke condensate (CSC) in short and long term exposure induced PARP-1 activity in vitro and adds on to the response after H_2O_2 trigger.

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Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe. Daten die aus anderen Quellen direkt oder indirekt übernommen wurden, sind unter Angabe der Quelle gekennzeichnet.

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Erklärung über Promotionsversuche

Hiermit erkläre ich, dass ich bisher keine früheren Promotionsversuche mit dieser oder einer anderen Dissertation unternommen habe.

Mohamed Hamdy Abdelrahim Ahmad

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