Signaling in the induction of genomic damage by endogenous compounds

Signalwege bei der Induktion von Genomschäden durch endogene Substanzen

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences, Julius-Maximilians-Universität Würzburg, Section Biomedicine

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Abbreviations

1400W	N-([3-(Aminomethyl)phenyl]methyl)ethanimidamide dihydrochloride
2-APB	2-aminoethoxydiphenyl borate
8-oxodG	8-hydroxydeoxyguanosine
A	adenine
AACOCF3	2-oxo-1,1,1-Trifluoro-6,9-12,15-heneicosatetraene
ACE inhibitors	angiotensin converting enzyme inhibitors
Ang II	angiotensin II
AT1 receptor	angiotensin II type 1 receptor
BAPTA	Ethylenedioxybis(o-phenylenenitrilo)tetraacetic acid
BNC	binucleated cells
BSA	bovine serum albumin
C	cytosine
Cand	candesartan
CFM	calcium free medium
CAT	catalase
CBPI	cytokinesis block proliferation index
DABCO	diazabicyclo octane
DAG	diacylglycerol
DAPI	diamidino phenylindole
DHE	dihydroethidium
DMEM	Dulbecco modified Eagle's minimal essentail medium
DMSO	dimethyl sulfoxide
DMTU	dimethylthiourea
DNA	deoxyribonucleic acid
DPI	diphenyleneiodonium chloride
DSB	double strand break
EDTA	ethylenediamine-tetraacetic acid disodium salt
ERK1/2	extracellular signal-regulated kinase
EtOH	ethanol
FCS	fetal calf serum
Fe^{2+}	ferrous ion
FITC	fluorescein isothiocyanat
FPG	formamidopyrimidine DNA glycosylase
FRAP	ferric reducing ability of plasma/ferric reducing antioxidant power
Fura 2-AM	Fura 2-acetoxymethyl ester
G	guanine
γ-H2AX	phosphorylated histone 2AX
GPX	glutathione peroxidase
GSH	glutathione, reduced form
GSSG	glutathione, oxidized form
GST	glutathione S-transferase
h	hour
HCl	hydrochloric acid
HClO	hypochlorous acid
H_2DCF-DA	2',7'-dichlorofluorescein diacetate
HEK-293	human embionic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK-2	human kidney 2 cell line

H_2O_2	hydrogen peroxide
hOGG1	8-oxodG DNA glycosylase 1
HRP	horse radish peroxidase
iNOS	inducible nitric oxide sythase
IP3	inositol triphosphate
IP3R	inositol triphosphate receptor
JNK	c-Jun N-terminal kinase
KCl	potassium chloride
KH_2PO_4	monopotassium phosphate
KOH	potassium hydroxide
LC-MS	liquid chromatography-mass spectrometry
LLC-PK1	porcine kidney cell line
L-NAME	N-nitro-L-arginine methyl ester
M	mol per liter
µM	micromole per liter
MAPK	mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney cell line
MEK	mitogen-activated protein kinase kinase
MeOH	methanol
min	minute
MN	micronuclei
NAC	N-acetyl-L-cysteine
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phoshate, reduced form
NaOH	sodium hydroxide
nm	nanometer
nM	nanomole per liter
NOX	NADPH oxidase subunit
NO	nitric oxide
NOS	nitric oxide sythase
O_2	molecular oxygen
$O_2^{\cdot -}$	superoxide anion
$^{\cdot}OH$	hydroxyl radical
$ONOO^-$	peroxynitrite
PBGD	porphobilinogen deaminase
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing tween
PCR	polymerase chain reaction
PI	propidium iodide
PI3K	phosphoinositide-3 kinase
PKC	protein kinase C
PLC	phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PTX	pertussis toxin
Rac	member of a family of hydrolases that bind and hydrolyze GTP
RAAS	renin-angiotensin-aldosterone system
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute medium

SOD	superoxide dismutase
STIM1	Stromal interaction molecule 1
T	thymidine
TBS	tris buffered saline
TEMPOL	4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl
TMB-8	8-(NN-diethylamino)octyl-3,4,5-trimethoxybenzoate
TPG	thapsigargin
Tris	trishydroxymethylaminomethane
U-73122	1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione
U-73343	1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedione

1 Introduction

1.1 Oxidative stress and its effects on biomolecules

Reactive oxygen species (ROS) are ions or small molecules that include oxygen ions, free radicals and peroxides. In mammalian cells ROS are continuously generated under physiological conditions. ROS are involved in the regulation of cell growth and differentiation, modulation of extracellular matrix, stimulation of some gene expression as well as serving as second messengers in signal transductions. On the other hand, the damaging effects of ROS have been well described. ROS can affect almost all biological macromolecules of cells (75). Their effects on DNA range from formation of oxidized bases to single and double strand breaks (68).

These effects are usually balanced by protective (antioxidant) mechanisms. Under physiological conditions, the amount of ROS which is present in the cell environment is easily scavenged by the antioxidant defense system of the body. This system comprises antioxidant enzymes, endogenous non-enzyme antioxidants and exogenous antioxidants from food intake. Superoxide dismutase which scavenges superoxide radicals, catalase, the enzyme responsible for scavenging H_2O_2 and glutathione peroxidase are among the most important examples of the enzymatic antioxidants. One of the most important non-enzymatic molecules which serves as a part of the antioxidant defense mechanism is glutathione (GSH), a tripeptide containing a L-cysteine, a L-glutamic acid and a glycine. The reduced thiol group of the cysteine acts as a strong antioxidant. Glutathione can be oxidized by reactive oxygen species or other oxidants to oxidized glutathione (GSSG). The oxidized form is more difficult to detect in the cells because the enzyme which reduces this form to GSH again (glutathione reductase) is constitutively active and inducible upon oxidative stress. In fact, the ratio of oxidized to reduced form of glutathione is a measure of redox balance in cells. In addition to GSH, some protein components and a variety of lipophilic compounds help maintaining the redox equilibrium in cells.

Exogenous antioxidants are another part of the defense mechanism. Among them are some vitamins and their precursors or derivatives as well as plant derived polyphenols. One example of the several compounds, generally known as vitamin E is α-tocopherol which is a powerful antioxidant. The precursor of vitamin A, β-carotene, is another exogenous antioxidant which has undergone several investigations for its beneficial effect on cancer incidence. But probably vitamin C is

the best known antioxidant among the public and also has shown positive effect in animal studies. In addition to vitamins, some other components of the human diet can work as antioxidants. Pelargonidin, a common anthocyanidin, chlorogenic acid, one of the most abundant polyphenols, epigallocatechin gallate, which is mostly found in green tea and resveratrol, a phytoalexin which is abundant in red wine are among the phytochemicals which have been proven to exert antioxidant capacity. Altogether, the components of the antioxidative defense mechanism are usually able to keep the redox balance in cells or in the body.

Reactive oxygen species can occur in the body from food intake or smoking, exposition to harmful radiation, activation of signal transductions pathways which result in production of ROS or by metabolism of some compounds. When the concentration of a compound which can be metabolized to form ROS is elevated due to a pathophysiological condition, more ROS is produced from oxidative metabolism. This results in a demand for higher activation of the antioxidative defense system. If the defense system fails to maintain the redox balance, the cells are exposed to oxidative stress, which can be defined as the condition characterized by an imbalance between steady-state concentrations of pro- and antioxidants in favor of the former resulting in an overall increase in the amount of cellular ROS. As noted above, ROS are able to damage bio-macromolecules. ROS reaction with biological membranes results in lipid peroxidation, a process which gives rise to the formation of a variety of reactive electrophiles such as epoxides and aldehydes. Malondialdehyde (MDA) for example is a by-product of lipid degradation which is commonly used as a marker of oxidative stress. Oxidative damage to a protein, especially in its active site can induce the loss of the biochemical function of the protein. When the catalytic function of the protein is lost, it may undergo selective degradation. Loss of sulfhydryl (–SH) groups, formation of carbonyls, disulfide crosslinks, methionine sulfoxide, dityrosine cross-links, nitrotyrosine, and glycoxidation and lipid peroxidation adducts, are among the oxidative damages which ROS can cause to proteins (110). It is suggested that oxidative damage to proteins plays a crucial role in senescence-associated loss of physiological functions, thus assigning ROS-induced protein damage to age-related diseases (111).

Reactive oxygen species can directly produce single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. It is estimated that in a given cell an amount of 10^5 oxidative lesions per day are formed

(38). Many forms of oxygen radicals are capable of forming oxidative base lesions. The hydroxyl radical in particular is able to form a number of lesions to DNA bases. This radical is one of the most reactive kinds of oxygen radicals which has a short half life and reacts quickly with cell components. To react directly with the DNA molecules, hydroxyl radicals have to be formed adjacent to the nucleic acid material. One of the most abundant DNA lesions caused by hydroxyl radical is oxidation of guanine at the C8 position which results in the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) (20), an important biomarker of oxidative stress (77). Due to base mispairing 8-oxodG can lead to G:C to T:A transversions after replication, representing a point mutation which is widely found in mutated oncogenes and tumor suppressor genes (53, 82, 107).

1.2 Oxidative stress in human conditions

A number of reports in the literature implicate excessive oxidative stress and/or inadequate antioxidant defense in the pathogenesis of cancer, age-related disorders, type 2 diabetes, autoimmune diseases, reproductive disorders, cardiovascular and neurodegenerative diseases (4, 13, 31, 41, 103, 105-106, 118).

1.2.1 Renin-angiotensin-aldosterone-system and DNA damage

The renin-angiotensin-aldosterone system is an endocrine hormone system which maintains the blood pressure through vasoconstriction and modulation of salt and water homeostasis. Higher activation of this system results in hypertension and also in renal diseases. Epidemiological studies have revealed higher cancer incidences and also higher cancer mortalities in hypertensive individuals (47). Phaeochromocytoma, a rare tumor of the adrenal medulla is associated with hypertension (103). Several other studies have demonstrated that hypertension is associated with colorectal neoplasia (14). On the other hand, several experimental and epidemiological studies have demonstrated an effect of angiotensin converting enzyme inhibitors on cancer, although there are some other reports which could not find any correlation (66). A study in rats revealed that the amount of urinary 8-oxodG in stroke-prone spontaneously hypertensive rats was not different from the normotensive Wistar-Kyoto (WYK) rats at six weeks of age, but became higher than control after the development of severe hypertension (89). Another study showed a significant increase in the total chromosomal damage and the percentage of abnormal metaphases between spontaneously hypertensive and normal WKY rats (3). Furthermore, it has been reported that lymphocytes of hypertensive patients

show higher genomic damage than those of control individuals (29, 50, 64, 117). Several studies have indicated that activation of the renin-angiotensin-aldosterone-system can lead to the formation of ROS (70, 105-106). The formed ROS is possibly responsible for the observed DNA damage and also can contribute to the formation of malignancies. Most of the studies investigating the source of generated ROS were typically limited to the NADPH oxidase of the phagocytic cells which is a multisubunit enzyme comprising of two catalytic membrane-bound subunits gp91 and p22 and the cytosolic regulatory subunits p47phox, p67phox, p40phox, Rac1 or Rac2. In recent years, four homologs of gp91 (now known as Nox2) in non-phagocytic cells have been recognized and named Nox1, Nox3, Nox4 and Nox5 (73). NADPH oxidase is activated by phosphorylation of its regulatory subunit p47 which leads to the conformational changes in the cytosolic subunits. Then the subunits p47, p40, and p67 and possibly the small G-protein Rac1 migrate from the cytosol to the membrane and associate with the heterodimer gp91/p22 to facilitate electron transfer from NADPH to O_2. A schematic representation of phagocyte NADPH oxidase is depicted in Figure 1. Different tissues express isoforms of Nox family with different localization and different patterns of activation. Among the family of Noxs, Nox2 is the only one which secrets superoxide to the outside of the cell membrane (101). To investigate the pathway which is involved in the production of ROS we focused on angiotensin II (Ang II) as one of the important components of the renin-angiotensin-aldosterone-system. Via ROS generated by NADPH oxidase, Ang II regulates several pathways including protein tyrosine phosphatases and kinases, MAP kinases, transcription factors activation and calcium signaling (101, 113).

Ang II is one of the oldest known peptide hormones and a major regulator of blood pressure. Together with aldosterone, Ang II is the active substance of the renin-angiotensin-aldosterone system. The functional octapeptide form is produced in several steps from its precursor angiotensinogen which is continuously produced in the liver. In response to stimulation such as hypotension, decreased sodium levels or sympathetic activations, renin is released from the kidneys and cleaves angiotensinogen, producing the inactive decapeptide angiotensin I. Angiotensin I also undergoes cleavage by an enzyme called angiotensin converting enzyme (ACE) producing the active octapeptide form Ang II. (Figure 2)

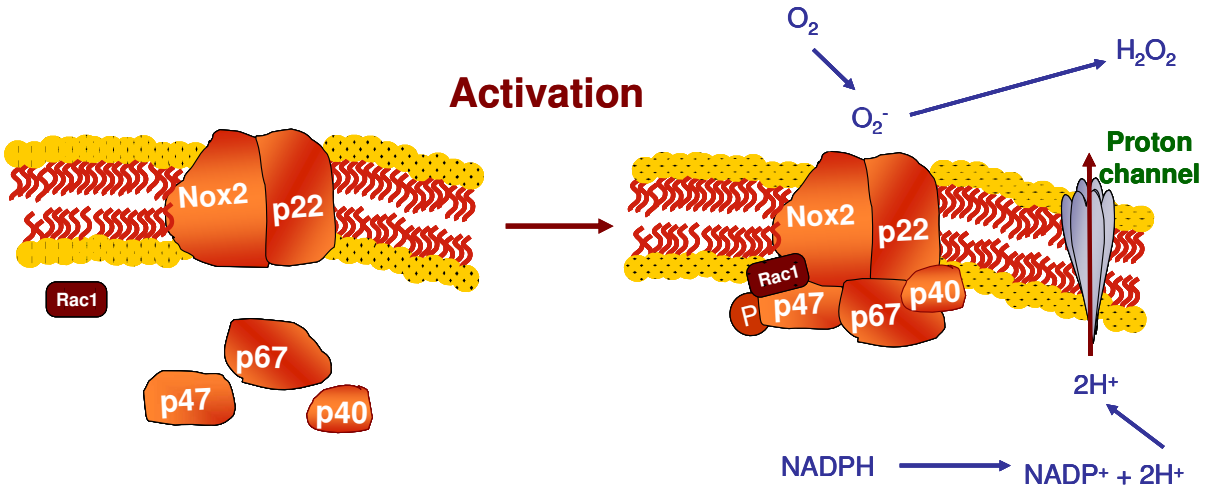

Figure 1 Schematic representation of phagocyte NADPH oxidase activation.

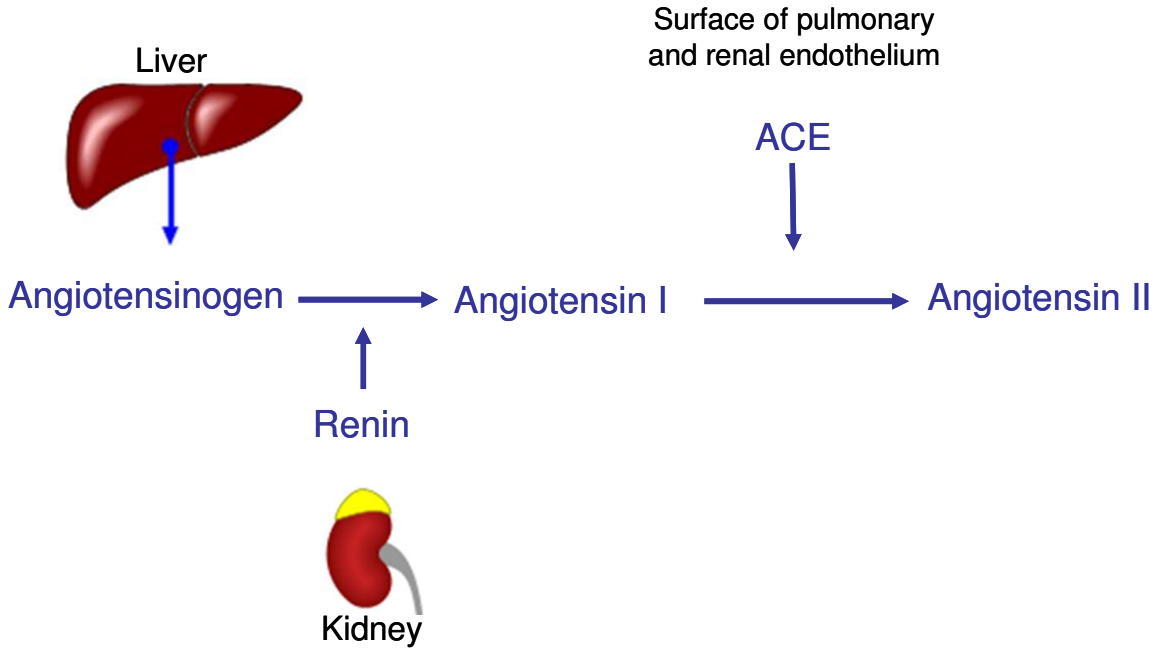

Figure 2 Formation of angiotensin II in the body.

Ang II mediates its effects in the cells via two receptors, Ang II type 1 receptor (AT1R) and Ang II type 2 receptor (AT2R), both G-protein-coupled receptors. AT1R is expressed in a wide variety of cells whereas AT2R is more predominant in fetal tissues. The AT1R mediates the major cardiovascular effects of Ang II. Some of these effects include vasoconstriction, secretion of aldosterone, renal sodium reabsorbtion, cell growth, fibrosis and thrombosis. The AT2R is involved in mechanisms such as vasodilatation, anti-inflammation, inhibition of cell growth, development of fetal tissues and apoptosis (109). Recently, our group has shown that perfusion of the isolated mouse kidney with concentrations of Ang II expected in

the kidney of hypertensive subjects is capable of damaging DNA in the primary kidney cells (104). This effect could be prevented using the AT1R antagonist candesartan which points to a role of the AT1R-dependent signaling in the genotoxicity of Ang II.

An earlier study in our group has shown that treatment of several cell lines with properties of proximal tubules with Ang II leads to DNA damage (105). Also chromosomal aberrations were increased dose dependently after long-term incubation with Ang II. Furthermore, the genotoxic effect of Ang II could be prevented using the AT1R antagonist candesartan, but the AT2R antagonist PD123319 could not impede the Ang II-induced DNA damage significantly. In addition to single strand breaks and chromosomal aberrations, the formation of double strand breaks, abasic sites and 8-oxodG base modifications after treatment with Ang II was also confirmed (104). All these kinds of damages could be prevented using candesartan to block the AT1R signaling.

So far no investigations have been conducted to scrutinize the pathways involved in the genotoxicity of Ang II.

1.2.2 Oxidative DNA damage and aging

There is considerable evidence that ROS production plays a role in both *in vitro* senescence and *in vivo* aging (84, 88). Cells obtained from laboratory mice have been reported to reach senescence after 4-5 cell divisions under normal cell culture conditions. However, when the oxygen content of the cell surroundings was reduced from 21 % to 3 %, the senescence was substantially delayed (94). Senescence-associated formation of γ-H2AX foci, an indicator of DNA double strand breaks, is reported to be decreased when the cells are transferred to 3 % oxygen atmosphere or treated with antioxidants indicating the role of ROS in aging associated DNA double strand formation. It is also reported that the life span of mice treated with antioxidant drugs increases up to 25 % (33) while mice lacking the enzyme superoxide dismutase 1 exhibit a 30 % decrease in life expectancy (32).

Telomeric DNA which contains TTAGGG repeats in vertebrates is another target of ROS induced damage. Telomeric DNA becomes shorter after each cell division. Below a threshold length, telomeric proteins dissociate and leave the telomers uncapped which results in cell growth arrest. Guanine, which is found in clusters in telomeric repeats, is the most easily oxidized nucleotide. Oxidized bases destabilize telomeric DNA resulting in accelerated telomere shortening even when DNA

replication does not take place. In contrast to the formation of other oxidative damage which can potentially be repaired, generally, somatic cells do not have telomerase activity to reverse the loss of telomeric repeats which makes the telomere loss irreversible (45, 88).

1.2.3 Oxidative stress and Parkinson's disease

It is generally accepted that the pathogenesis of Parkinson's disease (PD) centers on the formation of reactive oxygen species and the onset of oxidative stress leading to oxidative damage to the substantia nigra region in the brain. Extensive postmortem studies have provided evidence to support the involvement of oxidative stress in the pathogenesis of PD. This has been shown by visualizing oxidative damage to lipids, proteins and DNA. Also, the activity of antioxidant systems, namely superoxide dismutase and GSH levels, has been shown to be decreased in this disease (54). Although GSH depletion alone does not result in damage to nigral neurons, it may increase susceptibility to subsequent exposure to free radicals. Central to many of the processes involved in oxidative stress and oxidative damage in PD are the actions of monoamine oxidase (MAO), the enzyme which is responsible for the enzymatic oxidation of dopamine resulting in the production of H_2O_2 as a by-product (34) (Figure 3).

Lymphocytes can take up, store and metabolize catecholamines (7, 27). It is reported that the dopamine content and tyrosine hydroxylase immunoreactivity are reduced in peripheral blood lymphocytes in the early stages of PD (18). On the other hand, dopamine content and metabolism in the peripheral lymphocytes of PD patients are influenced by L-Dopa administration (102). PD patients receiving a high dose of L-Dopa had a significantly higher content of dopamine in their lymphocytes than PD patients who received a low dose of L-Dopa, or the healthy control. Therefore, peripheral blood lymphocytes can serve as a simple, easy-to-obtain and useful tool to study the potential genotoxic effect of dopamine.

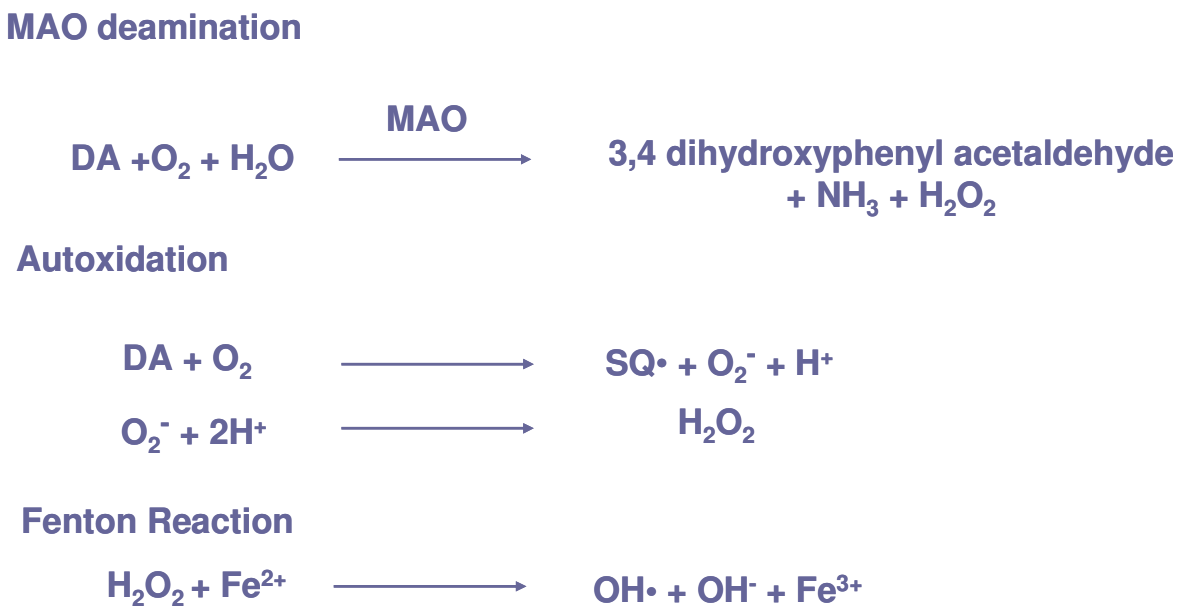

Figure 3. Oxidation of dopamine via MAO or autoxidation.

Cytogenetic analysis has revealed a high frequency of micronuclei (a measure of chromosomal aberrations, see 1.3) in blood lymphocytes of patients with untreated PD (77), which is of high importance because it shows that these patients not only suffer from damages in their central nervous system (CNS), but also outside of the CNS and especially in blood cells. Specific staining for centromeres demonstrated that most of the formed micronuclei in these patients consisted of acentric fragments (centromere-negative micronuclei) which points to chromosome breakage as the cause of micronuclei formation. As ROS are capable of inducing chromosomal damages, it can be hypothesized that the higher frequency of observed acentric micronuclei can emerge from abnormally high oxidative stress in these patients.

Another study has demonstrated elevated amounts of oxidative DNA damage in the lymphocytes of PD patients before L-Dopa therapy (26). This has been proven for single and double strand breaks as well as formamidopyrimidine DNA glycosylase (FPG) sensitive sites.

The exact mechanism of dopamine-induced damage to genetic material was not clear before the start of this project. We investigated the genotoxic effect of high dose L-Dopa therapy on the lymphocytes of PD patients and also confirmed the occurrence of DNA damage after dopamine treatment *in vitro* and then tried to reveal the mechanism by which dopamine exerts its genotoxic effect.

1.3 Quantification of genomic damage

Among the variety of damages that ROS can cause to biological molecules, the damage to genetic material is of great importance because if not repaired, this kind of damage can eventually be inherited to the next generation of the cells. A number of methods are described in the literature to identify and quantify genomic damage. Among them, the comet assay and the micronucleus frequency test are widely used in genotoxicity testing, human biomonitoring and epidemiological studies.

The comet assay was first introduced in 1984 by Östling and Johanson (93) as a single-cell gel electrophoresis method and later modified by Singh et al. (108). This method is a simple, effective and quantitative method which allows evaluation of strand breaks of DNA at the level of individual cells. This method is suitable for the evaluation of single and double strand breaks as well as a group of other damages generally known as alkali labile sites (incomplete excision repair sites, and apurinic or apyrimidinic sites, which are alkali labile and are translated into a strand break when exposed to the alkaline buffers of the comet assay) in a variety of biological samples such as cultured cells, blood lymphocytes and tissues. In this method the cells are embedded in an agarose gel on microscope slides and then are lysed in a high salt and detergent solution. This lysis process removes membranes, cytoplasm and also histones but leaves supercoiled DNA and some DNA-associated proteins. In the alkaline version of the comet assay, the next step is the denaturation of supercoiled DNA, which takes place in an alkaline solution. After that, an electrophoresis is conducted in the same high pH solution which allows the migration of broken DNA strands and relaxed DNA toward the positive pole of the electrophoresis field resulting in a comet shape formation of damaged cells. After this step the cells can be stained and the amount of migrated DNA can be quantified as a measure of DNA damage (Figure 4).

The standard protocol of the comet assay can also be modified using different enzymes which are involved in the repair of some specific kind of DNA alterations. As an example, the use of formamidopyrimidine DNA glycosylase (FPG), which is able to detect the major purine oxidation product 8-oxodG as well as other altered purines is one of the most common methods to detect 8-oxodG base modifications. The same procedure is applied using endonuclease III for detecting oxidised pyrimidines and AP sites, 3-methyladenine DNA glycosylase for 3-methyladenine (alkA),

endonuclease V (UV endonuclease) for cyclobutane pyrimidine dimmers and AP sites and uracil DNA glycosylase to detect uracil in DNA.

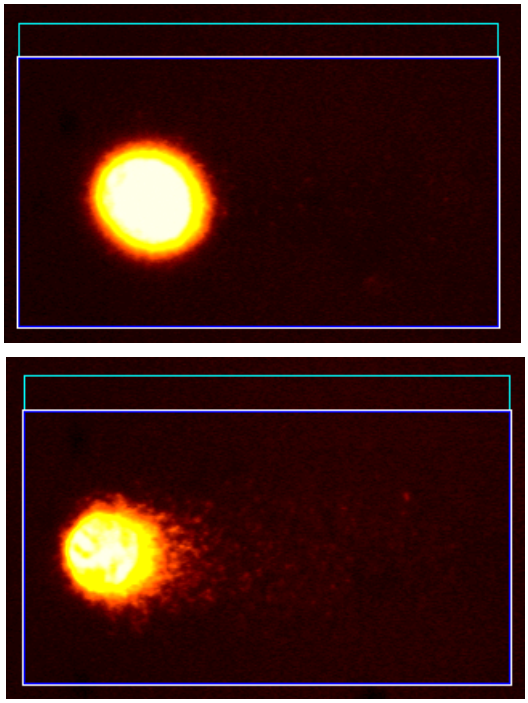

Figure 4. Two representative cells after comet assay. Cells are embedded in agarose and exposed to an electrical field. From cells with damaged DNA (lower picture), more DNA can migrate than from cells with intact nuclear DNA (upper picture).

The micronuclous frequency test is a method for quantifying heritable genomic damage. This method is suitable for detecting chromosomal aberrations and breakage in dividing cells which are obtained from a variety of sources such as cultured cells, blood lymphocytes or buccal mucosa cells. Micronuclei which are formed as a result of chromosomal breakage or mitosis disturbances are analyzed as an endpoint in routine genotoxicity testing of substances and human cytogenetic biomonitoring. A micronucleus which is formed during mitosis contains chromosome fragments or whole chromosomes which are unable to migrate to the spindle poles during anaphase. Later in telophase, these structures get their own nuclear membrane and resemble a small nucleus, a micronucleus (35). A schematic picture of micronucleus formation and representative cells containing micronuclei are illustrated in Figure 5. Because formation of micronuclei only occurs when cells go through cell division, it is important to observe only the population of the cells which are actively dividing during or after the desired treatment. One possibility to achieve this goal is to perform the cytokinesis-block micronucleus technique using cytochalasin B. This inhibitor of actin polymerization blocks the separation of

daughter cells but not of daughter nuclei, yielding binucleated cells. By limiting the analysis to such binucleated cells, it can be ensured that these cells have actively divided since the treatment.

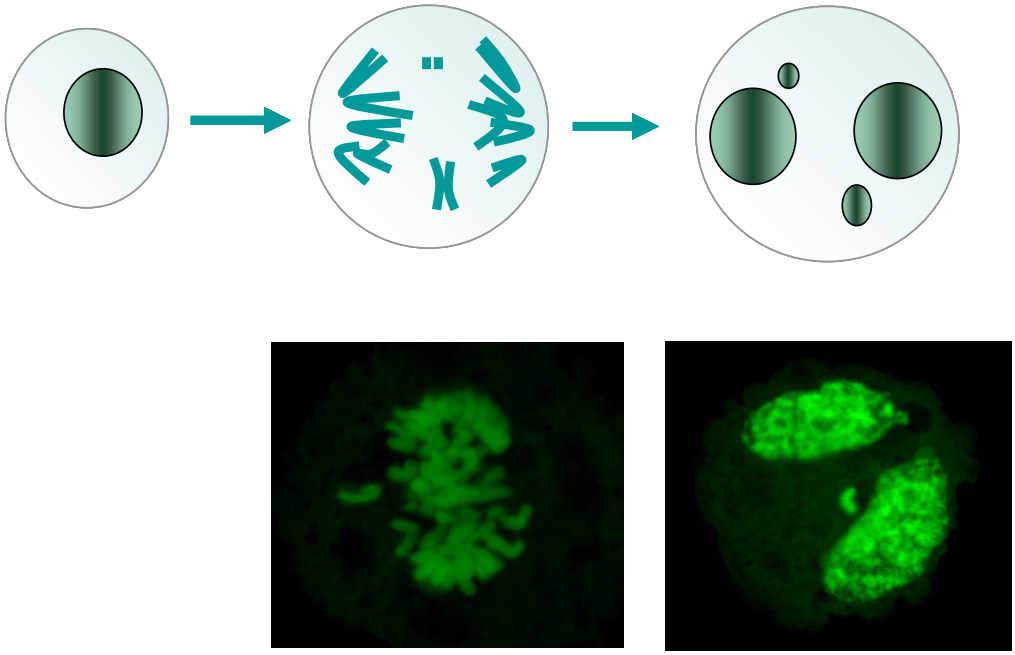

Figure 5. A micronucleus is a broken part of a chromosome or a whole chromosome which is unable to migrate toward the spindle poles during mitosis. Above is the schematic ilustration of micronuclei formation. The lower part of the picture shows micronuclei under the microscope.

Recently, micronucleus formation in peripheral blood lymphocytes has been correlated with cancer risk (13, 85). The authors suggest that the micronucleus frequency in peripheral blood lymphocytes can be a predictive indicator of cancer risk, implying that increased micronucleus formation is associated with early events in carcinogenesis. However, it is important to emphasize that this finding pertains to the risk of a group and not of individuals. An ongoing study is being conducted to investigate the potential association between micronucleus frequency in blood lymphocytes and cancer morbidity, as well as susceptibility to other degenerative diseases such as cardiovascular disease and Alzheimer disease, both of which are associated with increased micronuclei frequency in peripheral blood lymphocytes (85).

2 Objectives

A large body of evidence has accumulated for the damaging effects of ROS on DNA. Chromosome aberrations, DNA single and double strand breaks, base modifications and other kinds of DNA damage which may occur due to exposure to ROS may be involved in the process of malignancy development.

Increased levels of ROS production have been observed as a consequence of several diseases. One example of such these diseases is hypertension which is becoming more endemic especially in developed countries and in some cases is developed due to the elevation of Ang II levels. Another example is Parkinson's disease in which the prescription of the dopamine precursor L-Dopa seems to affect the redox balance in the patients.

The aim of this study was to characterize the genomic damage induced by Ang II and dopamine and the pathways which lead to the genotoxicity of these two compounds.

For Ang II first the genotoxic potential of the compound had to be confirmed in cell lines of human kidney using the comet assay and the micronucleus frequency test. The dependence of the observed genotoxicity on the production of ROS was to be demonstrated. Next, the source of produced ROS had to be identified and the pathway which starts with the stimulation of AT1R and ultimately results in the production of ROS had to be scrutinized.

In order to investigate the genotoxicity of dopamine, potential oxidative stress and chromosomal aberrations in PD patients under L-Dopa therapy had to be evaluated to question if L-Dopa conversion to dopamine in the circulating blood can affect the peripheral blood lymphocytes. Also, dopamine-induced DNA damage and apoptosis *in vitro* and the dependence on ROS formation were to be confirmed. Furthermore, the role of receptor signaling and uptake of dopamine by the dopamine transporter had to be elucidated.

3 Materials and Methods

3.1 Chemicals and reagents

If not mentioned otherwise, all chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). Cell culture media and reagents were obtained from PAA Laboratories GmbH (Pasching, Austria) and Invitrogen Life Technologies (Darmstadt, Germany). Epi life® calcium free medium was obtained from Cascade Biologics (Portland, OR, USA). The ERK activation inhibitor peptide was purchased from Calbiochem (Darmstadt, Germany). Pertussis toxin and thapsigargin were purchased from Enzo life sciences (Lörrach, Germany). VAS 2870 was a gift from Vasopharm GmbH (Würzburg, Germany). Halt^{TM} protease and phosphatase inhibitor single-use cocktail was purchased from Thermo scientific (Schwerte, Germany).

3.2 Antibodies

The anti α-Tubulin antibody (T6199) was purchased from Sigma-Aldrich (Taufkirchen, Germany). The antibodies against p47phox (sc-14015) and Nox2 (sc-130543) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Phospho-PKC (pan) (βII Ser660) antibody was purchased from Cell Signaling Technology Inc (Beverly, USA). Mouse monoclonal anti-phosphoserine (0023-100/PSER-16B4) was obtained from Biomol. Anti-AKT pS473 antibody (22650) was purchased from Rockland (Gilbertsville, PA, USA). Secondary antibodies were from Santa Cruz Biotechnology (sc-2004, sc-2005, sc-2006, sc-2020). Protein A/G PLUS-agarose (sc-2003) for immunoprecipitation was also obtained from Santa Cruz Biotechnology.

3.3 Oligonucleotides

The primers for PCR and qPCR were designed using the program Primer3 (http://frodo.wi.mit.edu/primer3) and then ordered from MWG Biotech (Ebersberg, Germany). The siRNA oloigonucleotides for Nox2 (sc-35503) and Nox4 (sc-41586) and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology.

3.4 Human subjects

18 PD patients were enrolled in the study of the effect of L-Dopa therapy. Patient characteristics are summarized in Table 1. Patients were treated with L-Dopa in a dose range of 188 to 1850 mg/day and dopa decarboxylase inhibitor, either carbidopa (25 to 425 mg/day) or benserazid (47 to 187 mg/day) or both.

	PD patients	Control
Number of participants	18	12
Sex (male/female)	12/6	1/11
Smokers/non-smokers	1/17	1/11
Age at study in years (range)	71.2 ± 3.2 (65–78)	68.8 ± 4.9 (61–80)
Age at onset in years (range)	61.0 ± 7.8 (47–77)	Not applicable
Daily dose of L-Dopa in mg/day (range)	808.68 ± 476.11 (188-1850)	Not applicable
Duration of L-Dopa treatment in years (range)	9.8 ± 5.2 (1.0–20)	Not applicable
Anti-oxidants/vitamin supplementation intake (yes/no)	3/16	2/10
Other medications (anti-diabetics; antihypertensive; thyroid management, etc.) (yes/no)	11/8	7/5

Table 1. Characteristics of the patients and control group.

Further medications included catechol-O-methyl transferase (COMT) inhibitors, dopamine agonists, antipsychotic drugs and MAO inhibitors (Table 2.).

Combination therapy for PD patients	No. of patients
L-Dopa (1200 mg/day) + dopa decarboxylase inhibitor (300 mg/day)	3
L-Dopa (600 mg/day) + dopa decarboxylase inhibitor (150 mg/day) + COMT inhibitor (1000 mg/day)	2
L-Dopa (587.5 mg/day) + dopa decarboxylase inhibitor (110.15 mg/day) + dopamine agonist (20.10 mg/day)	3
L-Dopa (850 mg/day) + dopa decarboxylase inhibitor (212.48 mg/day) + antipsychotic (50 mg/day)	1
L-Dopa (775 mg/day) + dopa decarboxylase inhibitor (193.75 mg/day) + COMT inhibitor (950 mg/day) + dopamine agonist (2 mg/day)	4
L-Dopa (975 mg/day) + dopa decarboxylase inhibitor (182.81 mg/day) + COMT inhibitor (800 mg/day) + antipsychotic (106.25 mg/day)	3
L-Dopa (559.375 mg/day) + dopa decarboxylase inhibitor (117.38 mg/day) + MAO-B inhibitors (5.5 mg/day) + dopamine agonist (34.85 mg/day)	2

Table 2. List of the medications taken by the Parkinson's patients in the study.

Life-partners of PD patients were also enrolled in this study, as a matched control group. Later 8 more male controls were also included to balance the male to female ratio in the control group. The study was approved by the University of Würzburg ethics committee and was carried out according to the declaration of Helsinki. A written consent was obtained from all participants prior to enrollment in the study.

3.5 Cell culture

HK-2, a human kidney cell line with many properties of proximal tubular cells, was obtained from Dr. G. Garibotto, Nephrology Division, Department of Internal Medicine and Urology Division, University of Genoa, Genoa, Italy and cultured in DMEM/F12 medium supplemented with 5 % fetal calf serum, 2 mM of L-glutamine, 1 % antibiotics, 10 µg/l epidermal growth factor, 5 µg/l hydrocortisone, 5 µg/l sodium selenate, 5 ng/l bovine pituitary extract, 5 mg/l transferrin, 5 mg/l insulin and 5 ng/l T3.
Human embryonic kidney cells HEK-293 cells obtained from ATCC were maintained in DMEM medium with high glucose content supplemented with 10 % fetal calf serum, 2 mM of l-glutamine, 0.4 % antibiotics and 2.5 % HEPES.
The porcine epithelial cells with the characteristics of distal tubules, LLC-PK1 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in DMEM medium (1 g/l glucose) supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 2.5 % HEPES and 0.4 % antibiotics.
PC12, a rat pheochromocytoma cell line with many properties of primary sympathetic neurons, was obtained from Dr. P. Tas, Department of Anesthesiology, University of Würzburg, Germany, and cultured in RPMI 1640 supplemented with 10 % horse serum, 5 % fetal calf serum, 2 mM L-glutamine and 1 % antibiotics.
The human B-lymphoblastoid cell line TK6 was obtained from Dr. W.J. Caspary, NIEHS, RTP, USA and maintained in RPMI 1640 medium supplemented with 10 % horse serum and antibiotics.
NRK-52E (NRK), an epithelial rat kidney cell line with proximal tubular properties, was obtained from ECACC (European Collection of Cell Culture, Salisbury, UK) and grown in DMEM medium (4.5 g/l glucose) supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 1 % non essential amino acids and antibiotics.
HL-60, a human promyelocytic cell line was kindly donated by Prof. Schinzel, Vasopharm, Wuerzburg, Germany, and was grown in RPMI 1640 medium supplemented with 10 % fetal calf serum, 1 % glutamine and antibiotics.
MDCK, Madin-Darby Canine kidney cells were purchased from ATCC and maintained in MEM with Earl's salts supplemented with 10 % fetal calf serum and 2 mM L-glutamine.
MDCK-DAT cells, MDCK cells transfected with the human dopamine transporter DAT (23) were a gift from Prof. G. Rudnick, Department of Pharmacology, Yale University School of Medicine (Atlanta, Georgia, USA) and were maintained in the same

medium as MDCK cells. Occasionally, 0.9 g/L of G418 was added to the medium to ensure the presence of the transfected gene.

All the cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 and routinely split two to three times per week (PC12 two times per week) to keep them in exponential growth.

For experiments related to dopamine genotoxicity, if not mentioned otherwise, all genotoxicity tests were performed in PC12 cells. MDCK and MDCK-DAT cells were used to investigate the effect of DAT transfection on dopamine-induced genotoxicity. The immunocytochemistry staining (DAT-localization) was also performed in MDCK-DAT cells because PC12 cells did not grow well on the coverslips. The quantification of dopamine in cell lysate was also conducted in MDCK-DAT cells due to inherent ability of PC12 cells to produce dopamine (7, 37) which interfered with our measurements.

3.6 Collection of blood samples and Isolation and culture of human peripheral blood lymphocytes

Blood samples were collected from patients and healthy controls via an indwelling cannula and collected in coded tubes containing heparin. Samples were transported from the Leopoldina Hospital, Schweinfurt (Germany) to the Department of Toxicology at the University of Wuerzburg (Germany) at room temperature within 2-3 hours and processed immediately.

Human peripheral blood lymphocytes (HPBL) were isolated by standard density gradient centrifugation using FicoLite H (Linaris-H, Wertheim, Germany). Blood was layered (1:1) onto FicoLite and centrifuged at 1600 rpm (370 g) for 30 min. at room temperature. The HPBL layer was transferred to a new tube and the cells were washed twice (1300 rpm; 250 g; 10 min) with RPMI 1640. HPBLs were cultured at a cell density of 1 x 10^6 cells/ ml in 5 ml of culture medium at 37 °C in a humidified, 5 % CO_2 incubator. The RPMI 1640 culture medium was supplemented with 15 % fetal calf serum, 1 % L-glutamine, 1 % Na-pyruvate, 0.4 % penicillin/streptomycin and 1 % non essential amino acids. To stimulate the lymphocytes for proliferation, PHA (phytohemagglutinin, final concentration 10 µg/ml) was added. The lymphocytes were then investigated for micronuclei frequency.

3.7 Comet Assay

The alkaline version of the comet assay was performed in all the experiments. After treatment, the cells were harvested and 20 µl of the treated cell suspension were mixed with 180 µl of 0.5 % low melting agarose and added to fully frosted slides that had been covered with a bottom layer of 1 % normal melting point agarose. The slides were incubated in lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris and 1 % Triton X-100, 10 g/l N-lauroylsarcosine sodium adjusted to pH 10 with NaOH) at 4 °C. After at least one hour the slides were washed and then placed in the electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH > 13.0) for 20 min. Then the electrophoresis was conducted for 20 min at 25 V (1.1 V/cm) and 300 mA. The slides were neutralized in 0.4 M Tris buffer (pH 7.5) and then dehydrated in methanol for 10 min. in -20 °C. The slides were then left in 37 °C incubator to dry and stored in room temperature afterward. Before evaluation 20 µl of propidium iodide (CAS Nr. 25535-16-4) solution (20 µg/ml) was added to each slide. Images of 50 randomly selected cells (25 per replicate slide) for each sample were analyzed with a fluorescence microscope (Labophot 2, Nikon, Germany) at 200-fold magnification using an image-analysis software (Komet 5, BFI Optilas, Germany). The percentage of DNA in the tail was used to quantify DNA migration.

3.8 Micronucleus frequency test

$1x10^5$ cells/ml were incubated with test substances in 5 ml medium. After the desired incubation time the medium was removed and replaced by fresh culture medium containing cytochalasin B (2 µg/ml). This inhibitor of actin polymerization blocks the separation of daughter cells but not of daughter nuclei, yielding binucleated cells. By limiting analysis to such binucleated cells, it can be ensured that these cells have actively divided since the treatment. After a further 20 hours (for blood lymphocytes and most of the cell lines) or 48 hours (PC12), cells were harvested, applied onto glass slides by cytospin centrifugation and fixed in methanol (-20 °C) for at least two hours. Before counting, cells were stained for 3 minutes with acridine orange (62.5 µl/ml in Sørensen buffer, pH 6.8), washed twice with Sørensen buffer (15 mM Na_2HPO_4 and 15 mM KH_2PO_4, pH 6.8) and mounted for microscopy. From each of two slides, 1000 binucleated cells were evaluated for cells containing micronuclei and the average was calculated. We rarely observed more than one micronucleus per cell under these conditions. In addition, the cytokinesis-block proliferation index, CBPI (number of mononucleated cells + 2 x number of binucleated cells + 3 x number of

multinucleated cells) / (sum of mononucleated, binucleated and multinucleated cells), was determined from 1000 cells of each sample. For substance combinations, concentrations which were described as effective in the literature and had been found not toxic in preliminary experiments were applied.

3.9 Ferric reduction antioxidant power (FRAP)

To exclude interference of potential antioxidant capacity of the compounds used for pharmacological inhibition with ROS-induced genotoxicity quantification, all the inhibitors and antagonists were tested for antioxidant activity using the ferric reducing antioxidant power (FRAP) method (6) which determines the reduction of a ferric tripyridyltriazine complex to its colored form ferrous, in the presence of antioxidants. Briefly, 20 µl of sample was added to 180 µl of water. Next, 600 µl of the FRAP reagent (1:1:10 mixture of 10 mM ferric tripyridyltriazine, 20 mM ferric chloride and 300 mM acetate buffer) was added and the absorption at 593 nm was measured after 3 min. The results were quantified according to a standard curve produced using different concentration of ferrous sulphate.

3.10 Apoptosis assay

Cells were incubated with the test substance in 24 well plates, which contained coverslips only for adherent cells (NRK). PC12 cells are weakly adherent to glass coverslips and were treated like suspension cells in this assay. After treatment, the medium was replaced with the fresh medium and cells were further incubated for 24 hours. This duration had been found optimal for detection of apoptosis in our cell lines in preliminary experiments. Then, live, unfixed cells were stained by applying 5 µM of DEVD-NucView 488 (Biotium, Inc., Hayward, USA). After 30 min cells were washed with PBS once. TK6 and PC12 cells were then resuspended in 20 µl of PBS for microscopic analysis, while coverslips with adherent cells (NRK) were mounted on slides using 20 µl PBS. The caspase-3 substrate DEVD-NucView 488 can rapidly cross the cell membrane to enter the cell cytoplasm, where it is cleaved by caspase-3 to release a high-affinity DNA dye. The released DNA dye migrates to the cell nucleus to stain the nucleus brightly green upon 488 nm excitation. 500 cells per treatment were analyzed combining bright field for the detection of all cells with fluorescent illumination for detection of apoptotic cells.

3.11 Measurement of the cellular superoxide anion concentration

To evaluate the release of superoxide anion, the cell-permeable fluorogenic probe dihydroethidium (DHE) was used. One day before the experiment, 2 x 10^4 cells were seeded on ∅12 mm coverslips in 6-well plates in 3 ml medium. After treatment the medium was removed and cells were washed twice with PBS. Fresh medium was added containing 5 µM DHE (Merck Biosciences GmbH, Schwalbach, Germany) and the cells were incubated for 30 min. at room temperature in the dark while gently shaking. After washing with PBS, the coverslips were mounted on a slide and observed under an Eclipse 55i microscope (Nikon GmbH, Düsseldorf, Germany) and a Fluoro Pro MP 5000 camera (Intas Science Imaging Instruments GmbH, Göttingen, Germany) at 200-fold magnification.

3.12 SDS-PAGE and Western blot

After treatment, the cells were harvested and lysed in homogenisation buffer (0.2 M mannitol, 50 mM saccharose, 10 mM HEPES, pH: 7.5). The homogenization process was facilitated by disruption of the cell membranes mechanically and the yielded suspension was then centrifuged at 14000 rpm for 30 min. at 4 °C. The protein-containing supernatant was transferred to a clean tube and the concentration of protein in this solution was determined using Bradford's method. Generally, 50 µg of protein per sample was loaded on a discontinuous acrylamide gel. After electrophoresis the gel was blotted on the PVDF membrane. The membrane was blocked overnight in either 5 % nonfat milk powder or 2 % bovine serum albumin in TBS-T buffer (5 mM TRIS, 150 mM NaCl, 0.05 % Tween-20) and then was incubated with primary antibody. After washing the excess of primary antibody, the horse radish peroxidase (HRP) conjugated secondary antibody was added followed by washing. After incubation with HRP substrate, the membrane was exposed to an X-ray sensitive film and the film was developed afterward.

3.13 Immunoprecipitation assay

The cells were seeded in T25 flasks one day prior to the experiment. At the day of the experiment the cells were treated and then washed with PBS. RIPA buffer supplemented with protease/phosphatase inhibitor was then used for 10 min. at 4 °C with gentle shaking to disrupt the cells. This process was facilitated by aspiration through a 21 gauge needle and the yielded suspension was then centrifuged at 10000 g for 10 min. at 4 °C. The supernatant was then transferred to a new tube and incubated with the first antibody for 1 hour at 4 °C with gentle shaking. Afterward,

Protein A/G PLUS-Agarose was added and the tube was incubated overnight at 4 °C with shaking. The immunoprecipitates were collected by centrifugation at 2500 rpm for 5 min. at 4 °C. The pellets were washed with RIPA buffer and then resuspended in homogenization buffer. The yielded samples were then used for SDS-PAGE and Western blot as mentioned above.

3.14 Calcium release measurement

1 x 10^5 cells were cultured on coverslips in 6-well plates and treated the day after. The cells were then washed and incubated in new medium containing 5 µM of Fluo-4 AM (Invitrogen, Darmstadt, Germany). After 15 min. the cells were washed in PBS and examined and photographed by Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany) which was equipped with LAS AF software (Leica, Wetzlar, Germany). The pictures were then analyzed using ImageJ software which could be downloaded free of charge from the following web address: http://rsbweb.nih.gov/ij.

3.15 SiRNA transfection

The cells were seeded in antibiotic free medium in 6-well plates one day prior to the transfection. At the day of the experiment, the cells were washed with Transfection Medium (sc-36868, Santa Cruz Biotechnology). SiRNA was diluted to 100 µM in Transfection Medium. This solution was named solution A. Transfection Reagent (sc-29528, Santa Cruz Biotechnology) was also diluted to 100 µM with Transfection Medium and named solution B. Solution A and B were gently mixed and incubated for 45 min. at room temperature and then 800 µM of Transfection Medium was added to this mixture. The cells were then covered with this solution and incubated for 18 hours at 37 °C. After this time, either the medium was changed and the cells were treated with angiotensin or the RNA/protein was extracted for confirming down-regulation of the desired target.

3.16 Subcellular protein extraction

ProteoExtract ® subcellular proteome extraction kit (Calbiochem, Darmstadt, Germany) was used to extract proteins from cytosol and membrane. The cells were cultured in T25 flasks. The day after, the cells were treated and then washed two times with wash buffer for 5 min. at 4 °C. Then extraction buffer I supplemented with protease inhibitor cocktail was added to the cells with gentle agitation at 4 °C. After 10 min. the supernatant was transferred to a clean tube and regarded as cytosolic protein fraction. Extraction buffer II supplemented with protease inhibitor cocktail was

added to the remaining of the cells and incubated at 4 ℃ for 30 min. The supernatant containing membrane/organelles was then transferred to another clean tube.

3.17 RNA isolation, PCR and Real-time PCR

The expression of mRNA was detected using the reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from the cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and 2.0 µg of RNA was used for cDNA synthesis using Verso cDNA Synthesis Kit (Thermo scientific, Schwerte, Germany). The following primers were used for amplification of p47 and the house keeping gene β-actin: p47 forward 5'-CCA CCT CCT CGA CTT CTT CA-3', p47 reverse: 5'-GAC ACG TCT TGC CCT GAC TT-3' (528 bp), β-actin forward: 5'-CTC TTC CAG CCT TCC TTC CT-3', β-actin reverse: 5'-AGC ACT GTG TTG GCG TAC AG-3' (610 bp). Annealing temperatures for p47 and β-actin were 54 ℃ and 56 ℃ respectively. For amplification of Nox2 and Nox4 genes the following primers were used: Nox2 forward: 5'-TGC AGC CTG CCT GAA TTT CAA C-3', Nox2 reverse: 5'-GAG GCA CAG CGT GAT GAC AAC-3' (391 bp), Nox4 forward: 5'-CTG GTG AAT GCC CTC AAC TT-3', Nox4 reverse: 5'-CTG GCT TAT TGC TCC GGA TA-3' (556 bp). Annealing temperatures for Nox2 and Nox4 were 56 ℃ and 52 ℃ respectively. The PCR was performed using REDTaq^{TM} ReadyMix^{TM} PCR Reaction Mix (Sigma-Aldrich, Taufkirchen, Germany) as Taq polymerase enzyme and thermal cycler PTC 200 MJ research (Watertown, MA, USA). The following primers were used for amplification of the dopamine receptors and the house keeping gene porphobilinogen deaminase (PBGD): DAT forward 5'-CTG ACC AAC TCC ACC CTC AT-3', DAT reverse 5'-CAC AGG TAG GGA AAC CTC CA-3' (146 bp), PBGD forward 5'-ACA ACC GCG GAA GAA AAC-3', PBGD reverse, 5'- AGC ATC GCT ACC ACA GTG TC-3' (101 bp). Annealing temperature was 54 ℃. Real-time PCR was conducted on the Light Cycler ® 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using ABsolute QPCR SYBR Green Mix solutions (Thermo Scientific, Schwerte, Germany) and the data were analyzed with the Light Cycler ® 480 software release 1.5.0 SP3 (version 1.5.0.39).

3.18 Immunocytochemistry staining

For immunocytochemistry staining the cells were cultured on coverslips and treated the day after. After treatment the cells were washed with PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS/Mg/Ca) and fixed in cold methanol for 10 minutes. The cells

were then rehydrated for 5 min. in PBS/Mg/Ca and then incubated in permeabilization buffer (PBS/Mg/Ca plus 0.3 % Triton X-100 and 0.1 % bovine serum albumin) for 15 minutes. The blocking of unspecific proteins was performed in 16 % FCS, 0.3 % Triton X-100, 0.45 M NaCl and 20 mM sodium phosphate pH 7.4. After blocking, the cells were incubated with monoclonal anti-DAT antibody (Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:100 in blocking buffer with gentle shaking for 1 hour. After three times washing with permeabilization buffer, FITC labelled goat anti rat antibody (Santa Cruz Biotechnology, Heidelberg, Germany) was added (1:200 in blocking buffer) for one hour. The cells were then washed three times with permeabilization buffer followed by a final washing step with 5 mM sodium phosphate, pH 7.5 for 5 minutes. The coverslips were mounted on slides using Confocal-Matrix ® (Micro Tech Lab, Graz, Austria) and examined and photographed by Leica TCS SP5 confocal microscope and analyzed later with ImageJ software. The membrane to cytosol ratio of DAT was quantified. Because the membrane localization of DAT in polar cells like MDCK-DAT is not homogenous (49) we decided to measure the fluorescence intensity of four different regions of the membrane. For each cell, the fluorescence intensity of an 8 x 8 pixel box from four regions of the cell membrane at 12, 3, 6, and 9 o'clock of the cells was measured. The fluorescence intensity of four similar boxes from the cytosol region in close vicinity to the membrane boxes was also quantified. Then, the ratio of the average intensity of the membrane boxes to the average intensity of the cytosol boxes was calculated for each cell. For each treatment 50 cells were analyzed. This procedure is illustrated in Figure 6.

3.19 Measurement of released H_2O_2 in medium

H_2O_2 measurement was conducted according to the method described by Pick et al (98) with some modifications. PC12 cells were cultured the day before the experiment. The cells were washed with PBS and then covered with phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer, pH: 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 19 U/ml horse radish peroxidase). The cells were then treated with PBS (negative control), 100 µM of dopamine or 1.56, 3.13, 6.25 and 12.5 µM of H_2O_2 (for standard curve) for 30 min. At the end of treatment the cells were collected and centrifuged. The supernatant was collected in a new tube and 1 N NaOH was added. The absorbance of this solution was read at 600 nM. To account for directly dopmanine-mediated absorption at the applied wavelength, 100 µM of

dopamine was added to the supernatant of a negative control sample and the measured absorbance (never more than a few percents of the total absorbance) was subtracted from absorbance of the dopamine treated samples. The pellet was used for determination of protein concentration using Bradford's method. The amount of released H_2O_2 was calculated from the standard curve and was related to protein content of each sample.

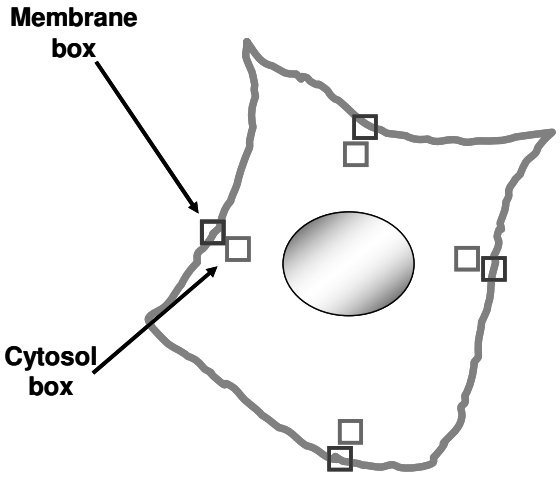

Figure 6. Schematic representation of a cell to show the boxes used for quantification of localization of the dopamine transporter (DAT) in the cell membrane and the cytosol.

3.20 Statistics

Statistical calculations were performed using Statistica 8 (StatSoft (Europe) GmbH, Hamburg, Germany). If not mentioned otherwise, data from at least 3 independent experiments ± standard deviation are depicted. Statistical significance among multiple groups was tested with Kruskal-Wallis test. Individual groups were then tested using the Mann Whitney U-test and results were considered significant if the p-value was ≤ 0.05. For quantification of immunocytochemical data (DAT localization) and calcium measurement, student's T-test was used. The p-value of ≤ 0.05 was considered significant.

4 Results

4.1 Angiotensin II

4.1.1 Genotoxicity of Ang II

The genotoxicity of Ang II in LLC-PK1, HEK-293 cells and HK-2 cells was evaluated. Two methods were used to detect Ang II-induced DNA damage, the comet assay and the micronucleus test. Both methods revealed a significant increase of DNA damage after incubation of cells with Ang II (Figure 7).

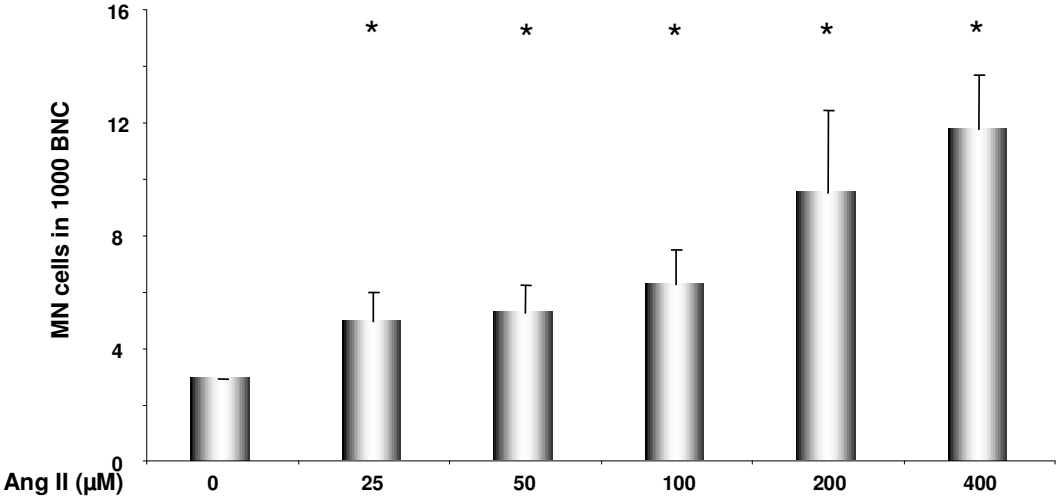

Figure 7 Ang II-induced DNA damage measured by micronucleus frequency test after incubating LLC-PK1 cells with the indicated concentrations of Ang II for 4 h. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang 0 nM (Mann-whitney U-test).

To verify how much time is needed for Ang II to exert its genotoxic effect, a time course experiment was conducted in HEK-293 cells. The cells started to show a genotoxic response in the comet assay after 30 min. which was significant after 60 min. of treatment (Figure 8).

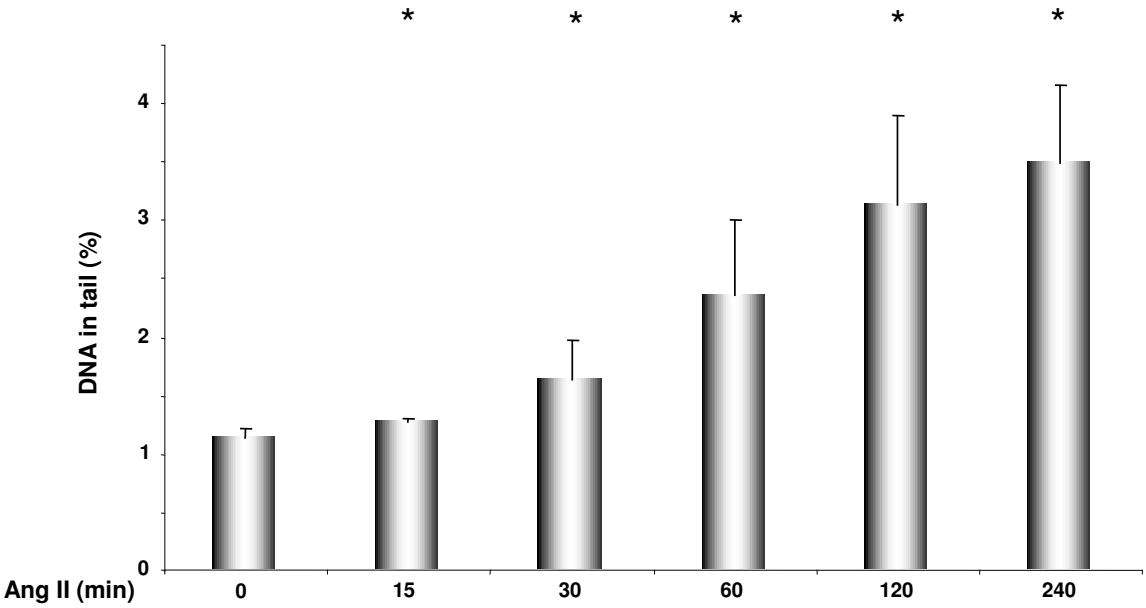

Figure 8 Ang II-induced DNA damage measured with the comet assay after incubating HEK-293 cells with 200 nM of Ang II for the indicated time. * p ≤ 0.05 vs. Ang 0 min (Mann-whitney U-test).

4.1.2 AT1R antagonist candesartan

Hypothesizing that the genotoxic effects of Ang II are mediated by the Ang II type 1 receptor (AT1R), we tested the specific AT1R antagonist candesartan (Cand). Cand completely prevented the Ang II-induced DNA damage in HK-2 cells in the comet assay (Figure 9) as well as in the micronucleus frequency test (not shown). This suggests a fundamental role of the AT1R in Ang II-induced DNA damage.

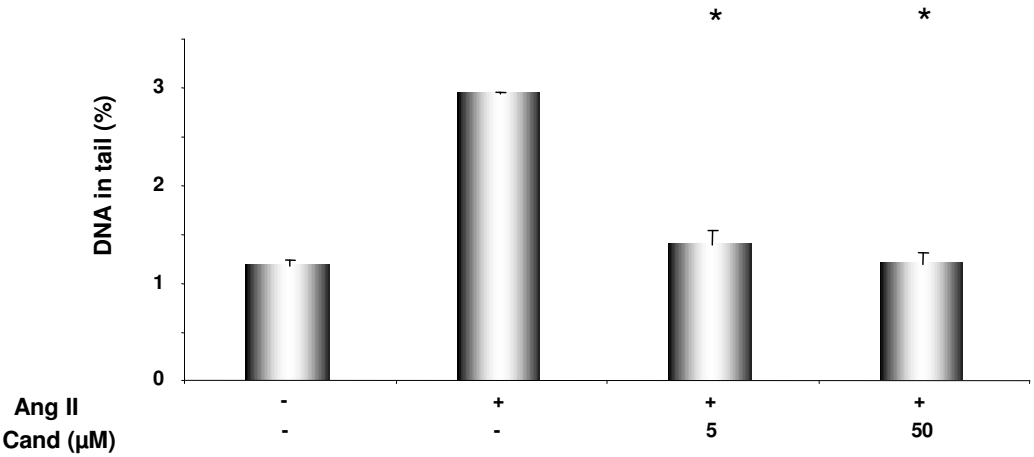

Figure 9 DNA damage measured with the comet assay after treatment of HK-2 cells with 200 nM Ang II with or without co-incubation with the indicated concentrations of the AT1R antagonist candesartan for 4 h. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

4.1.3 ROS production and antioxidants

It was shown in our group that treatment of cells with Ang II leads to the production of reactive oxygen species measurable with flow cytometry (105). To reveal which kind

of reactive oxygen species are formed after Ang II treatment, LLC-PK1 cells were stained with the superoxide specific dye dihydroethidium. The cells were then inspected under the fluorescence microscope. The Ang II-treated cells showed a higher production of superoxide in comparison to the control sample. This effect could be prevented using the superoxide radical scavenger TEMPOL (Figure 10).

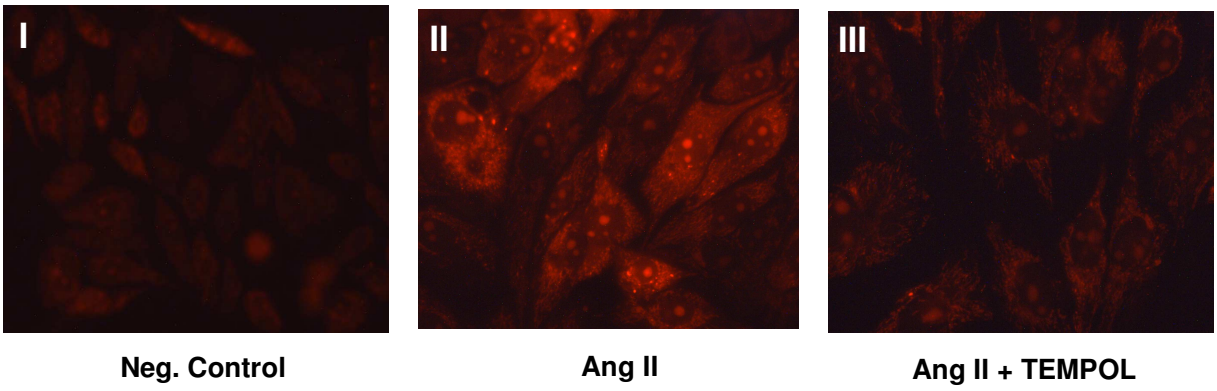

Figure 10 Induction of superoxide radical production visualized with help of the superoxide specific dye dihydroethidium in LLC-PK1 cells. Cells were treated with I) PBS, II) 200 nM Ang II and III) 200 nM Ang II + 50 µM of the superoxide radical scavenger TEMPOL.

To confirm which ROS exactly are responsible for the genotoxicity of Ang II, we investigated the effect of two antioxidant enzymes as well as of two antioxidant compounds with different antioxidant properties. Our results showed that catalase, the enzyme which removes an excess of hydrogen peroxide can reduce DNA damage caused by Ang II in HEK-293 cells. Furthermore, pre-incubation of cells with catalase (allowing for catalase uptake by the cells) had a more efficient protective effect (Figure 11).

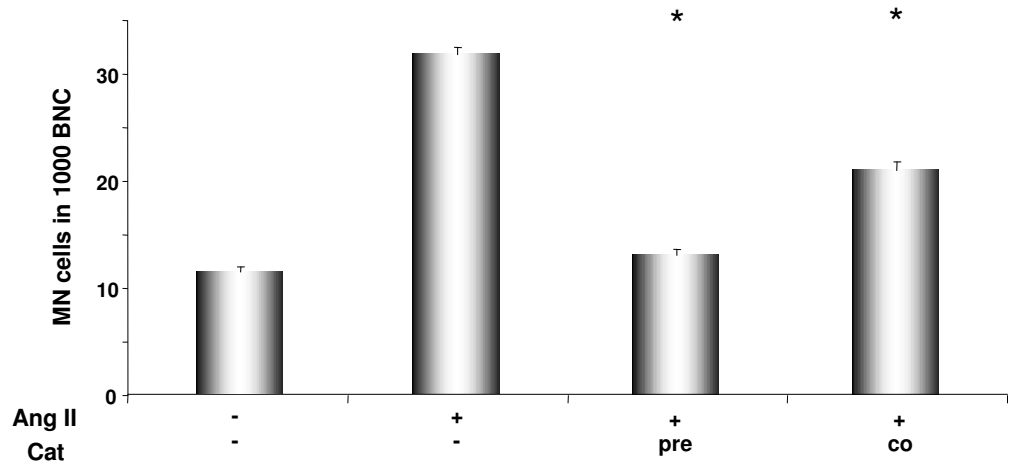

Figure 11 Micronucleus formation after incubation of HEK-293 cells with 200 nM Ang II and 300 U of the hydrogen peroxide scavenger enzyme catalase for 4 h. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

Superoxide dismutase (SOD), the enzyme which conducts the dismutation of superoxide to hydrogen peroxide caused no reduction of DNA damage either with

pre- or co-incubation (Figure 12). This enzyme, which cannot enter the cells nevertheless proved its potency in reducing the DNA damage caused by PMA. PMA is known to induce extracellular superoxide release. However, TEMPOL, a mimetic of SOD which can cross the cell membrane, was able to protect the cells against genotoxic effects of Ang II completely (Figure 13). This led us to the conclusion that the radicals which are responsible for DNA damage are formed and act inside the cells and are not secreted out of the cells. This point will be later discussed with regard to the NADPH oxidase enzyme isoforms.

To determine the contribution of hydroxyl radicals in Ang II induced DNA damage, N,N′-dimethylthiourea (DMTU), an antioxidant which shows hydroxyl radical scavenging properties was used. The data in Figure 14 illustrate that DMTU was able to decrease the genotoxic effects of Ang II, but not completely.

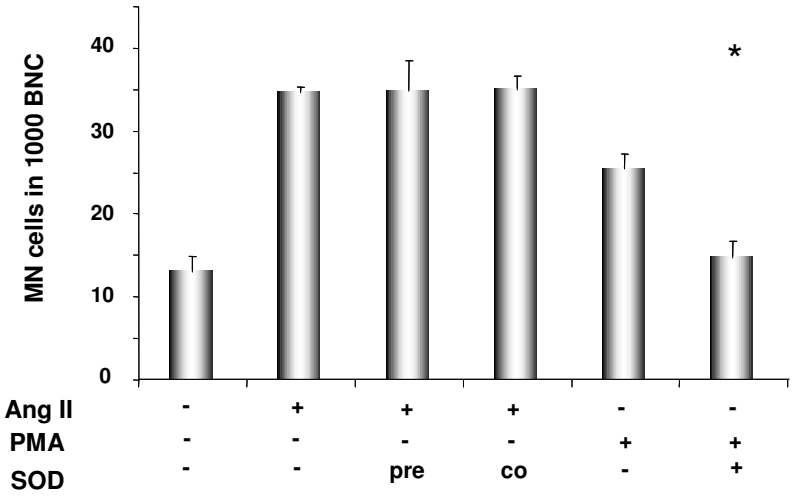

Figure 12 Micronucleus formation after incubation of HEK-293 cells with 200 nM Ang II and 300 U of the superoxide scavenger enzyme superoxide dismutase (SOD) for 4 h. 32 µM PMA served as the positive control. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. PMA treated sample (Mann-whitney U-test).

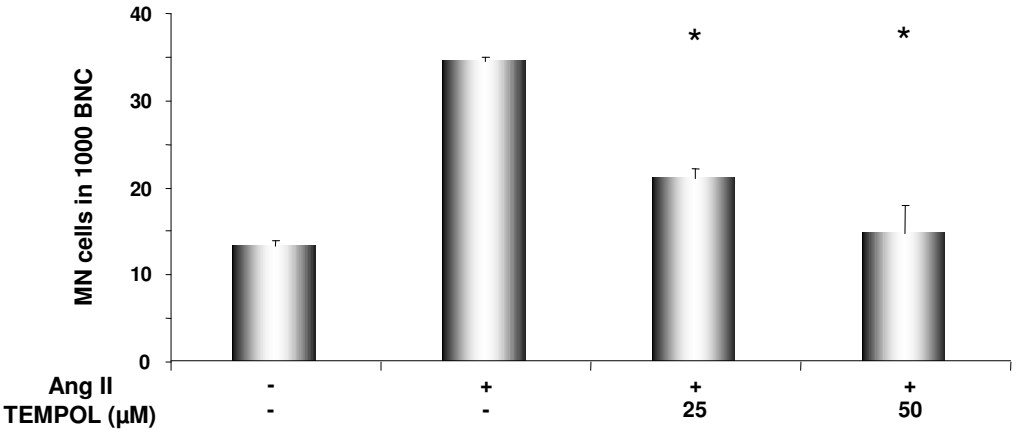

Figure 13 Micronucleus formation after incubation of HEK-293 cells with 200 nM of Ang II and the indicated concentrations of the superoxide dismutase mimetic TEMPOL for 4 h. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

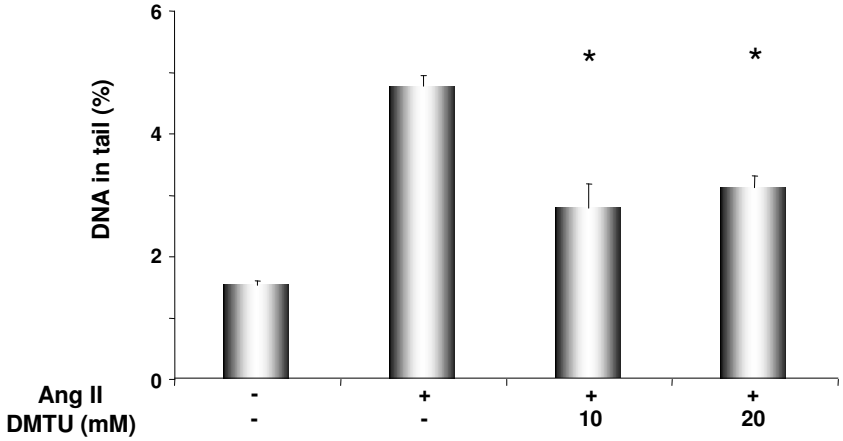

Figure 14 DNA damage measured by comet assay after treatment of HEK-293 cells with 200 nM Ang II with or without the indicated concentration of hydroxyl radical scavenger DMTU for 4 h. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

4.1.4 Inhibition of NOS and its effect on the genotoxicity of Ang II

Ang II is known to increase nitric oxide synthase (NOS) and NADPH oxidase activity as two possible sources of ROS production. To verify if the NOS family is involved in Ang II-induced genotoxicity, L-NAME as a general inhibitor of the family and 1400W, a specific inhibitor of inducible NOS were added to the cells together with Ang II. The depicted data in Figure 15 and Figure 16 show that none of the inhibitors were able to protect the cells against the genotoxic effect of Ang II, implying that NOS does not play a role in the genotoxicity of Ang II.

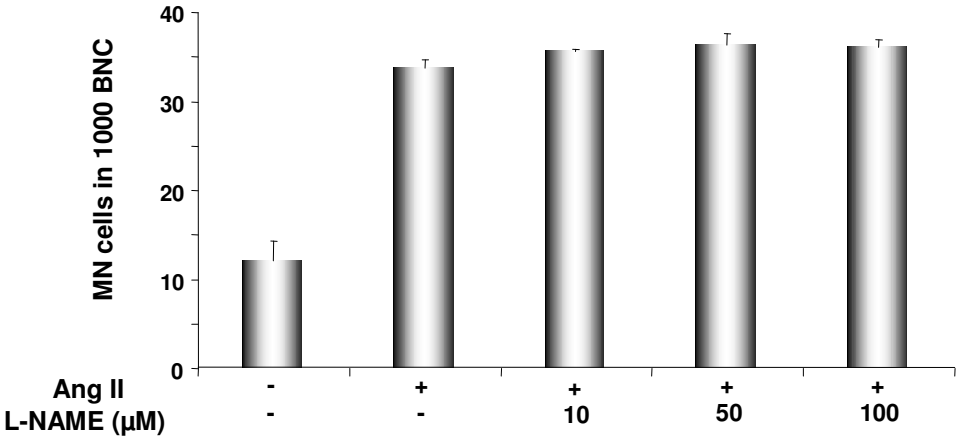

Figure 15 Micronuclei formation after treatment of HEK-293 cells with 200 nM of Ang II with or without co-incubation with the indicated concentrations of NOS inhibitor, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) for 4 h. MN = micronuclei, BNC = binucleated cells.

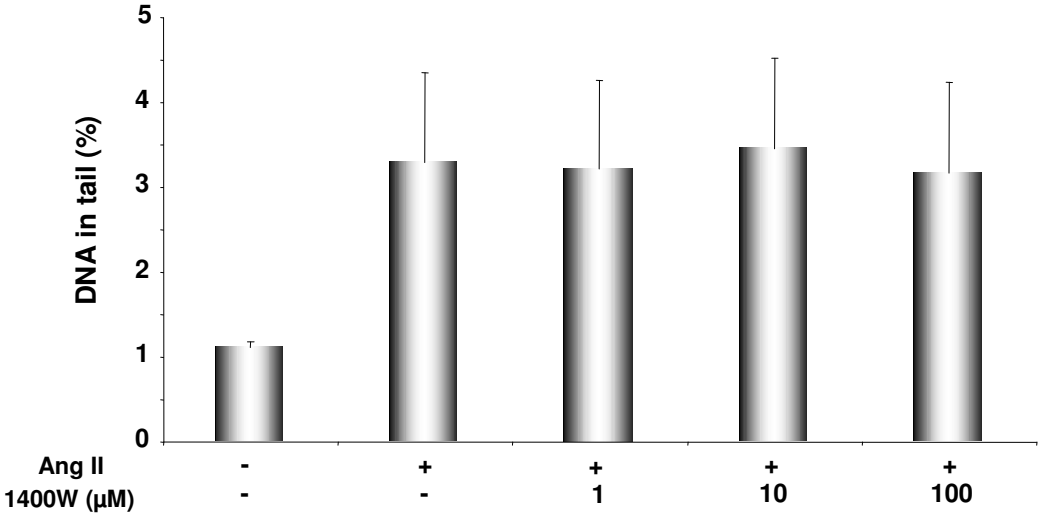

Figure 16 Micronuclei induced by 4 h treatment of HK-2 cells with 200 nM Ang II and the indicated concentrations of the specific inducible NOS inhibitor 1400W. MN = micronuclei, BNC = binucleated cells.

4.1.5 The effect of NADPH oxidase inhibition on the genotoxicity of Ang II

NADPH oxidase was also targeted with the same strategy using diphenyleneiodonium chloride (DPI), an inhibitor of flavoprotein enzymes such as NADPH oxidase and VAS 2870, a novel inhibitor of NADPH oxidase. As depicted in Figure 17, DPI was able to reduce the DNA damage in the cells, leading us to the conclusion that superoxide radicals which are produced by this enzyme may play a role in the genotoxicity of Ang II. VAS 2870 which is supposed to exclusively inhibit NADPH oxidase reduced the DNA damage caused by Ang II to the control level (Figure 17) which confirms the hypothesis that NADPH oxidase is the source of ROS which is responsible for genotoxicity of Ang II.

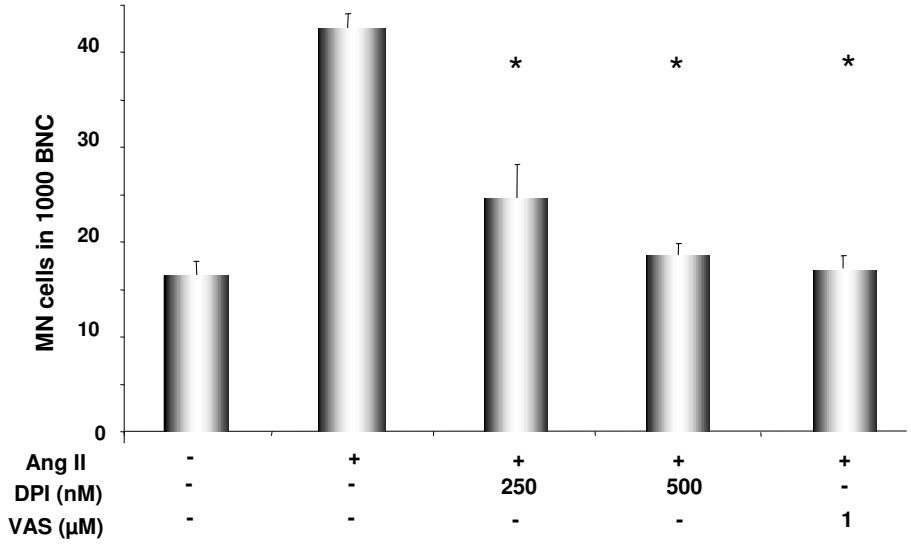

Figure 17 DNA damage measured in the micronucleus frequency test after 4 h treatment of HK-2 cells with 200 nM Ang II with or without co-incubation with the indicated concentrations of the flavoprotein enzyme inhibitor diphenyleneiodonium chloride (DPI) and with the NADPH oxidase inhibitor VAS 2870 (VAS). MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

To investigate the activation of NADPH oxidase, migration of the p47 subunit from cytosol to the membrane was monitored. A higher proportion of p47 was observed in the membrane after 1 hour of Ang II treatment. However, the presence of unspecific bands in the Western blot hindered us from quantification of the bands. Therefore, phosphorylation of p47 after Ang II treatment was assessed using immunoprecipitation. The results showed a higher phosphorylation of p47 after 1 and 2 hours incubation with Ang II (Figure 18).

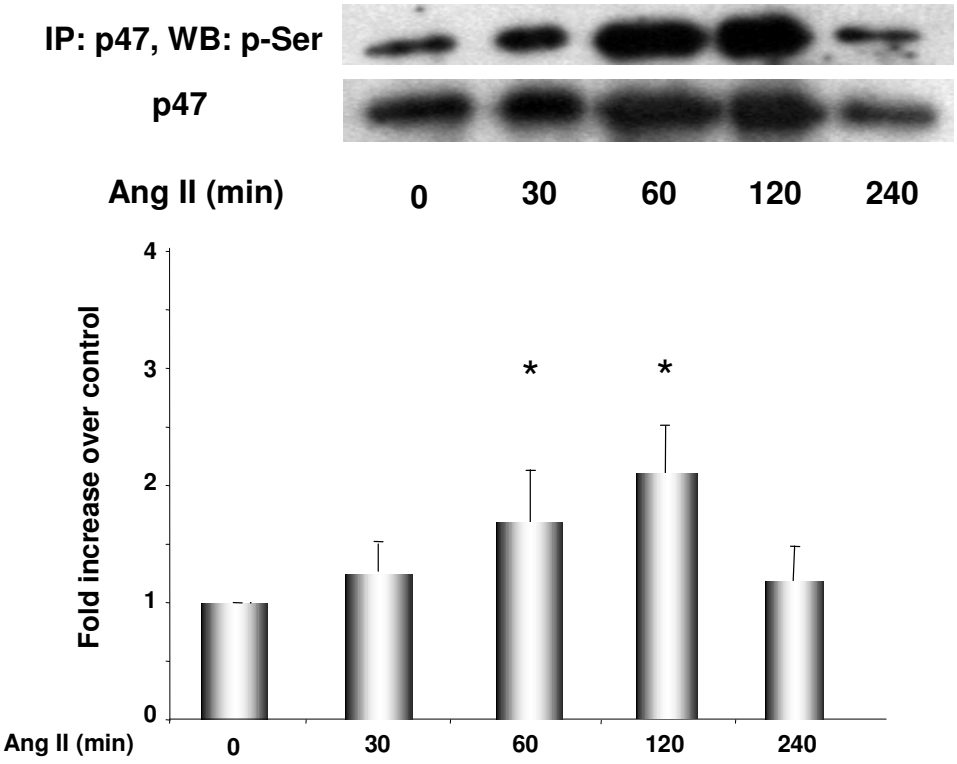

Figure 18 Activation of NADPH oxidase in HK-2 cells investigated by phosphorylation of its p47 subunit after the indicated duration of 200 nM Ang II treatment.

In kidney, mostly two isoforms of Nox family called Nox2 and Nox4 are present. Nox2 is the only member of Nox family which secrets superoxide to the outside of the cell membrane (101). Before, we showed that incubation of the cells with Ang II and superoxide dismutase is not able to reduce the genotoxic effect of Ang II, while TEMPOL which is cell membrane permeable reduces the effect. The fact that only cell permeable TEMPOL could prevent the effect of Ang II, was a hint for the involvement of Nox4. It is known that phosphorylation of Rac1 is necessary for activation of Nox2, but Nox4 does not need Rac1 for its activation. Rac1 is phosphorylated by PI3K to be activated. We inhibited PI3K with the two specific inhibitors wortmannin and LY-294002 to prevent Nox2 activation. As illustrated in Figure 19 none of these compounds prevented the induction of DNA damage by Ang II as measured in the comet assay. To ensure that the used concentration of wortmannin was enough to inhibit the activity of PI3K, the phosphorylation of Akt, another substrate of this enzyme was measured in western blot. The same concentration of wortmannin which was used in our genotoxicity tests completely hindered phosphorylation of Akt.

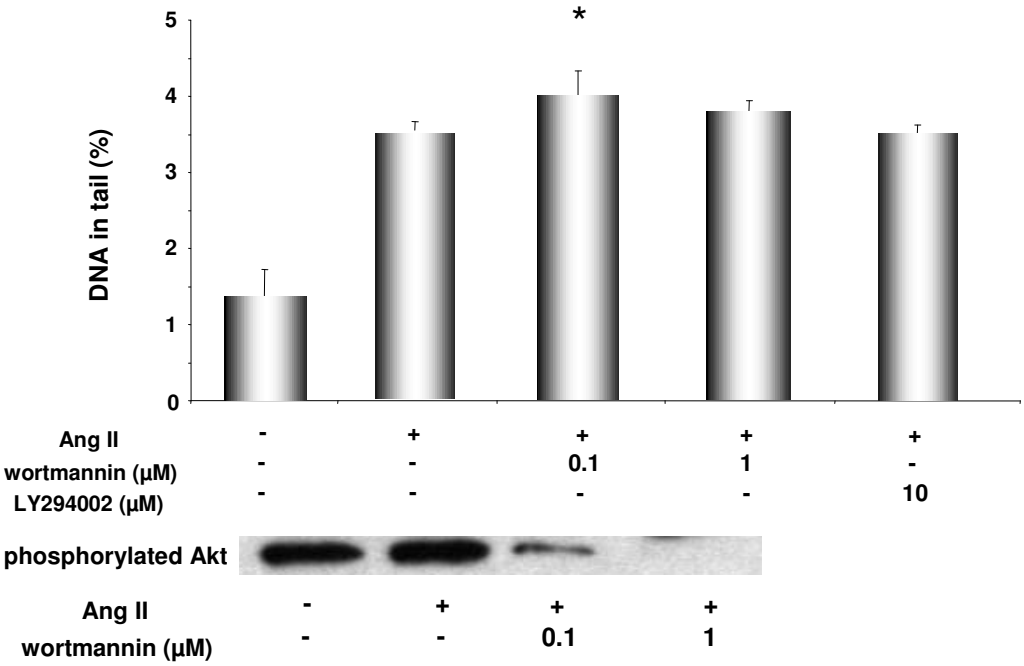

Figure 19 DNA damage measurement after 4 h treatment of HK-2 cells with 200 nM Ang II and the two PI3K inhibitors wortmannin and LY 294002. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test). The phosphorylation of Akt, a downstream substrate of PI3K, was assessed to ensure that the used concentration of the inhibitors were able to inhibit PI3K activity.

We also inhibited Nox2 and Nox4 isoforms using siRNA strategy. The downregulation of Nox2 and Nox 4 was confirmed by RT-PCR and also in Western blot. The cells were then treated either with PBS as the negative control or with Ang II. The data represented in Figure 20 and Figure 21 show that the cells that were treated with Nox2-siRNA did not show any difference to the cells which served as the control. However, Nox4-siRNA downregulation hindered DNA damage formation induced by Ang II.

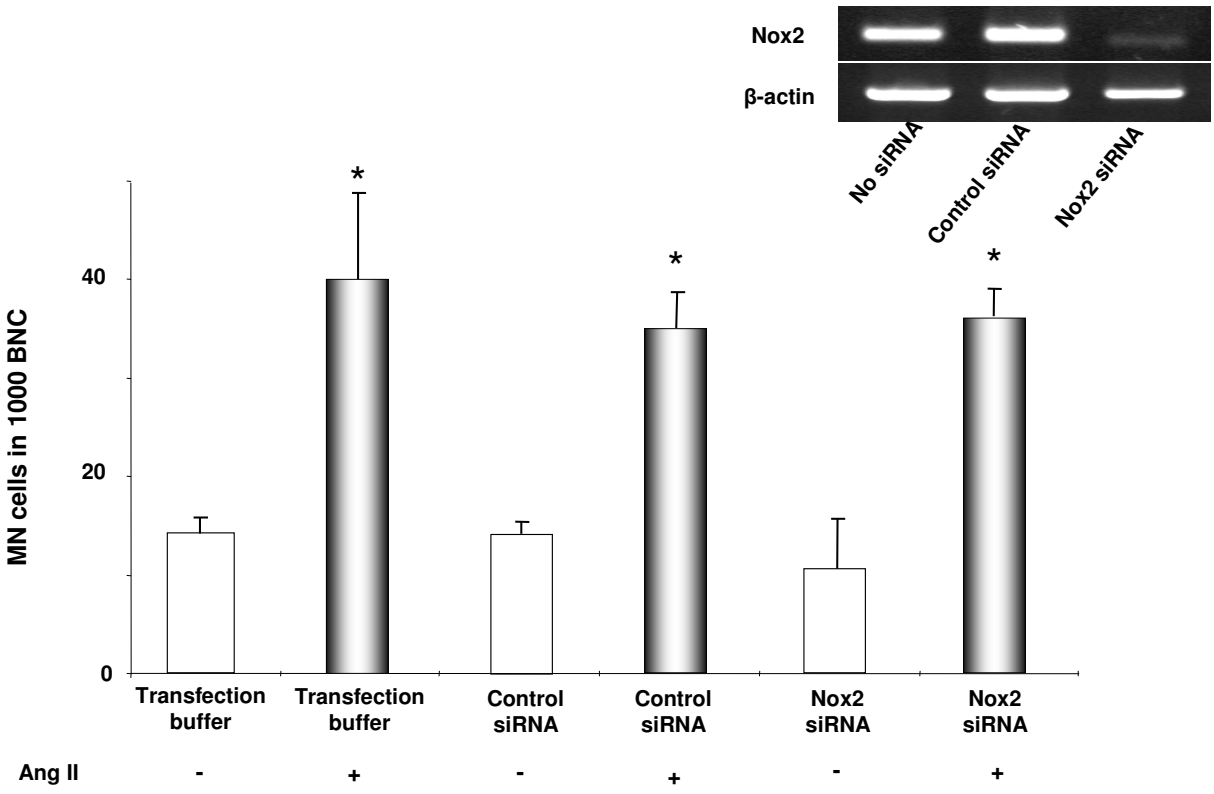

Figure 20 DNA damage measured by the micronucleus frequency test after 200 nM Ang II treatment for 4 h in HK-2 cells transfected with Nox2-siRNA. MN = micronuclei, BNC = binucleated cells. The success of siRNA transfection was confirmed by RT-PCR. * p ≤ 0.05 vs. Transfection buffer treated sample (Mann-whitney U-test).

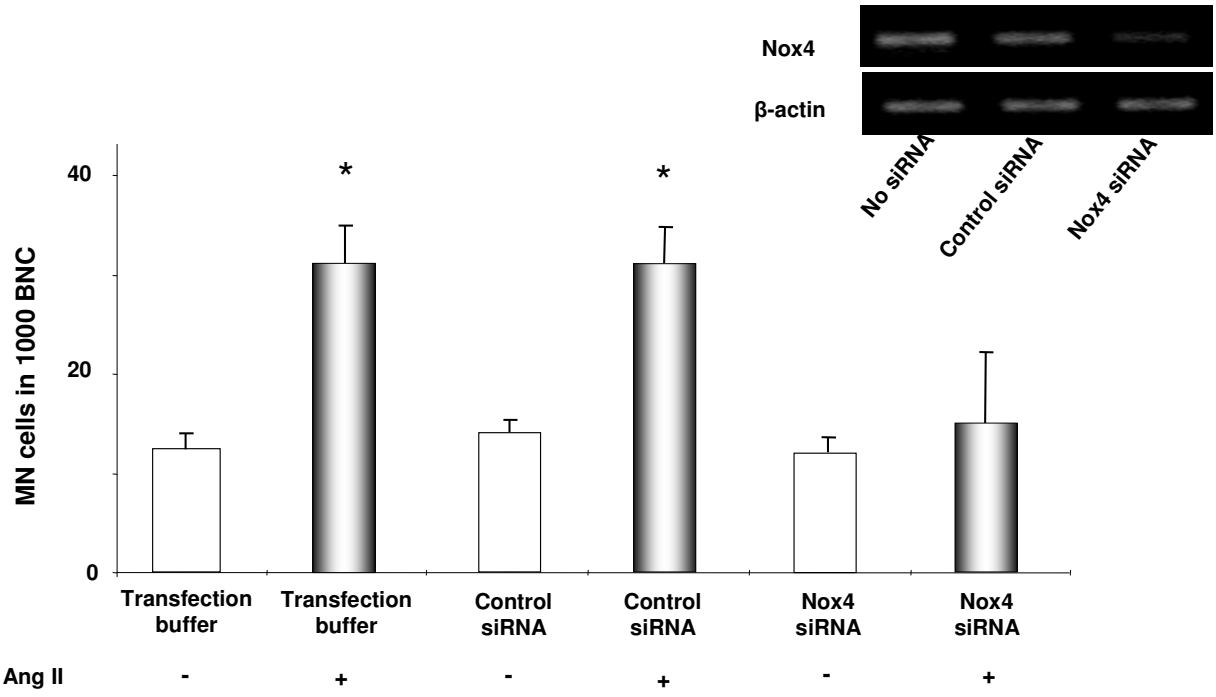

Figure 21 DNA damage measured by the micronucleus frequency test after 200 nM Ang II treatment for 4 h in HK-2 cells transfected with Nox4-siRNA. MN = micronuclei, BNC = binucleated cells. The success of siRNA transfection was confirmed by RT-PCR. * p ≤ 0.05 vs. Transfection buffer treated sample (Mann-whitney U-test).

4.1.6 The effect of protein kinase C inhibition on the genotoxicity of Ang II

One potential mechanism to induce NADPH oxidase activation is via protein kinase C (PKC). PKC goes through an auto-phosphorylation process when it is activated. PKC β II for example becomes phosphorylated at serine 660 when it is activated. With the aid of an antibody which recognizes different isoforms of PKC only when they are phosphorylated at carboxy terminal residues homologous to serine 660 of PKC β II, we checked if PKC is activated after Ang II treatment. Figure 22 illustrates our results from Western blot analysis, showing a higher activation of PKC after Ang II treatment. Exploring the role of PKC in the induction of DNA damage by Ang II, sphingosine and rottlerin, two specific inhibitors of PKC were used. Both, comet assay and micronucleus frequency test, showed a decrease in DNA damage after incubation of the cells with these inhibitors together with Ang II (Figure 23).

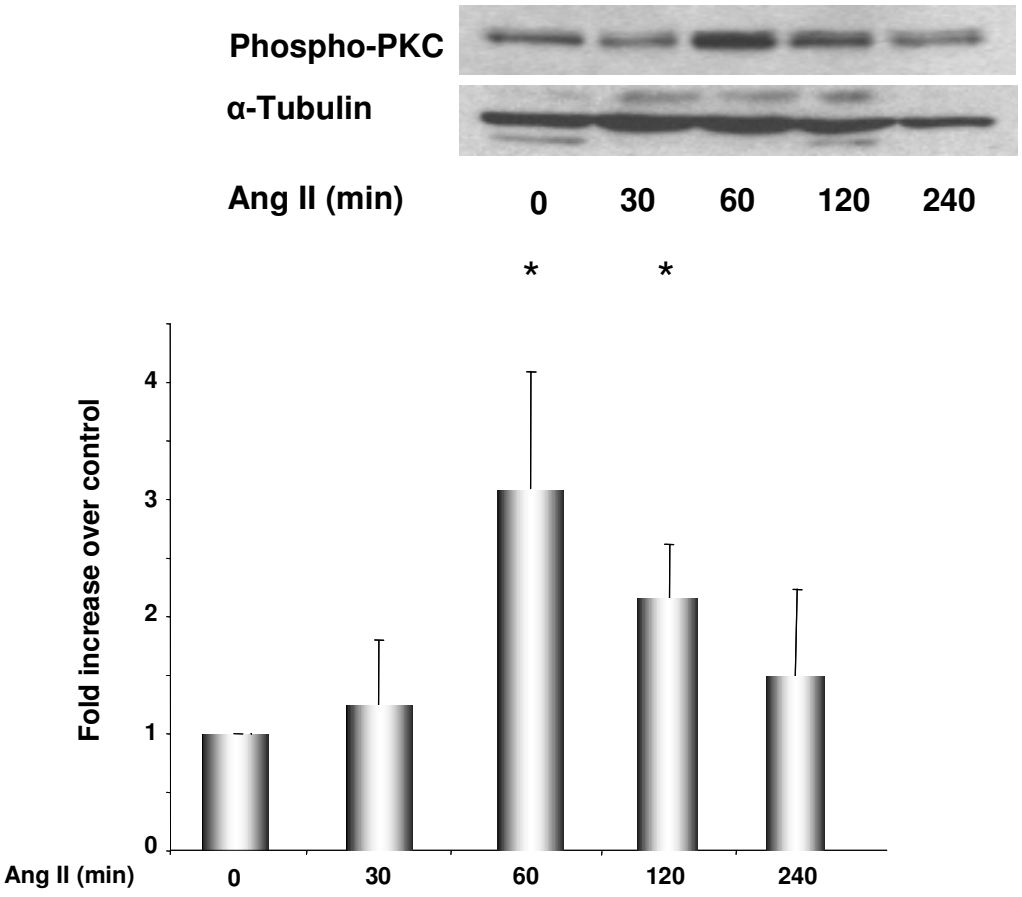

Figure 22 Western blot analysis of phosphorylation of protein kinase C after indicated period of 200 nM Ang II treatment in HK-2 cells. Phosphorylation of PKC was assessed by an antibody against phospho- Ser 660 PKC. * p ≤ 0.05 vs. untreated sample (Mann-whitney U-test).

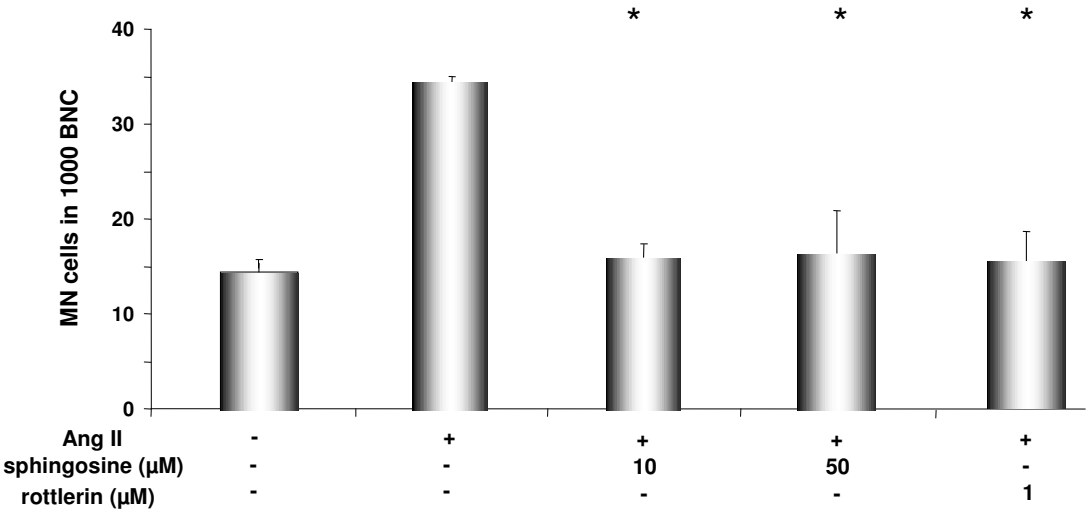

Figure 23 DNA damage measured with the comet assay after treatment of HK-2 cells for 4 h with 200 nM Ang II with or without co-incubation with the indicated concentrations of PKC inhibitors, sphingosine and rottlerin. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

4.1.7 Inhibition of phospholipases and their effects on Ang II-induced DNA damage

Phospholipase D (PLD) produces phosphatidic acid which is short-lived and is converted to diacylglycerol (DAG) which in turn can activate PKC. PLD inhibition by 1-butanol failed to impede Ang II-induced DNA damage in our cells (Figure 24).

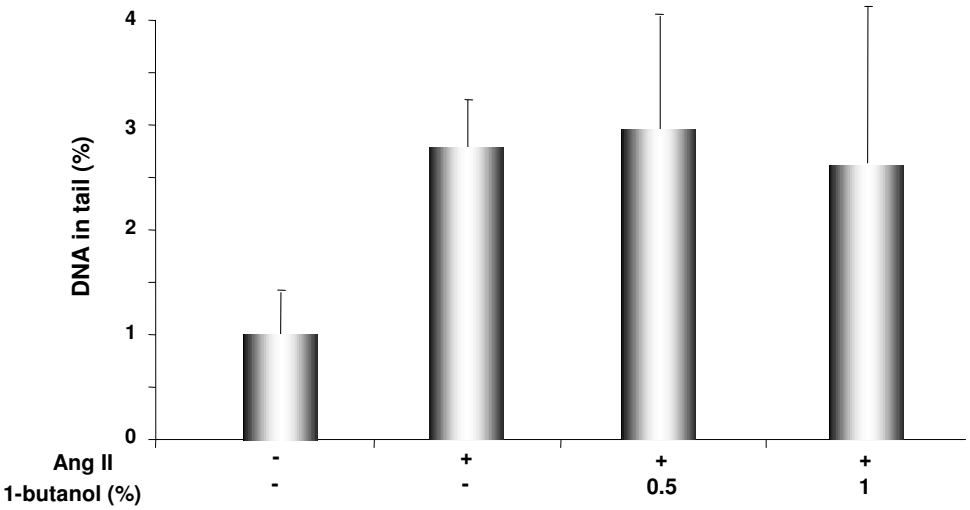

Figure 24 DNA damage induced by 4 h treatment of HK-2 cells with 200 nM Ang II and the indicated concentrations of 1-butanol, a specific inhibitor of PLD.

Involvement of phospholipase C (PLC) as the activator of PKC was investigated using the inhibitor U-73122, which successfully protected the cells against Ang II-induced DNA damage (Figure 25), while U-73343, the inactive analogue of the inhibitor failed to show the similar effect at both tested concentrations.

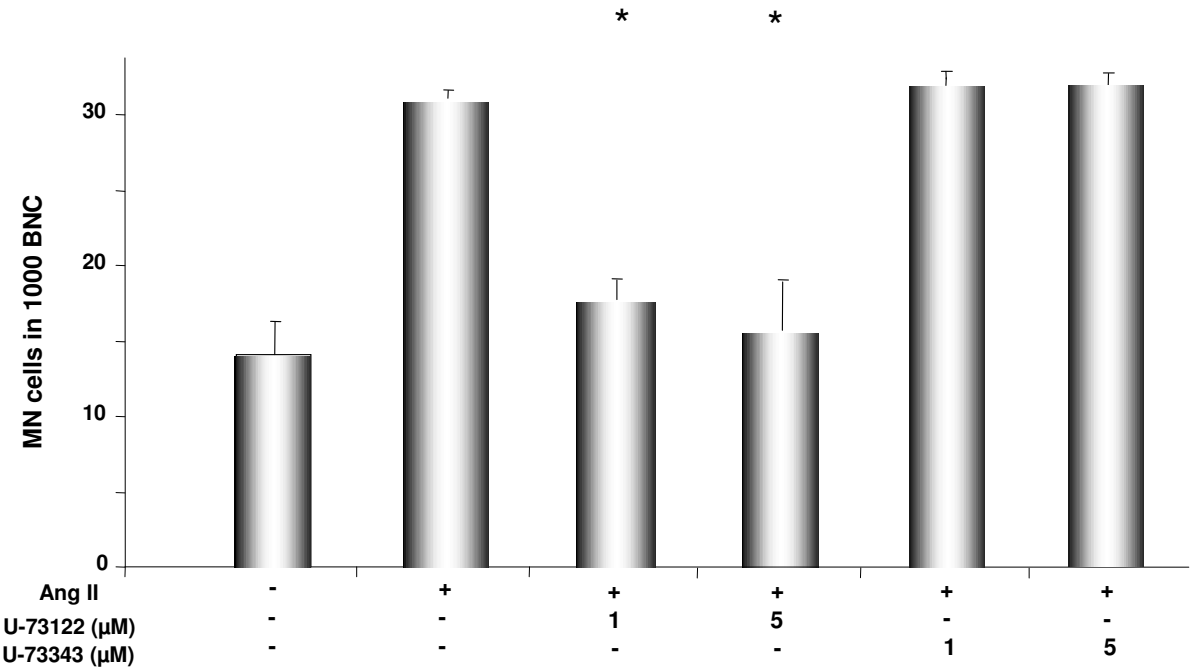

Figure 25 Effect of the phospholipase C inhibitor U-73122 on the micronucleus formation induced by 4 h treatment of HK-2 cells with 200 nM Ang II. The same concentrations of an inactive analog of phospholipase C inhibitor U-73343 was also investigated to confirm the specificity of the inhibitor. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

4.1.8 The effect of $G_{\alpha\text{-}q/11}$ and $G_{\beta\gamma}$ inhibition on Ang II-induced genotoxicity

AT1R is a G-protein coupled receptor (GPCR). $G_{\alpha\text{-}q/11}$ is coupled to AT1R which later binds to $G_{\beta\gamma}$ and activates PLC. We used GP-2A, a competitive $G_{\alpha\text{-}q}$ inhibitor and gallein a $G_{\beta\gamma}$ inhibitor to confirm that the signaling via AT1R and the subsequent pathway which involves G-proteins are responsible for the observed genotoxicity of Ang II. Inhibition of both G-protein types resulted in a decrease of Ang II-induced DNA damage to the control level (Figure 26).

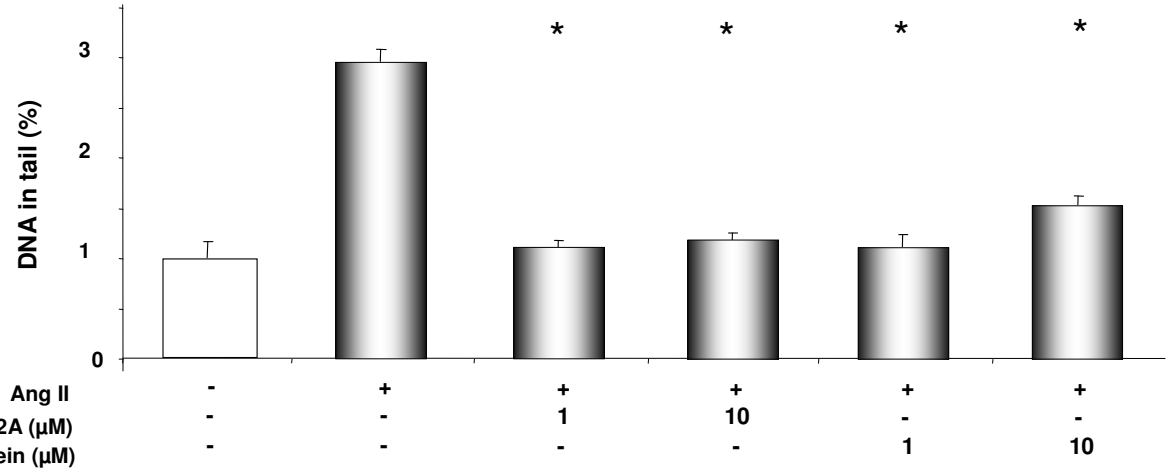

Figure 26 DNA damage measured by the comet assay after 4 h treatment of HK-2 cells with 200 nM Ang II with or without co-incubation with the indicated concentrations of the $G_{\alpha\text{-}q}$ G-protein inhibitor GP-2A or $G_{\beta\gamma}$ inhibitor gallein . * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

Also, the effect of $G_{\alpha i/o}$ inhibition on the genotoxicity of Ang II was investigated using pertussis toxin. The data presented in Figure 27 confirm the role of pertussis toxin sensitive $G_{\alpha i/o}$ subunits of G-proteins in genotoxicity of Ang II.

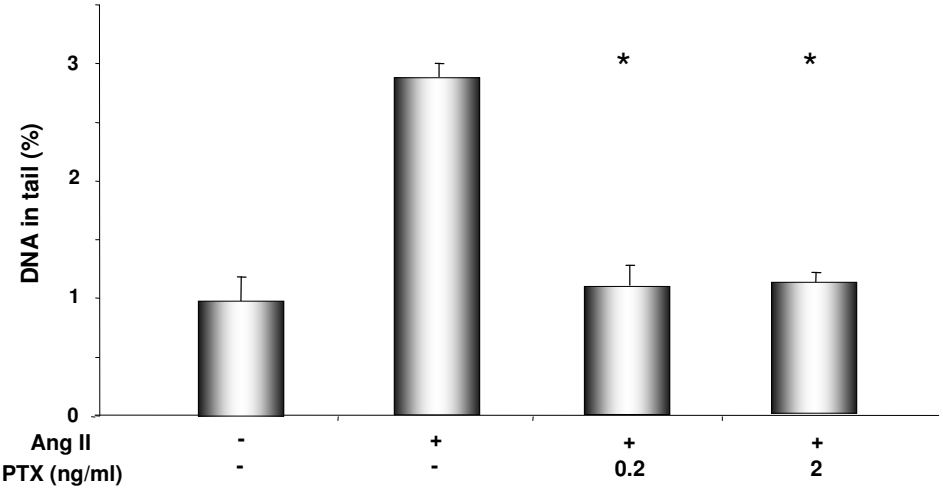

Figure 27 DNA damage measured by the comet assay after 4 h treatment of HK-2 cells with 200 nM Ang II with or without co-incubation with the indicated concentrations of the $G_{\alpha i/o}$ G-protein inhibitor pertussis toxin. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

4.1.9 Involvement of calcium in Ang II-induced DNA damage

Figure 28 illustrates a schematic representation of PKC activation by calcium. The involvement of calcium in the activation of PKC by Ang II was investigated. Ang II treatment resulted in calcium release which could be chelated by the calcium chelator BAPTA-AM (Figure 29). This effect of BAPTA-AM also prevented DNA damage induced by Ang II (Figure 30).

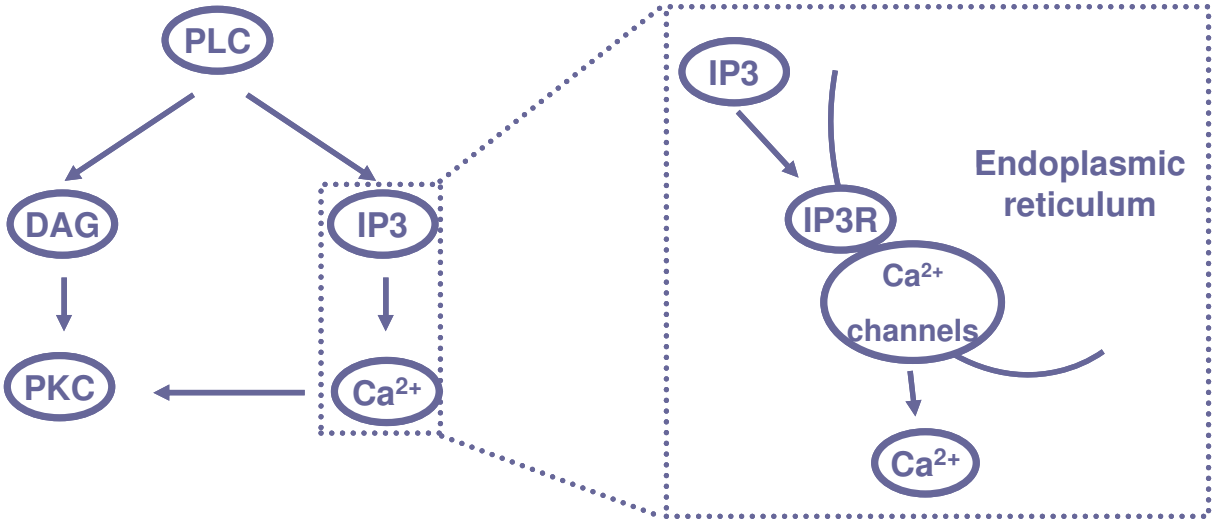

Figure 28 Schematic representation of PKC activation by PLC and involvement of calcium. PLC acts on PIP2 in the membrane and produces IP3 and DAG. DAG can directly activate PKC. IP3 binds to its receptor on endoplasmic reticulum and causes release of calcium from ER calcium stores. The released calcium can activate PKC.

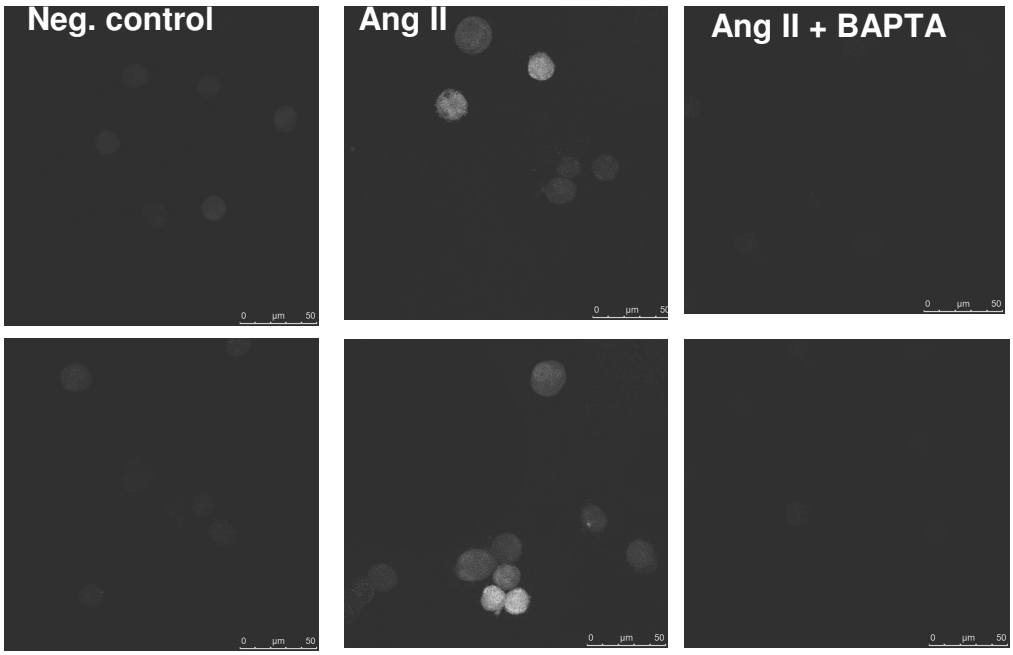

Figure 29 Calcium release in HK-2 cells in response to 200 nM Ang II treatment for 30 min, visualized with help of the fluorescent calcium binding dye Fluo-4. The released calcium can be chelated by BAPTA which also leads to decreased DNA damage induced by Ang II.

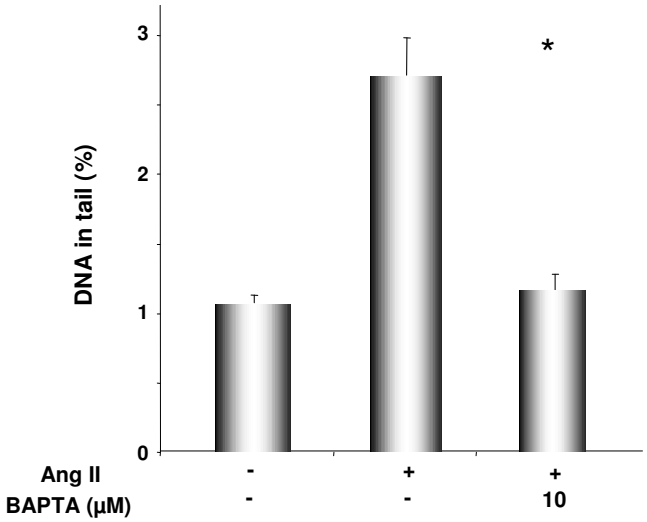

Figure 30 DNA damage measured in the comet assay after 4 h treatment of HK-2 cells with 200 nM Ang II with or without co-incubation with the calcium chelator BAPTA-AM. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

To distinguish between the source of calcium which is necessary for the activation of PKC and the consequently observed DNA damage after Ang II treatment, a variety of compounds were used. First, thapsigargin was used to deplete the calcium stores of the cells. Thapsigargin is a plant derived compound which inhibits a class of enzymes known as sarco/endoplasmic reticulum Ca^{2+}-ATPase. Inhibition of these enzymes blocks the ability of the cells to pump calcium into the sarcoplasmic and endoplasmic reticula which causes these stores to become depleted. We pre-treated our cells with thapsigargin for 30 min, followed by 4 hours of Ang II treatment. In this manner, Ang II failed to induce DNA damage in the comet assay. The number of micronuclei

was slightly increased after thapsigargin and Ang II treatment in comparison to the negative control, but this was significantly lower than the number of micronuclei induced by Ang II alone (Figure 31).

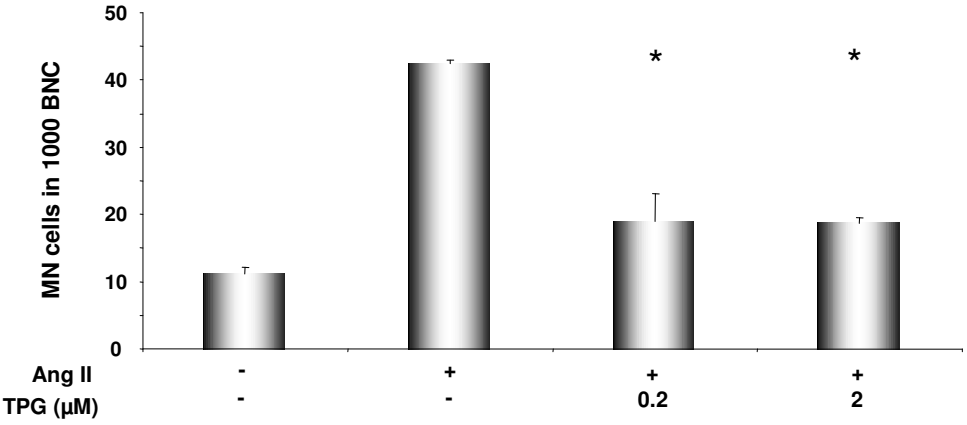

Figure 31 Induced micronuclei after 4 h treatment of HK-2 cells with 200 nM Ang II with or without 30 min pre-incubation with the calcium depletor thapsigargin. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

The calcium channels on the endoplasmic reticulum are IP3 receptor (IP3R)-associated. Therefore, we used a blocker of these channels (2-APB) as well as an IP3R antagonist (TMB-8). Both of these compounds successfully reduced the damage by Ang II in the comet assay and in the micronucleus frequency test (Figure 32 and Figure 33), confirming the role of endoplasmic calcium stores in the genotoxicity of Ang II.

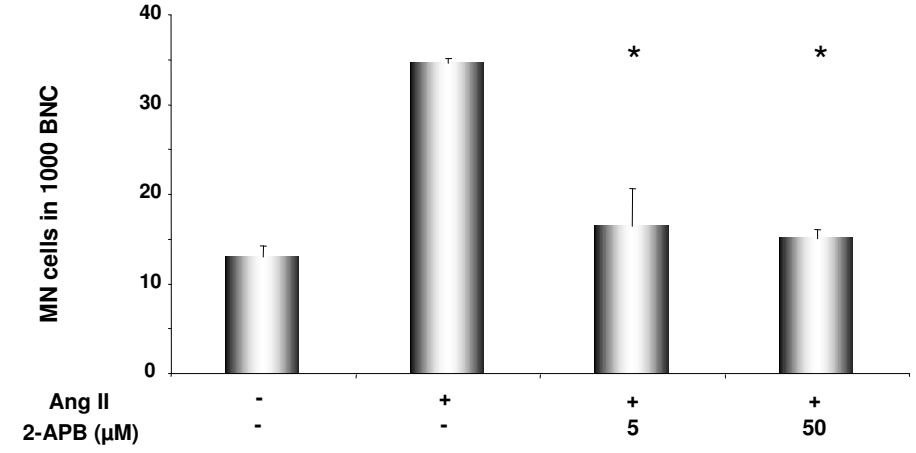

Figure 32 Micronuclei formation in response to 200 nM Ang II and the ER calcium channel blocker 2-APB for 4 h in HK-2 cells. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

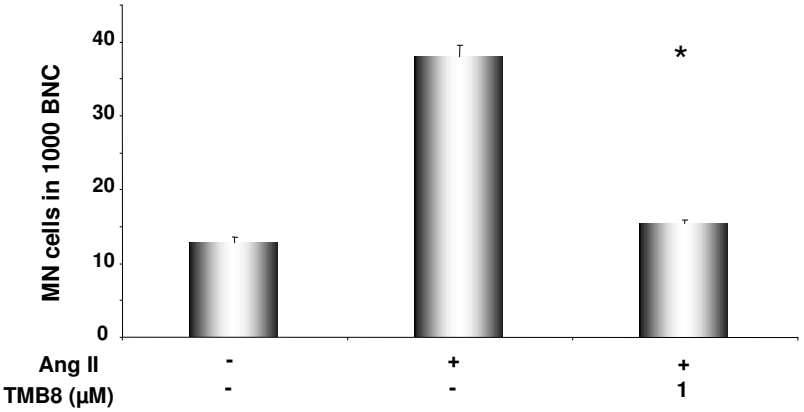

Figure 33 Micronuclei formation in response to 200 nM Ang II and the IP3R antagonist TMB8 for 4 h in HK-2 cells. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

In the next step, the potential role of extracellular calcium was investigated. For this purpose, the cells were washed 30 min. before the experiment and then calcium free medium was added to the flask. Treatment of the cells with Ang II in calcium free medium failed to induce damage assessed by comet assay and MN test (Figure 34).

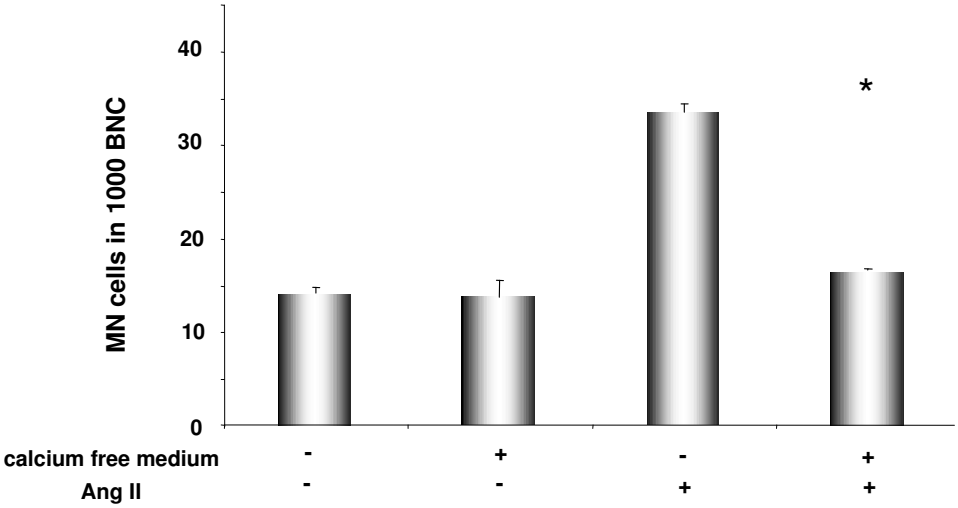

Figure 34 Micronuclei formation in HK-2 cells in response to 4 h treatment with 200 nM Ang II in calcium free medium. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang II treated sample in normal medium (Mann-whitney U-test).

Furthermore, amlodipine, an inhibitor of membrane calcium channels impeded the genomic damage caused by Ang II (Figure 35). In summary, these results pointed to the role of extracellular calcium ions as well as intracellular calcium stores.

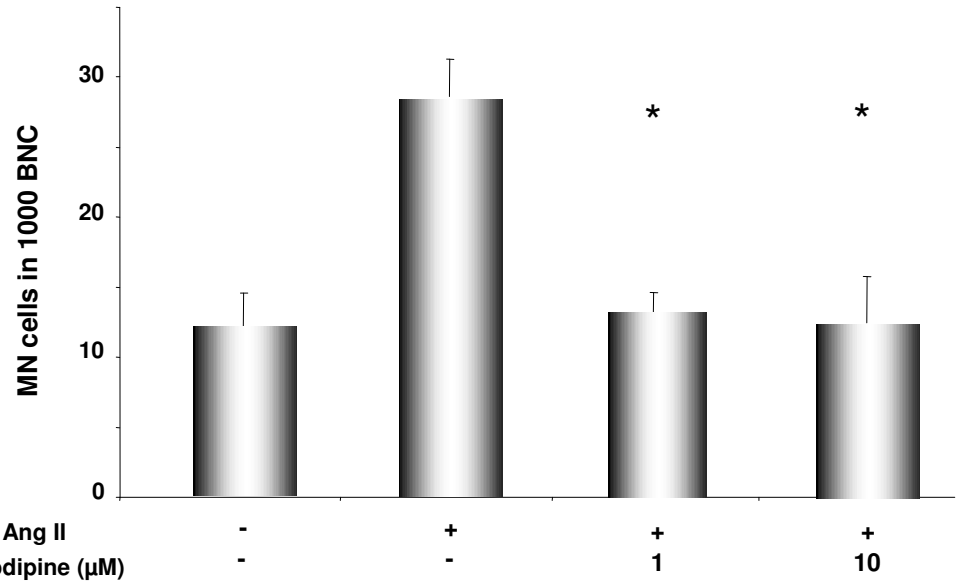

Figure 35 Micronuclei formation in HEK-293 cells in response to 4 h treatment with 200 nM Ang II and the indicated concentration of membrane calcium channel blocker amlodipine. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. Ang II treated sample (Mann-whitney U-test).

To confirm that the observed effect of these compounds on genotoxicity is due to the intervention in calcium concentrations inside the cells, the cells were stained for calcium using Fluo-4 and then visualized under a confocal microscope. The pictures were quantified by measuring the average of the fluorescence intensity in 200 cells. Ang II treatment resulted in the increase of fluorescent intensity up to two fold of the negative control. This increase was absent when the cells were treated in calcium free medium or after treatment with Ang II and either of BAPTA-AM, thapsigargin, TMB8 and 2-APB. The results of the quantification are shown in Figure 36.

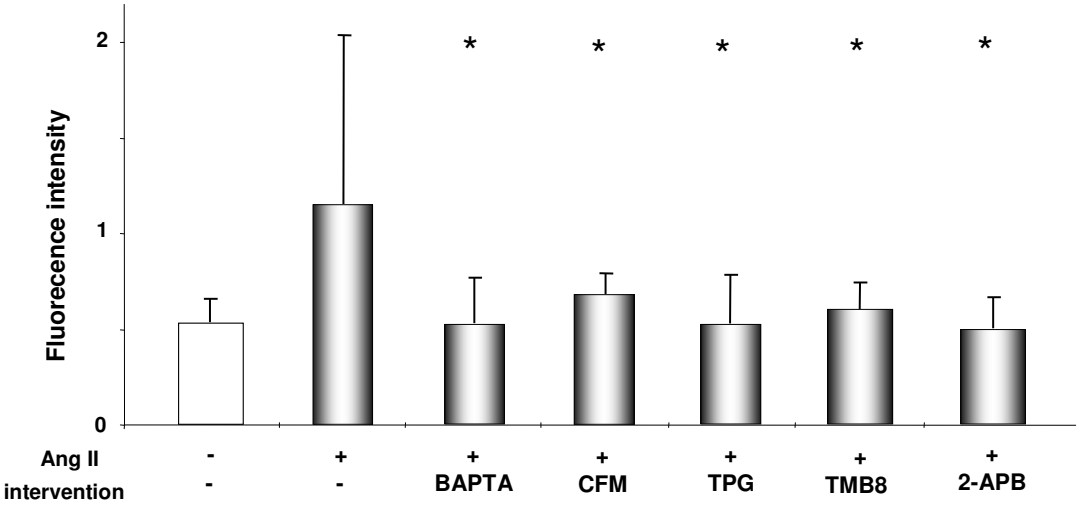

Figure 36 Quantification of calcium release in HK-2 cells, visualized by the fluorescent calcium binding dye Fluo-4 after 30 min. treatment with 200 nM Ang II and the indicated compounds to intervene calcium signaling. * p ≤ 0.05 vs. Ang II treated sample (Student's t-test).

4.2 Dopamine

4.2.1 Evidence of dopamine-induced genotoxicity in vitro

The genotoxicity of the neurotransmitter dopamine was investigated using the micronucleus frequency test in several cell lines of different tissue origin. The cell lines were selected from dopamine-responsive tissues. (A part of the results which are not presented here are included in reference (112)). While the human lymphoblastoid TK6 cells showed a significant response at 25 µM dopamine and higher, but not with the tested concentrations 6.25 and 12.5 µM, rat kidney NRK and rat pheochromocytoma PC12 cells exhibited clearly elevated micronucleus frequencies with all tested concentrations. The results of the micronucleus frequency test in NRK cells are depicted in Figure 37. PC12 cells revealed a more pronounced induction of micronuclei than TK6 and NRK cells. MDCK and LLC-PK1 cell lines did not show any increase in the number of micronuclei after treatment with different concentrations of dopamine (MDCK 6.25-100 µM, LLC-PK1 50-200 µM, data for LLC-PK1 cells not shown here).

4.2.2 Evidence of dopamine-induced apoptosis

Different concentrations of dopamine were used to investigate if this neurotransmitter can also induce apoptosis *in vitro*. We did not observe any induction of apoptosis with 100 µM dopamine, while 300 µM of dopamine caused significantly elevated

apoptosis frequencies in TK6 and PC12 cells (Table 3). H_2O_2 was used as a positive control which induced a significant number of apoptotic cells in TK6 and NRK cells. The positive control could not be quantified in PC12 cells due to excess toxicity/cell fragmentation.

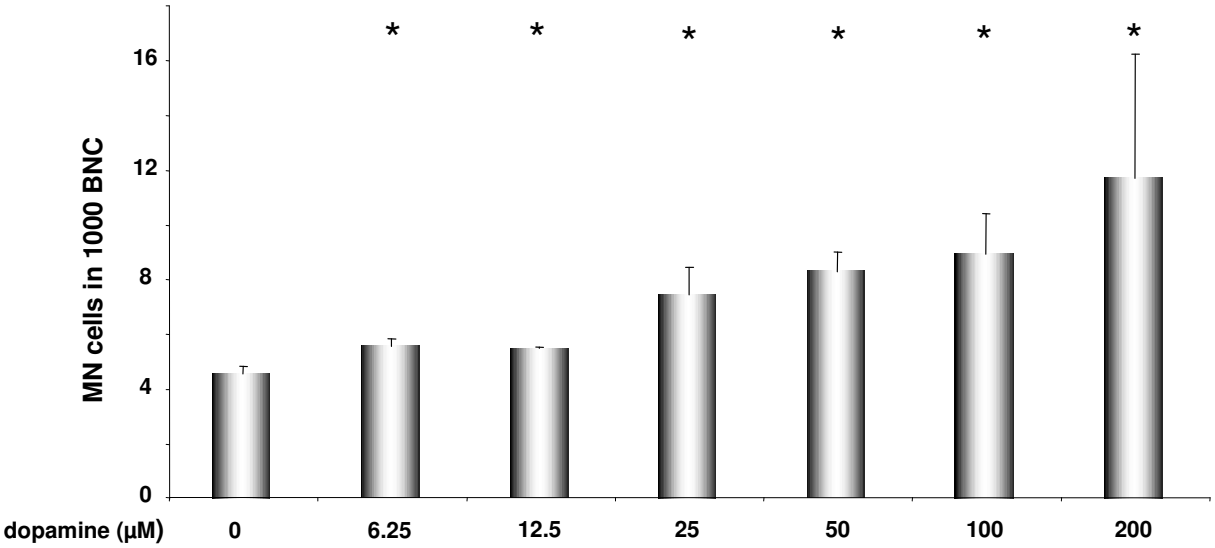

Figure 37 Dopamine-induced genotoxicity quantified by micronucleus frequency test in NRK cells after 4 h incubation with indicated concentrations of dopamine. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. untreated sample (Mann-Whitney U-test).

Cell line	Test substance	Concentration (µM)	% Apoptotic cells
TK6	Control	0	1.3 ± 0.4
	Dopamine	100	2.2 ± 1.1
		300	4.5 ± 0.5*
	H_2O_2	100	8.9 ± 3.6*
NRK	Control	0	0.7 ± 0.2
	Dopamine	100	0.9 ± 0.2
		300	0.9 ± 0.3
	H_2O_2	100	13.9 ± 1.0*
PC12	Control	0	1.3 ± 0.1
	Dopamine	100	1.9 ± 0.6
		300	5.3 ± 0.6 *

Table 3 Dopamine-induced apoptosis *in vitro*. Significance (p ≤ 0.05 in Mann-Whitney U-test versus the control of the same cell line) is indicated by an asterisk (*).

4.2.3 Role of dopamine receptor D1 in the genotoxicity of dopamine

PCR analysis confirmed the presence of D1 and D2 type receptors as well as of dopamine transporter (DAT) in PC12 cells (112). Therefore we decided to use these cells for experiments combining dopamine receptor antagonists and DAT inhibitors with dopamine. We investigated the role of D1R in the genotoxicity of dopamine. SCH 23390 was used as an antagonist of D1R in NRK and PC12 cells. In both cell lines (data from NRK cells are not shown) the antagonist reduced the genotoxic effect of dopamine (Figure 38).

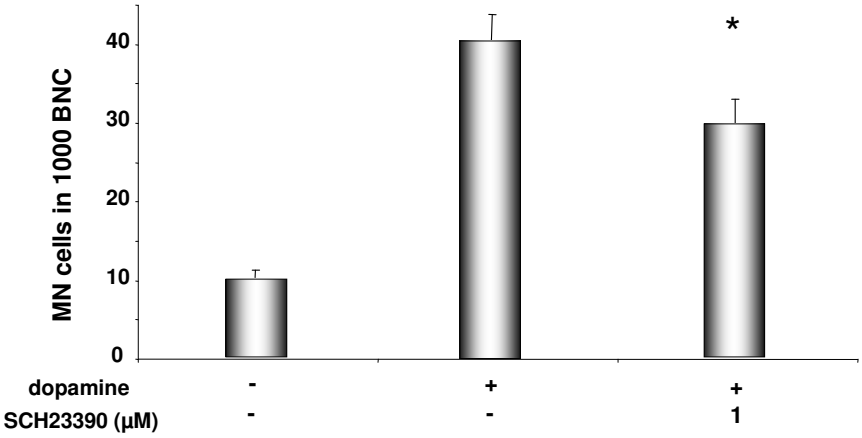

Figure 38 Micronuclei formation in response to 4 h incubation with 100 µM dopamine together with D1R inhibitor SCH 23390. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. dopamine treated sample (Mann-Whitney U-test).

We also questioned if signaling from D1R alone leads to a DNA damage response in the absence of dopamine. For this reason we used a potent agonist of D1R, SKF 38393 to see if activation of D1R can increase micronucleus formation in PC12 cells. No increased formation of micronuclei was observed when the cells were treated with this agonist at concentrations of up to 120 µM (Figure 39).

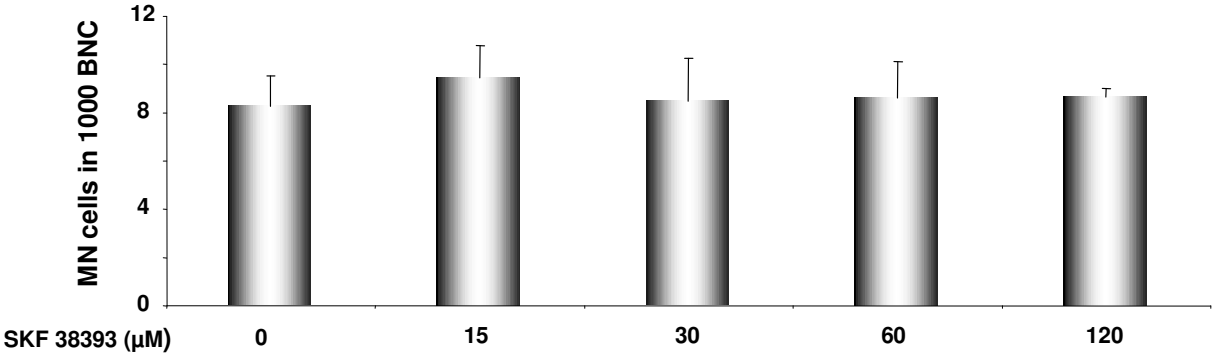

Figure 39 Micronuclei induced by 4 h treatment with the indicated concentrations of the D1R agonist SKF 38393 in PC12 cells. MN = micronuclei, BNC = binucleated cells.

4.2.4 Role of dopamine receptor D2 in the genotoxicity of dopamine

The D2 receptor antagonist sulpiride (Figure 40) reduced the genotoxic effect of dopamine significantly. The G-protein coupled receptor D2R is coupled with the $G_{\alpha i/o}$ protein, which is sensitive to the inhibitor pertussis toxin. This compound was applied to block the signaling from D2R in PC12 cells. Figure 41 shows that pertussis toxin significantly reduced the genotoxicity of dopamine in the micronucleus frequency test. These findings revealed a role for the D2 receptor as a mediator of dopamine-induced genotoxicity.

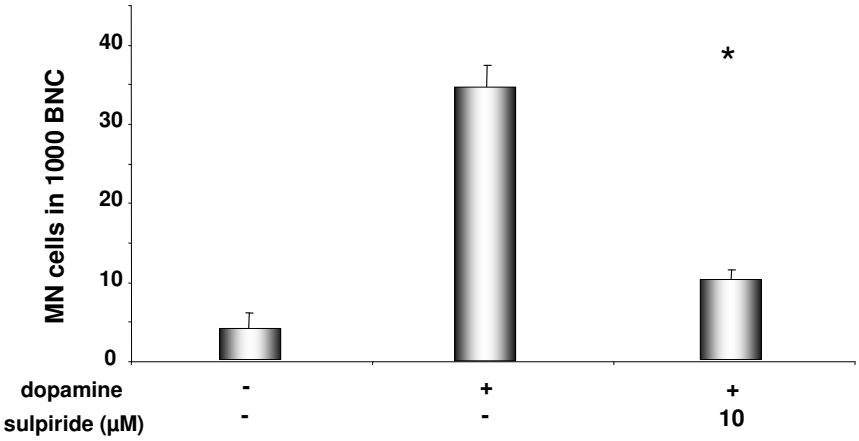

Figure 40 Micronuclei formation after 4 h treatment of PC12 cells with 100 µM dopamine with or without co-incubation with the D2R antagonist, sulpiride. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. dopamine treated sample (Mann-Whitney U-test).

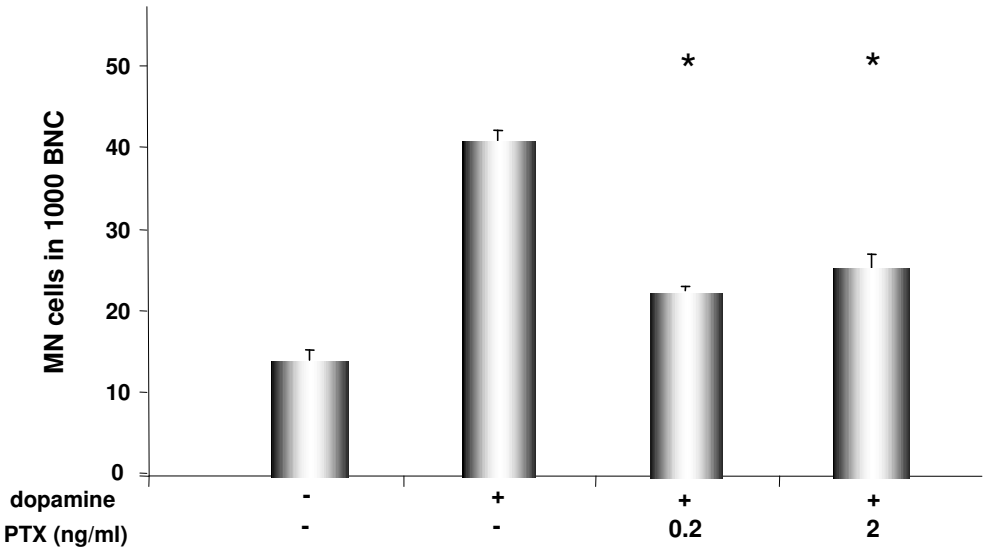

Figure 41 DNA damage measured by micronucleus frequency test after 4 h treatment of PC12 cells with 100 µM dopamine with or without co-incubation with the indicated concentrations of the $G_{\alpha i/o}$ G-proteins inhibitor pertussis toxin (PTX). MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. dopamine treated sample (Mann-Whitney U-test).

To investigate if activation of D2R signaling alone is enough to cause genotoxic damage even in the absence of dopamine, we stimulated D2R with quinpirole, a widely used D2R and D3R agonist. As illustrated in Figure 42, the indicated concentrations of quinpirole of up to 50 µM did not induce the formation of micronuclei.

We performed Real-time analysis of DAT expression after quinpirole treatment to question if D2R activation with quinpirole in the concentrations which failed to induce micronucleus formation affects the DAT expression level. PC12 cells were treated with quinpirole, total RNA was extracted and cDNA was produced. Afterwards the expression of DAT was quantified (Figure 43). Our results show that the expression of DAT increased significantly after 2 and 4 hours.

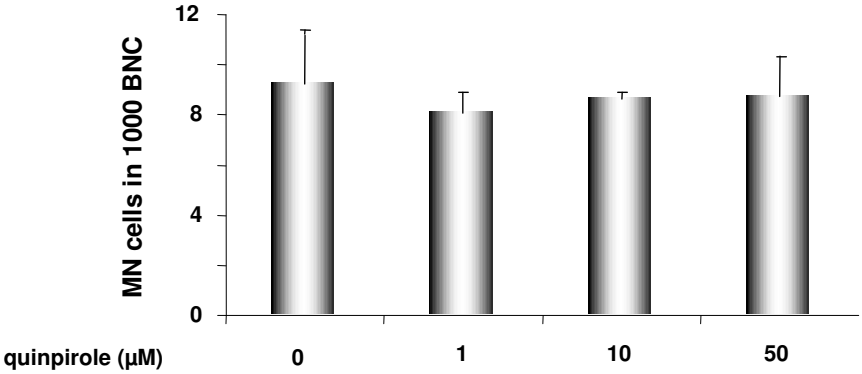

Figure 42 Micronuclei induced by 4 h treatment of PC12 cells with the indicated concentrations of the D2R agonist quinpirole. MN = micronuclei, BNC = binucleated cells.

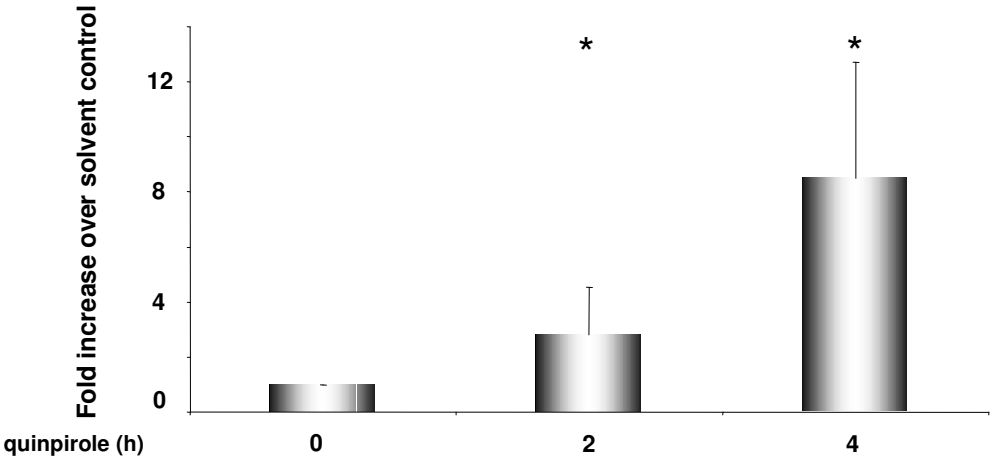

Figure 43 Expression of dopamine transporter after indicated periods of treatment with 50 µM D2R agonist quinpirole quantified by q-PCR. * p ≤ 0.05 vs. untreated sample (Mann-Whitney U-test).

4.2.5 Role of DAT in the genotoxicity of dopamine

We applied two different approaches to check for the role of DAT in dopamine-induced genotoxicity. First we used pharmacological inhibitors of DAT in combination

with dopamine in PC12 cells. GBR 12909 and nomifensine were used to inhibit DAT in our cell line. The results show that inhibition of DAT with either of these two inhibitors resulted in the formation of a lower number of micronuclei in comparison to the dopamine treated sample (Figure 44).

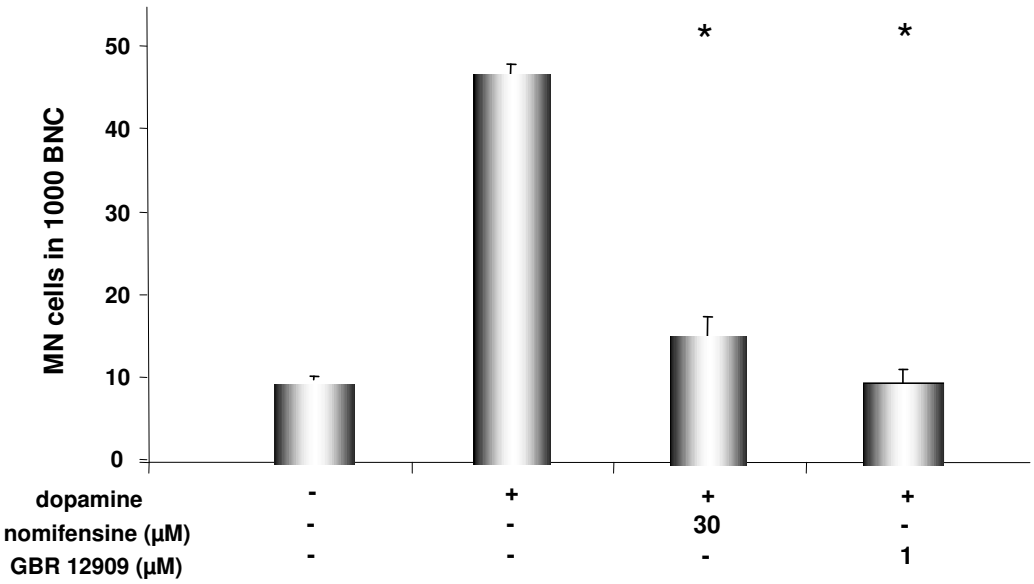

Figure 44 Prevention of MN formation induced by 4 h treatment of PC12 cells with 100 µM dopamine after co-incubation with the two DAT inhibitors nomifensine and GBR 12909. MN = micronuclei, BNC = binucleated cells. * p ≤ 0.05 vs. dopamine treated sample (Mann-Whitney U-test).

As mentioned before (4.2.1), the MDCK cell line did not show any DNA damage response when treated with different concentration of dopamine. Therefore, we used MDCK-DAT cells which were transfected with the human DAT gene, to investigate if the presence of the DAT gene can change the response of MDCK cells to dopamine. The activity of the transfected gene was confirmed elsewhere (34). As depicted in Figure 45, MDCK-DAT cells which where able to take up dopamine showed a dose-dependent micronucleus formation response whereas MDCK cells failed to show any DNA damage response.

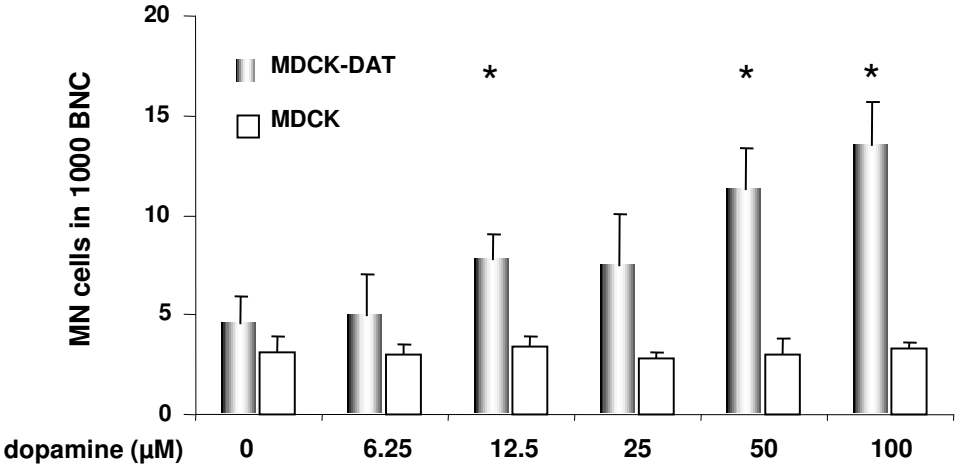

Figure 45 Dopamine-induced genotoxicity quantified by the micronucleus frequency test in MDCK and MDCK-DAT cells after 4 h incubation with the indicated dopamine concentrations. MN cells = micronucleated cells, BNC = binucleated cells. * p ≤ 0.05 vs. untreated sample of the same cell line (Mann-Whitney U-test).

4.2.6 Genotoxicity of dopamine is mediated via ROS production

Since dopamine has been reported to exert oxidative stress in mammalian cells, we tested whether a known antioxidant could reduce the micronucleus formation after dopamine treatment (Figure 46). The superoxide radical scavenger TEMPOL as well as the hydroxyl radical scavenger dimethylthiourea (DMTU) were in fact able to reduce micronucleus induction in PC12 cells significantly, but not completely.

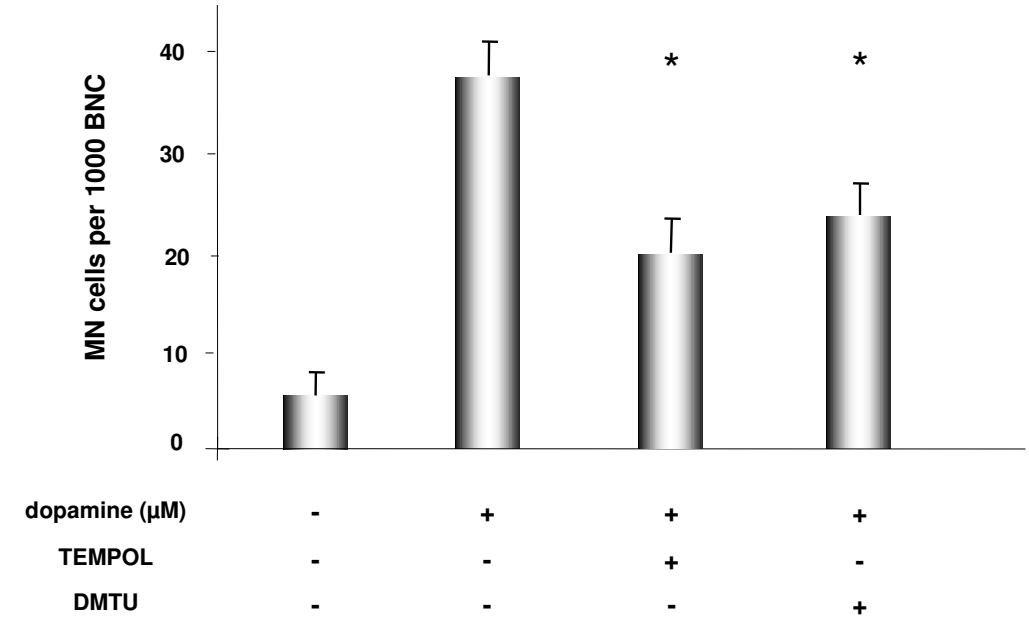

Figure 46 Effects of 4 h incubation with 50 µM of the superoxide scavenger TEMPOL or 10 mM of the hydroxyl radical scavenger DMTU on the micronucleus induction by 100 µM dopamine. MN cells = micronucleated cells, BNC = binucleated cells. * p ≤ 0.05 vs. dopamine treated sample (Mann-Whitney U-test).

4.2.7 Monoamine oxidase as a potential source of ROS formation

After uptake in cells, dopamine is either metabolized by the enzyme monoamine oxidase (MAO), other oxidizing enzymes or undergoes autoxidation. To distinguish whether any of these options is relevant in the genotoxicity of dopamine, we inhibited MAO with 50 µM of the MAO inhibitor trans-2-phenylcyclopropylamine hydrochloride (PCPA) and also with 1 µM of the MAO B specific inhibitor Ro 16-6491 in PC12 cells (Figure 47 and Figure 48 respectively). Both compounds reduced the number of micronuclei induced by dopamine, indicating that oxidation of dopamine by MAO is likely to play a role in the genotoxicity of dopamine. Once dopamine is metabolized by MAO, semiquinones, quinones and ROS are produced, which all are potentially able to damage cellular DNA. We used another substrate of MAO, 1-phenylethylamine (PEA) with a similar structure to dopamine, which can be metabolized to ROS but not to semiquinones or quinones (Figure 49). PEA induced micronuclei in PC12 cells, though not as many as dopamine, implying that ROS formation plays a role in dopamine genotoxicity, but semiquione- and quinone-formation may also contribute.

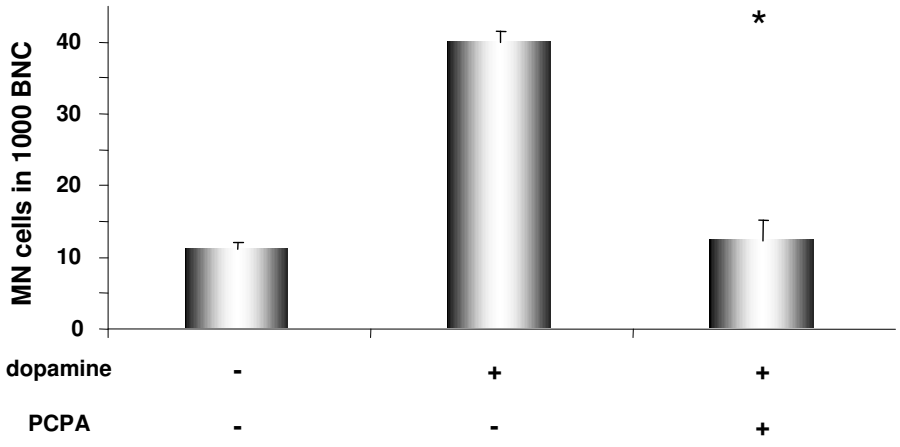

Figure 47 Micronucleus induction after 4 h treatment of PC12 cells with 100 µM dopamine with or without co-incubation with 50 µM of the monoamine oxidase inhibitor trans-2-phenylcyclopropylamine hydrochloride (PCPA). MN cells = micronucleated cells, BNC = binucleated cells. * p ≤ 0.05 vs. dopamine treated sample (Mann-Whitney U-test).

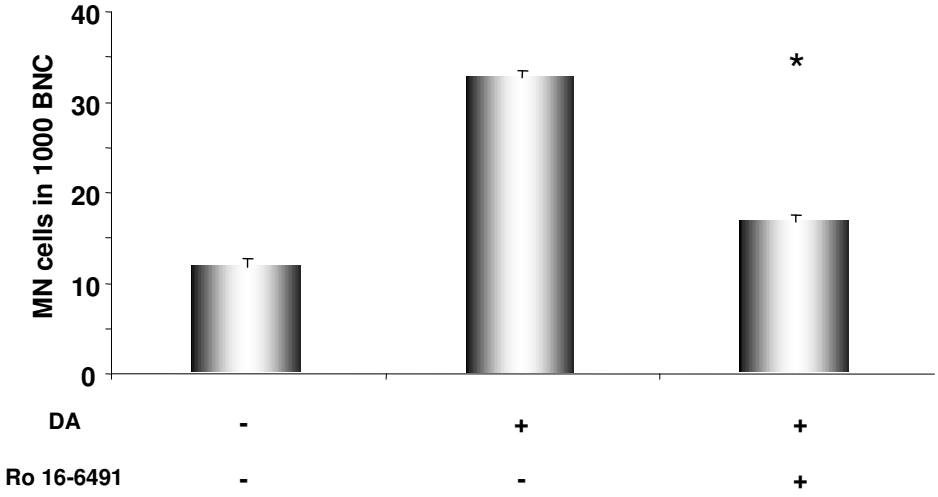

Figure 48 Micronucleus induction after 4 h treatment of PC12 cells with 100 µM dopamine with or without co-incubation with 1 µM of the MAO B specific inhibitor Ro 16-6491. MN cells = micronucleated cells, BNC = binucleated cells. * p ≤ 0.05 vs. dopamine treated sample (Mann-Whitney U-test).

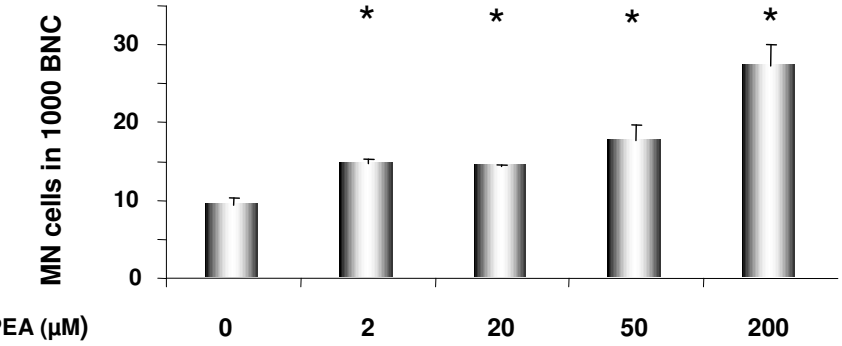

Figure 49 Micronucleus induction by the indicated concentration of monoamine oxidase substrate 1-phenylethylamine (PEA) for 4 h in PC12 cells. MN cells = micronucleated cells, BNC = binucleated cells. * p ≤ 0.05 vs. untreated sample (Mann-Whitney U-test).

4.2.8 Potential antioxidant capacity of deployed inhibitors

To ensure the absence of intrinsic antioxidant capacities of the inhibitors used to study the signal transduction pathways, which would give artefactual results, we tested the receptor antagonists and transporter inhibitors for their own potential antioxidative capacity (Figure 50). The formation of strand breaks as well as alkali labile sites can be monitored in the comet assay. In the combination with hydrogen peroxide, which is known to cause DNA-damage in the comet assay via oxidative stress, SCH 23390, sulpiride and nomifensine at the concentration used in the micronucleus test (30 µM for nomifensine) did not reduce the effect of hydrogen peroxide in the comet assay, while the positive control N-acetylcysteine (NAC), a precursor of glutathione which scavenges hydrogen peroxide, almost completely prevented comet formation. Interestingly, the DAT inhibitor GBR 12909 showed some

protective activity and thus revealed antioxidant capacity. A higher concentration of nomifensine (60 µM) also revealed a non-significant intrinsic antioxidant capacity.

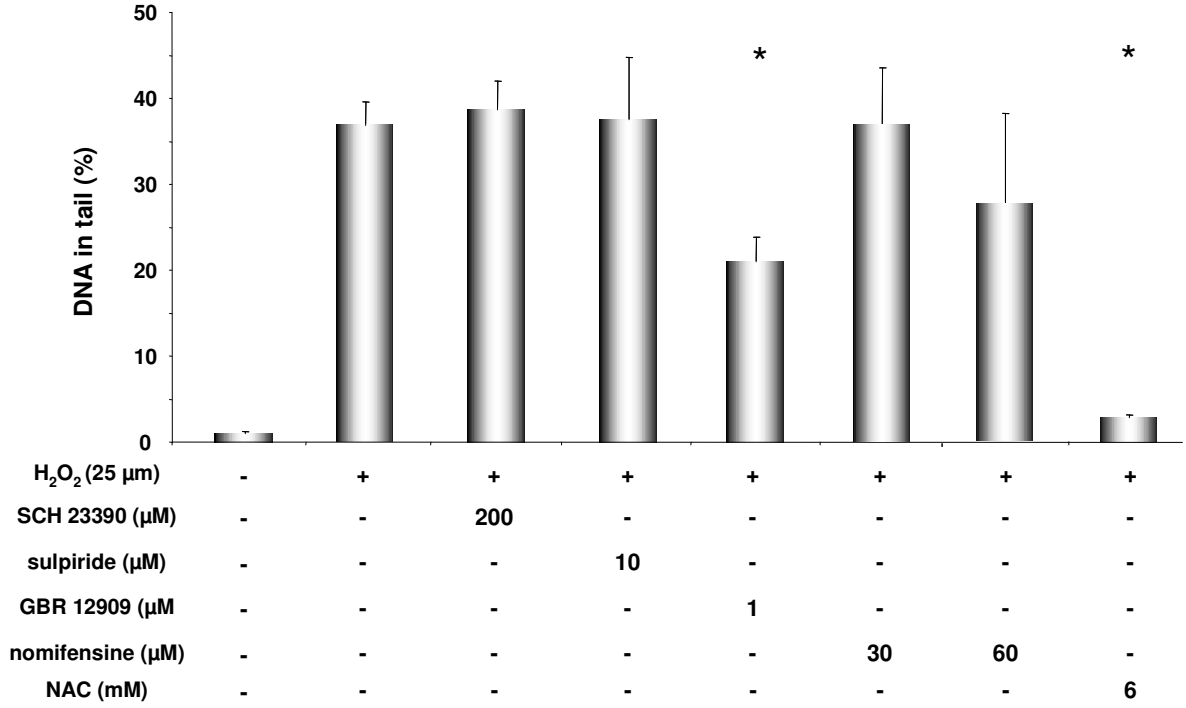

Figure 50 : Influence of the D1R antagonist SCH 23390, D2R antagonist sulpiride, the dopamine transporter inhibitors GBR 12909 and nomifensine and of the antioxidant N-acetylcysteine (NAC) (served as the positive control) on 30 min hydrogen peroxide (H_2O_2) induced DNA-damage in HL-60 cells detected in the comet-assay. * $p \leq 0.05$ vs. H_2O_2 treated sample (Mann-Whitney U-test).

4.2.9 Role of PI3K and ERK1/2 in the genotoxicity of dopamine

The role of PI3K and ERK1/2 inhibition was investigated, since it has been reported that activation of D2R can activate PI3K and ERK1/2 (17). A number of studies have revealed that the activity of these kinases can ultimately affect redistribution of DAT from the cytosol to the membrane. To elucidate the role of these kinases in the pathway of genotoxicity of dopamine, we inhibited PI3K and ERK1/2 and evaluated the dopamine-induced micronucleus formation in PC12 cells. PI3K was inhibited using the specific inhibitor wortmannin. Concentrations of 0.1 and 1 µM were able to decrease the number of dopamine-induced micronuclei (Figure 51).

Activation of ERK1/2 was inhibited using an ERK1/2 activation inhibitor peptide which was first developed by Kelemen et al (59). This membrane permeable peptide selectively binds to ERK and prevents its interaction with MEK (MAP kinase kinase). The inhibitory peptide was able to impede the damage to the genetic material which was induced by dopamine alone (Figure 52). Thus, PI3K and ERK1/2 both contribute to the genotoxicity of dopamine.

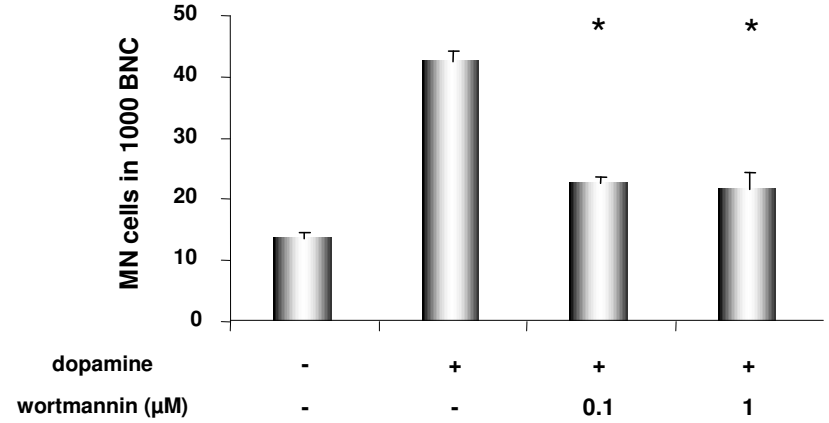

Figure 51 Micronucleus formation after treatment of PC12 cells with 100 µM dopamine for 4 h, with or without co-incubation with the indicated concentrations of the PI3K inhibitor wortmannin. MN cells = micronucleated cells, BNC = binucleated cells. * p ≤ 0.05 vs. dopamine treated sample (Mann-Whitney U-test).

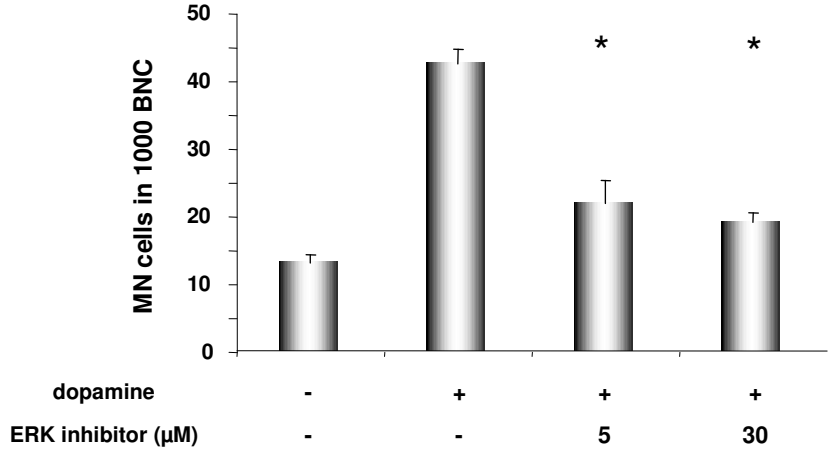

Figure 52 Micronucleus formation after treatment of PC12 cells with 100 µM dopamine for 4 h, with or without co-incubation with the indicated concentrations of an ERK activation inhibitor peptide. MN cells = micronucleated cells, BNC = binucleated cells. * p ≤ 0.05 vs. dopamine treated sample (Mann-Whitney U-test).

4.2.10 Immunocytochemical staining of DAT

In order to investigate the effect of dopamine on the localization of DAT and the role of D2R in this process, we stained the DAT in MDCK-DAT cells which were treated with dopamine with or without pre-incubation with the D2R antagonist sulpiride. Treatment of MDCK-DAT cells with 100 µM of dopamine for 5 and 15 min. (Figure 53b and Figure 53c respectively) led to an increase of DAT migration from the cytosol to the membrane in comparison to untreated cells (Figure 53a), where DAT was more distributed throughout the cytosol. Pre-treatment of the cells with 10 µM of sulpiride for 10 min. prevented this migration and resulted in an equal distribution of DAT in the cytosol and membrane regions (Figure 53d and Figure 53e). Figure 53f shows that treatment with 50 µM of the D2R agonist quinpirole for 15 min. resulted in

an increased localization of DAT in the cell membrane. The manner of quantification of DAT distribution between cytosol and membrane is explained in the materials and method section (3.19) (Figure 6) and the results of this quantification are illustrated in Figure 54.

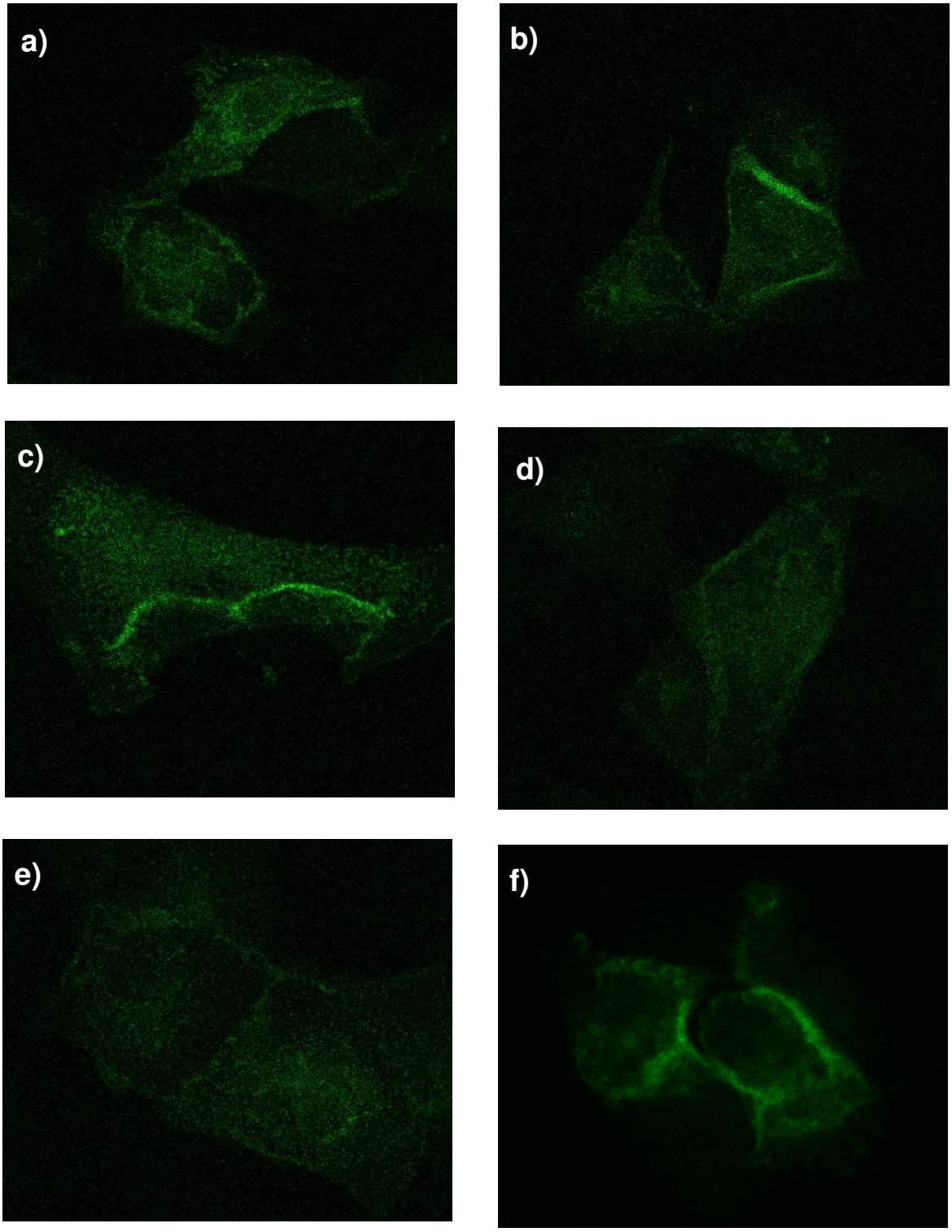

Figure 53 Immunocytochemical staining of MDCK-DAT cells for the localization of the dopamine transporter protein (DAT). (A) untreated cells, (B) dopamine 100 µM for 5 min, (C) dopamine 100 µM for 15 min, (D) 10 min. incubation with 10 µM of sulpiride followed by 5 min. incubation with 100 µM of dopamine, (E) 10 min. incubation with 10 µM of sulpiride followed by 15 min. incubation with 100 µM of dopamine, (F) quinpirole 50 µM for 15 min.

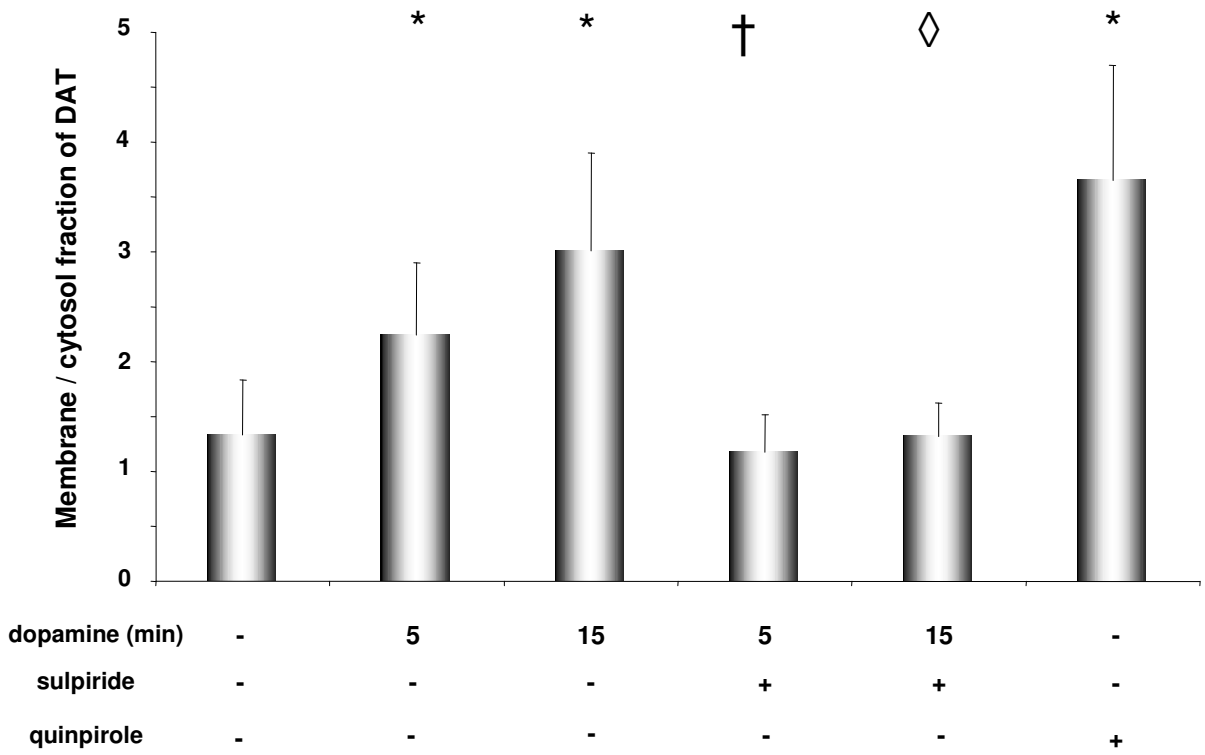

Figure 54 Quantification of the ratio of DAT localization in membrane and cytosol after treatment of the cells with 100 µM of dopamine with or without pre-incubation with 10 µM of the D2R antagonist sulpiride or after treatment with 50 µM of the D2R agonist quinpirole. * p ≤ 0.05 vs. untreated sample, † p ≤ 0.05 vs. 5-minute dopamine treated sample, ◊ p ≤ 0.05 vs. 15-minute dopamine treated sample (Student's t-test).

4.2.11 The effect of L-Dopa therapy in Parkinson's disease patients

To check if our results *in vitro* are of any relevance *in vivo*, we looked for a therapeutic model which results in higher concentration of dopamine *in vivo*. High dose L-Dopa therapy of Parkinson's disease patients provides a suitable model due to conversion of L-Dopa to dopamine also in the circulation system. We looked for the micronucleus formation in the peripheral blood lymphocytes of the L-Dopa treated patients and also of the control group. Demographic and clinical data of the subjects enrolled in this study are presented in Table 1 and Table 2. Figure 55 shows that no difference in the number of micronuclei between the patient and the control group was found. Micronucleus frequency is known to be influenced by gender with higher observed micronucleus formation in females. To account for a potential influence of gender, we added eight male individuals not suffering from PD to the micronucleus analysis of the control group. Together with these, the micronucleus frequency of the 18 patients (18.8 ± 7.1 micronucleated/1000 binucleated cells, 12 male/6 female, 71.1 ± 3.2 years) could then also be compared with an overall control group of 18

individuals (14.6 ± 8.3 micronucleated/1000 binucleated cells, 9 male/9 female, 63.4 ± 10.7 years). Still, no significant difference between the groups was found.

A comparison of the micronucleus values for patients receiving dopamine agonists (nine patients) and those who did not (nine patients) revealed slightly elevated values for patients with additional dopamine agonist therapy (Figure 56). A positive correlation of L-Dopa dose with micronuclei was found for the patients receiving dopamine agonists (Figure 57b), while micronucleus frequency showed no correlation with dose of L-Dopa in the patients without additional dopamine agonist therapy (Figure 57a).

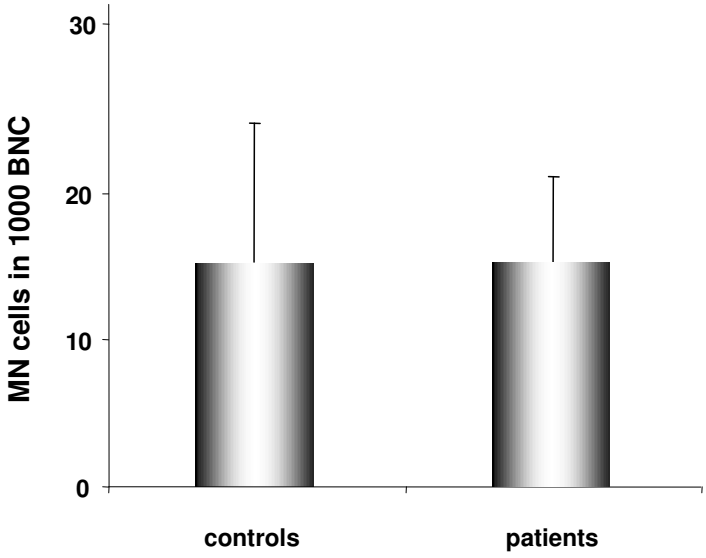

Figure 55 Micronuclei frequency in peripheral blood lymphocytes of healthy individuals (control) and Parkinson's disease patients after chronic L-Dopa treatment.

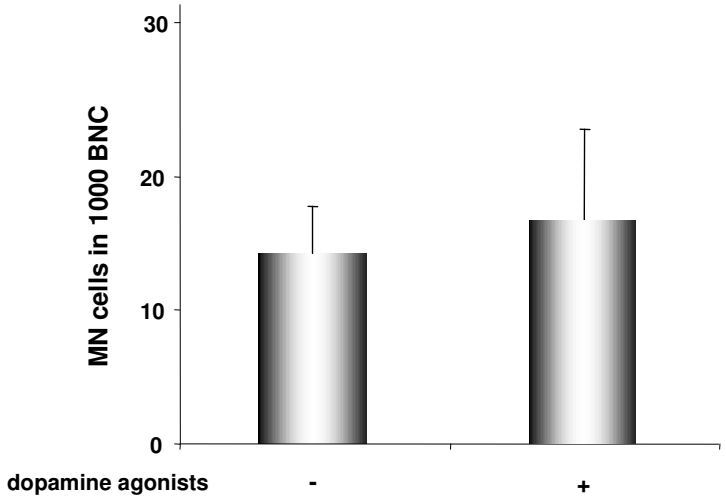

Figure 56 Micronuclei frequency in peripheral blood lymphocytes of Parkinson's disease patients after chronic L-Dopa treatment with or without additional dopamine agonist treatment.

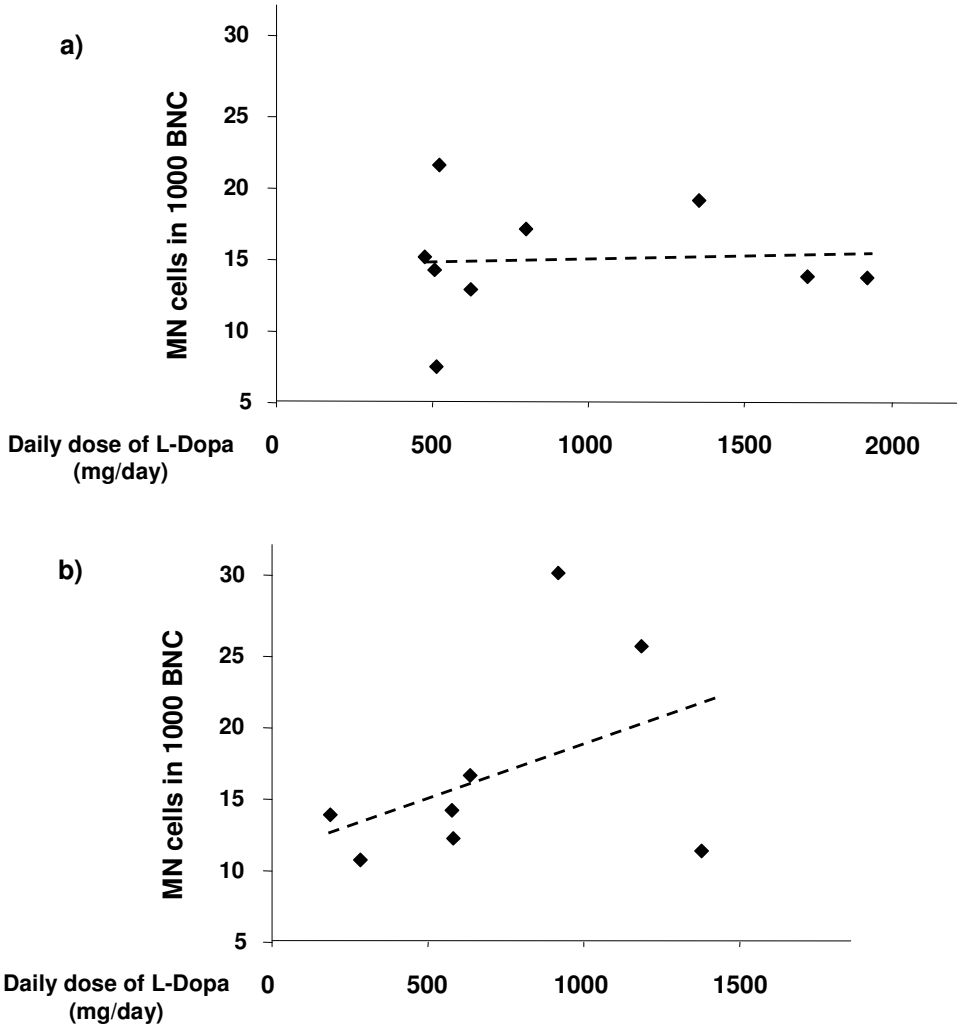

Figure 57 Correlation of micronucleus formation and the daily dose of L-Dopa in patients without (a) or with (b) additional dopamine agonist therapy.

5 Discussion

The damaging effects of ROS on biological molecules are well described. ROS are produced by both exogenous and endogenous sources. Potential exogenous sources comprise of metal ions, radiation, chlorinated compounds, barbiturates, phorbol esters and others (62). Some sources of endogenous ROS are oxidative phosphorylation, cytochrome P450 mediated metabolism, peroxisomes, inflammatory cell activation and ROS producing enzymes. In this work we focused on endogenous ROS which are produced by the ROS generating enzyme NADPH oxidase after Ang II treatment and by oxidative metabolism of dopamine by monoamine oxidase which leads to the formation of H_2O_2 as a by-product.

Under physiological conditions, the amount of ROS which occurs in the body is easily scavenged by components of the antioxidant defense system like antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase), endogenous antioxidants (glutathione) or exogenous antioxidants from food intake (vitamins, polyphenols, etc.). When the redox balance in the body is disturbed in favour of pro-oxidants due to a pathological condition, a demand for higher activation of the antioxidative defense system occurs. In this case, the success of the defense system depends on the amount of produced ROS, the efficacy of antioxidant enzymes and nutritional habits of the individual.

Accumulating evidence indicates a role of ROS in the pathology of cardiovascular diseases. The renin-angiotensin-aldosterone system (RAAS) is an important part of physiological and pathological responses of the cardiovascular system. The biologically active hormone of this system, Ang II, is not only responsible for vasoconstriction and blood regulation but is also implicated in inflammation, endothelial dysfunction, atherosclerosis, hypertension and congestive heart failure (76). Some of these effects of Ang II are mediated via production of ROS generated by membrane bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase localized in the vascular walls (46, 119).

Our group has shown that DNA damage was induced by Ang II in cell lines derived from kidney (105) as well as in perfused isolated mouse kidneys (104). The damage included single and double strand breaks, chromosomal aberrations, abasic sites and 8-oxodG base modifications. These effects of Ang II were mediated via its AT1 receptor. The purpose of this study was to scrutinize the pathway involved in the observed genotoxicity of Ang II. Here we confirmed the genotoxicity of Ang II in two

other kidney cell lines of human origin, HEK-293 and HK-2 cells. In HEK-293 cells, Ang II caused significant DNA damage after 1h of incubation. Using the superoxide specific dye dihydroethidium we showed that Ang II treatment led to superoxide anion production which was abolished by the membrane permeable superoxide dismutase (SOD) mimetic TEMPOL. The DNA damage caused by Ang II was also prevented by TEMPOL but not by addition of SOD itself. This led us to the conclusion that the produced ROS were responsible for the observed DNA damage and that SOD was not able to reduce damage due to its inability to cross the cell membrane. Combination of Ang II with catalase also prevented DNA damage to some extent. There is a report about the ability of cells to take up catalase. The fact that pre-incubation with catalase more efficiently prevented Ang II-induced damage than co-incubation is in agreement with this report. Pig kidney, LLC-PK1 cells, start showing DNA damage measured by comet assay after 15 minutes of Ang II treatment. Catalase was not able to protect the cells at this time point. With increased incubation time, catalase was probably taken up by the cells and exhibited its antioxidant capacity inside the cells where the ROS are produced (unpublished data). Because Ang II is reported to be able to induce the formation of 8-oxodG base modification which is formed by the hydroxyl radical, we also investigated if the hydroxyl radical scavenger DMTU can reduce the DNA damage induced by Ang II. DMTU decreased the effect of Ang II only to some extent. This can be either explained by the fact that the hydroxyl radical is very active and its migration distance in the cells is very limited which confines its interactions to the adjacent molecules or most of the damage induced by Ang II is formed by superoxide radicals and H_2O_2 instead of the hydroxyl radical.

The quest for finding the source of ROS formation led us to NADPH oxidase which is also expressed in kidney cells. We found upregulation and phosphorylation of the p47 subunit of NADPH oxidase after Ang II treatment. Using two inhibitors we showed that inhibition of NADPH oxidase completely prevented DNA damage by Ang II treatment. Three homologs of the gp91phox subunit of NADPH oxidase, Nox1, Nox2 and Nox4, are expressed in the kidney (40). Nox4 which has 35 % identity to Nox2 was initially characterized as kidney NADPH oxidase (42) and most probably is located on intracellular membranes (72). To distinguish whether only one or both of these isoforms are involved in the genotoxicity of Ang II, we tried to differentiate between Nox2 and Nox4 action. To the best of our knowledge, no pharmacological

isoform-specific inhibitor of Nox family has been developed so far (73). Using siRNAs, only inhibition of Nox4, but not of Nox2, abolished the effect. Except for Nox2, all other members of the Nox family release their superoxide product inside the cell. The results from our siRNA transfection and involvement of Nox4 explain the inability of SOD enzyme to prevent Ang II-induced DNA damage although the same concentration successfully protects the cells against the Nox2 activator PMA. In addition to this, we did not observe any reduction of the Ang II-induced genomic damage after inhibition of PI3K. PI3K is responsible for the phosphorylation of Rac1, which is necessary for activation of Nox2 (1) whereas at least in epithelial cells Rac1 is not required for activation of Nox4 (72). In agreement to our results, using Nox4 knockout mice, it has been shown that Nox4-mediated oxidative stress leads to neuronal damage and apoptosis. The same effect was not observed in mice with deficiency in Nox1 or Nox2 genes (63). In contrary to this and also to our results, recently it has been reported that Ang II induces superoxide anion production in a Nox2-dependent manner in a cell line with properties of macula densa (40). However, the mechanism of induction of superoxide formation may differ in cell lines of different origins.

In the next step we looked for the role of PKC as a potential kinase which can phosphorylate p47 resulting in the activation of NADPH oxidase. The activation of PKC was confirmed by assessing its phosphorylation after Ang II treatment. Further investigation showed that inhibition of PKC hinders Ang II from damaging the cells. PKC itself can be activated by products of phospholipases. We inhibited PLC and PLD and looked for DNA damage induced by Ang II.

Ang II can activate PLD resulting in hydrolysis of phosphatidylcholine (PC) to choline and phosphatidic acid (PA). PA is rapidly converted to diacylglycerol (DAG). DAG can ultimately activate PKC. No effect on the genotoxicity of Ang II after PLD inhibition was observed. We also inhibited PLC which is activated via stimulation of AT1R. Activation of PLC results in the production of inositol-1,4,5-triphosphate (IP_3) and DAG. IP3 binds to its receptor at endoplasmic reticulum (ER), opening a channel which allows calcium efflux into the cytoplasm. Both calcium and DAG are potential activators of PKC. A specific inhibitor of PLC could almost completely abrogate the DNA damage caused by Ang II whereas the inactive isoform of the same inhibitor failed to show any inhibitory effect. According to these results we conclude that PLD is not involved in the pathway of Ang II-induced genomic damage but PLC is.

The ER calcium channel blocker 2-APB and the IP3 receptor antagonist TMB8, as well as the calcium store depletory compound thapsigargin hindered the genotoxicity induced by Ang II, which clearly shows the role of ER calcium signaling in this pathway. We also observed that incubation of the cells with Ang II together with the calcium chelator BAPTA-AM, the membrane calcium channel blocker amlodipine and also treatment of the cells with Ang II in a calcium free medium protected the cells against the damaging effect of Ang II. The latter results point to requirement of extracellular calcium as mediator of Ang II genotoxicity. Using fluorescence microscopy, we demonstrated that all the above mentioned interferences also lead to decreased calcium release by the cells. Involvement of both intra as well as extracellular calcium sources in the genotoxicity of Ang II is probably explained by the role of STIM1 as a signaling molecule which may act to amplify and augment the calcium signal (99). In an ongoing experiment, we transfected the HEK-293 cells with wild type and mutant gene of STIM1 to check this hypothesis.

As mentioned above, PLC is activated by AT1R stimulation. AT1R is a GPCR which has been shown to be capable of coupling to various α-subunits (G_q, $G_{q/11}$, and $G_{i/o}$) (57, 91) as well as $G_{\beta\gamma}$ complexes (76, 114). In line with previous findings (105) the AT1R antagonist candesartan reduced the genotoxicity of Ang II in HK-2 cells almost to the control level which confirms that the genotoxicity of Ang II is mediated via AT1R signaling. Supporting results came from inhibition of $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G_{\beta\gamma}$. In all cases, inhibition of G-protein signaling abrogated the damage caused by Ang II.

In conclusion, we show here that Ang II is able to induce genomic damage in cell lines of kidney origin. The observed damage is associated with production of ROS. A decrease in Ang II-induced DNA damage was observed after inhibition of G-proteins, PLC, PKC and NADPH oxidase and interfering with intra- as well as extracellular calcium signaling. This leads to the the following current model of signaling in Ang II-induced DNA damage (Figure 58): binding of Ang II to the AT1 receptor activates PLC via stimulation of G-proteins, resulting in activation of PKC in a calcium dependent manner which in turn, activates NADPH oxidase. NADPH oxidase with involvement of its Nox4 subunit then produces ROS which cause DNA damage.

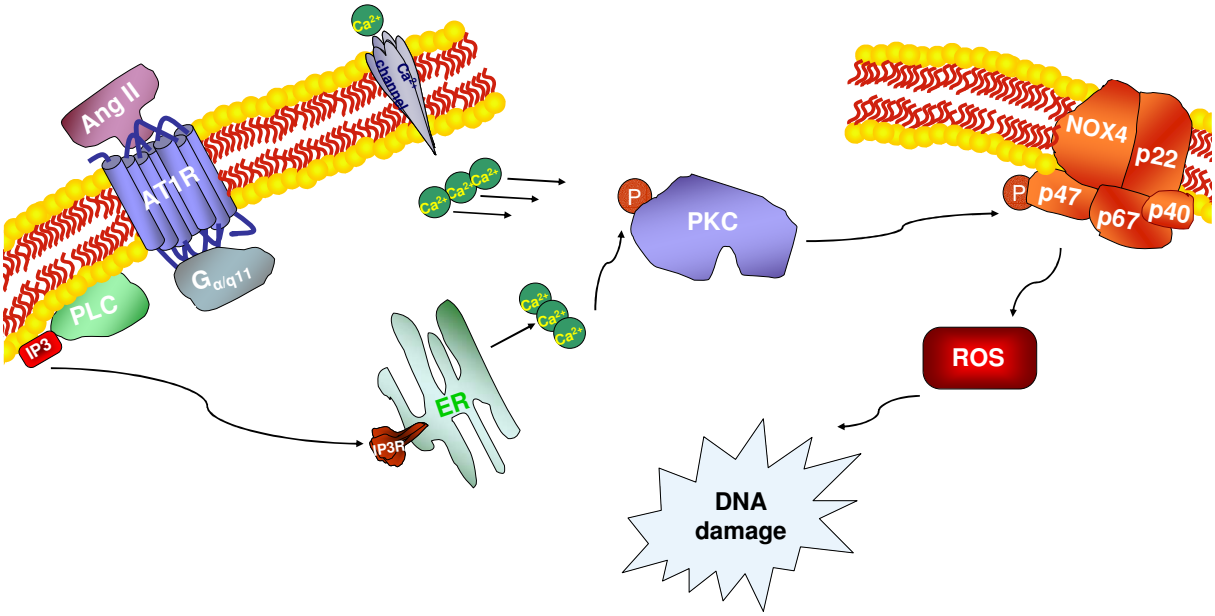

Figure 58 Model of signaling in Ang II-induced DNA damage. Binding of Ang II to the AT1 receptor activates PLC via stimulation of G-proteins, resulting in activation of PKC in a calcium dependent manner which in turn, activates NADPH oxidase. NADPH oxidase with involvement of its Nox4 subunit then produces reactive oxygen species which cause DNA damage. Ang II = angiotensin II, AT1R = angiotensin type 1 receptor, $G_{\alpha/q11}$ = G protein subunits $G_{\alpha/q11}$, PLC = phospholipase C, IP3 = Inositol triphosphate, ER = endoplasmic reticulum, PKC = protein kinase C, ROS = reactive oxygen species.

A large body of evidence links the production of ROS, subsequent oxidative stress and damage to biomolecules to the pathogenesis of age-related and chronic diseases (51, 62, 79, 97). It has been hypothesized that increased generation of ROS, elevated levels of oxidized DNA lesions or lower activity of host defense mechanisms is a driving force in the development of cancer in aged individuals (24). Some reports have shown that the repair activity decreases in higher age which can be associated with the accumulation of oxidatively damaged DNA and increased cancer risk. The link between the activity of the repair enzyme Ogg1 which repairs 8-oxodG and cancer is plausible because case-control studies have shown that groups with low repair capacity had a higher odds ratio for different kinds of cancer (41, 95-96).

The presence of increased oxidative stress and oxidative damage in neurodegenerative diseases has been well characterized in a large number of publications (22, 31, 44, 71, 81). Particularly, the nervous system is very sensitive to oxidative stress because of the high content of polyunsaturated fatty acids, its high oxygen consumption rate (brain for example accounts for 20-25 % of the total body oxygen consumption, but for less than 2 % of the body weight) and relatively fewer

antioxidant pathways (5, 15, 48). Catecholamines are an important source of ROS production in the CNS (16). For example, metabolism of dopamine, serotonin and norepinephrine by monoamine oxidase results in the production of H_2O_2 (23).

L-Dopa therapy has been the first line treatment for Parkinson's disease (PD) in the last three decades. PD is a neurodegenerative syndrome which together with Alzheimer's disease accounts for the large number of neurodegenerative disease cases. The disease is characterized clinically by resting tremors, bradykinesia, muscular rigidity and postural instability. The clinical course of PD progresses over several years. Most of the clinical symptoms are secondary to the degeneration of dopaminergic neurons in the pars compacta of the substantia nigra. The surviving neurons often contain cytoplasmic inclusions that consist of fatty acids, polysaccharides, proteins and sphingomyelin (16). Beside sporadic cases, genetic predisposition may also increase the risk of PD. Mutation in genes which encode for α-synuclein, ubiquitin carboxyl-terminal hydrolase L1 and parkin are among the risk factors for PD (48). A number of environmental factors such as metals, foods, pesticides, head injuries and infections have been evaluated for their contribution in PD susceptibility and in recent years several interactions between environmental agents and genetic polymorphisms of metabolic enzymes have been observed (25). Smoking and caffeine for example are consistently reported to be associated dose-dependently with risk of PD. The risk of the disease is also increasing with higher age and male gender (48).

Several lines of evidence point to a role of oxidative stress in the pathology of PD. For example, lipid peroxidation is significantly higher in the brain of PD patients (30). But interestingly, despite an abundant evidence for an involvement of oxidative insult at early stages of PD, interventions like administration of one or a few antioxidants have been, at best, modestly successful in clinical trials (69). It is hypothesized that the complexity of ROS metabolism accounts for the failure of these interventions, which demands more integrated approaches which not only enrich the exogenous antioxidants but also upregulate the complex endogenous antioxidant defence system (48).

The concentration of dopamine in the plasma (86) and also in the lymphocytes (102) of PD patients correlates significantly with the administered dose of L-Dopa. The suitability of lymphocytes as a peripheral marker of the central neuronal deficits in PD has been shown (4, 86). Decreased intracellular dopamine content has been

observed in the peripheral blood lymphocytes of early PD patients. Also, patients receiving L-Dopa have been found to have high dopamine levels in their lymphocytes (18). In the attempt to identify the potential effect of L-Dopa therapy on peripheral blood lymphocytes of PD patients, we recruited a group of PD patients and their life partners as the control subjects. Analysis of micronuclei frequency showed no significant difference in the number of micronuclei between patients and a control group. Females are known to have higher frequencies of micronuclei in their lymphocytes than males (36). The gender distribution was unequal between the groups in our study with more females in the control group because they were life partners of the male PD patients. For this reason we included data from six more male individuals to the control group but still did not observe any significant difference to the patient group. We also investigated the correlation between the micronucleus frequency and administered dose of L-Dopa in patients which received a dopamine agonist in addition to L-Dopa therapy. While no correlation was observed between micronucleus frequency and the L-Dopa dose in patients which were under L-Dopa therapy alone, the patients who received dopamine agonists in combination with L-Dopa showed an increased number of micronuclei with higher doses of administered L-Dopa. The effect of dopamine receptor signaling on dopamine-induced genomic damage will be discussed later. In the same study we reported an elevated amount of 8-oxodG formation, an important indication of oxidative stress, in lymphocytes of PD patients in comparison to the control group (92) which is in line with some post-mortem studies in brains of PD subjects (2, 87). The fact that despite observed elevated levels of oxidative stress we did not see any increase in the micronucleus formation can be explained by the inherent antioxidant capacity of L-Dopa in a cell free system (92).

In vitro we have confirmed the genotoxicity of dopamine in several cell lines of different tissue origin. We observed an increased number of micronuclei after dopamine treatment in rat epidermal kidney, NRK cell line with properties of proximal tubular cells, in rat pheochromocytoma PC12 cells and in the human B-lymphoblastoid cells, TK6 (112). We did not observe any genotoxicity of dopamine in the epithelial porcine kidney cell line LLC-PK1 or the dog kidney MDCK cells with properties of distal tubules . Reduction of cell proliferation assessed by cytochalasin-B block proliferation index was observed in TK6 and NRK cells and higher concentrations of dopamine caused apoptosis in TK6 and PC12 cells. PC12 cells

which express dopamine receptors and the transporter (112) showed a more pronounced genotoxic response in comparison to NRK and TK6 cells, which made them suitable for the following experiments.

An early report suggested that the genotoxcity of dopamine is related to the generation of ROS, namely superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radicals as a results of dopamine oxidation because they observed a reduction of DNA strand breaks after combination of dopamine with antioxidants, DMTU and SOD (80). In agreement to this, we also found a reduction in the dopamine-induced number of micronuclei in PC12 cells after treatment with dopamine and the radical scavengers TEMPOL and DMTU. This is in line with other reports, which demonstrated that the oxidation of dopamine via enzymatic pathways (for example by MAO) as well as autoxidation leads to the production of ROS (10, 16). We also demonstrated the release of H_2O_2 by PC12 cells into the culture medium after dopamine treatment. Our results using two different MAO inhibitors and dopamine in the micronucleus frequency test provide evidence that ROS production after MAO-dependent oxidation of dopamine plays the major role in the genotoxicity of dopamine, although autoxidation may add to this. MAO deaminates dopamine in the cytoplasm and produces H_2O_2 as by-product. H_2O_2 might damage the biological macromolecules of the cell. In the presence of ferrous ions, H_2O_2 is reduced to the highly reactive hydroxyl radicals through the Fenton reaction (8), which can attack nucleic acids, lipids and proteins in the cells directly or are converted to other radicals which all are potentially capable of damaging DNA as well as other biomolecules. The hydroxyl radical produces the altered DNA base 8-oxodG, which is an important biomarker for oxidative stress (75) and also the most abundant oxidative DNA lesion (20). Recently our group has shown in mass spectrometry measurements that treatment of PC12 cells with dopamine leads to a significant increase in 8-oxodG levels (34). These elevated levels of 8-oxodG were prevented when the MAO inhibitor PCPA was used in combination with dopamine, showing that dopamine-induced 8-oxodG formation in this cell line is MAO dependent. Due to base mispairing, 8-oxodG can lead to G:C to T:A transversions after replication, representing point mutations (20, 37). Prevention of 8-oxodG formation is so vital for the cells that even phylogenetically ancient organisms such as *Escherichia coli* contain the MutT enzyme, which has 8-oxodGTPase activity responsible for the removal of 8-oxodGTP from the nucleotide pool (78).

PEA has a structure similar to dopamine with the exception of two hydroxyl groups lacking in the phenyl ring. This difference in structure hinders PEA from producing quinones and semiquinones after being deaminated by MAO, but not from production of H_2O_2 (32). PEA was able to induce micronucleus formation in PC12 cells, suggesting that the genotoxicity of dopamine may be due to the production of ROS. Since the effect of PEA was not as high as that of dopamine, formation of quinones and semiquinones may also be involved. Also it should be considered that PEA may not have the same affinity to dopamine receptors and the transporter as dopamine does (see below).

In addition, we found a reduction of the genotoxicity of dopamine after co-treatment of the cells with dopamine together with D1R or D2R antagonists (SCH 23390 and sulpiride respectively). When used in high concentration, SCH 23390 is reported to have affinity to D2R as well (39). Unfortunately we could not find a more specific antagonist which exclusively affect the D1R. The use of siRNA down-regulation strategy or a D1R knock out cell line may be helpful to reveal the role of D1R in this pathway. Furthermore, some common molecular mechanism of D1R and D2R like activation of protein kinase B adds another level of complexity (9).

Confirming the involvement of D2R signaling in dopamine-induced DNA damage, a combination of pertussis toxin with dopamine treatment was performed which reduced the dopamine-mediated micronucleus formation. Pertussis toxin inhibits the $G_{\alpha i/o}$-coupled D2, D3, and D4 receptors (90), hence confirming the role of D2 like receptors in the dopamine-induced increase of micronuclei formation. In contrast, the D2 like receptor agonist quinpirole was not capable of inducing any genotoxic response in the absence of dopamine, which indicate that D2R signaling itself is not genotoxic, but is involved in the dopamine-induced genotoxicity. The inability of quinpirole to induce micronucleus formation cannot be attributed to low concentration of the compound because the same concentration was able to induce DAT migration to the membrane and also to upregulate DAT mRNA in Real-time PCR experiments.

Treatment of the cells with dopamine and either of two DAT inhibitors GBR 12909 and nomifensine also hindered the genotoxic effect of dopamine. Since dopamine is taken up into the cell via DAT, this would be in line with the idea that genotoxicity of dopamine occurs upon entry into the cell and subsequent oxidation inside the cell. Confirming results came from the gentoxicity assay in MDCK cells. MDCK-DAT cells which were transfected with human DAT (49) but not untransfected MDCK cells were

able to take up dopamine which was added to their medium (34) and then form micronuclei. This strong evidence of the role of DAT in dopamine-induced DNA damage is in line with our results from pharmacological inhibition of DAT using GBR 12909 and nomifensine.

The expression of D2R and DAT in axonal terminals is an anatomical hint for a potential interaction between these two proteins (52). Indications are that D2 antagonists decrease the half life of the DAT protein in responsive brain tissues (21, 61). Among neurotransmitters, transporter internalization and trafficking are common mechanisms of rapid regulation of transporter function. For example, it has been reported that PKC is involved in activation and cell surface expression of serotonin transporter (11, 100). Many other reports provide evidence about the role of kinases in the regulation of DAT (12, 65, 83, 118). Activation of PKC is reported to decrease the V_{max} of DAT with no change in the affinity of the transporter for dopamine (28) and also to redistribute the localization of DAT from cell membrane to the cytosol (67, 83). On the other hand PI3K and MAPK are reported to promote cell surface expression of DAT and therefore enhance dopamine uptake (19, 83). It has been suggested by others that stimulation of D2R leads to the activation of ERK and PI3K in a pertussis toxin sensitive manner (17). The latter implies the contribution of $G_{\alpha i/o}$ proteins which are coupled to D2R. Several reports have already proven the expression of ERK and PI3K in PC12 (43, 55, 58, 115) and MDCK cells (56, 60, 116) which made these two cell lines suitable for our study.

Inhibition of ERK activation was conducted by applying a short cell permeable peptide which corresponds to the N-terminus of MEK (MAP kinase kinase). This membrane permeable peptide selectively binds to ERK and prevents its interaction with MEK and thereby has no effect on JNKs (Jun N-terminal Kinase) or p38 MAP kinase which makes it more suitable for specific inhibition of ERK activation in comparison to the common pharmacological inhibitors (59). Inhibition of ERK activation reduced the genotoxic effect of dopamine, which can be attributed to the decreased ability of DAT to take up dopamine (44 % in MDCK-DAT cells upon ERK inhibition) (34). Inhibition of PI3K by wortmannin also reduced the dopamine-induced DNA damage and measurement of dopamine uptake after wortmannin treatment also showed a decrease to 59 % which may account for the impeded genotoxic damage.

We also observed a reduction of dopamine uptake after treatment with the D2R antagonist sulpiride (to 67 %). This reduction confirmed our results from

immunocytochemistry where we could show that sulpiride was able to reduce dopamine-induced migration of DAT from the cytosol to the membrane. This agrees with another report describing the effect of D2R antagonism on DAT localization using other methods (74).

Our results from reduction of DAT activity after PI3K inhibition are in accordance with some previous reports (19) but Bolan and co-workers (12) suggested that DAT regulation by D2R is PI3K independent. It is important to consider that our observation here is probably the mixed effect of D2/D3 receptors. Regulation of DAT by D2 and D3 receptors may take place at least in part through different mechanisms (118). The last report also stresses that inhibition of PI3K does not influence phosphorylation of MAPK induced by D3 signaling. Therefore, the effect of PI3K on DAT regulation can be considered MAPK independent. In our model we cannot distinguish between the roles of D2 or D3 receptors in the genotoxicity of dopamine. More investigations would have to be conducted to clarify this aspect.

Several other groups have measured the physiological and pathophysiological concentration of dopamine in plasma or other body fluids. The values for physiological concentrations lie usually in the nanomolar range but these values can change extremely under pathophysiological conditions. One study reports values around 20 and 35 µM in blood and cerebrospinal fluid respectively of elderly individuals which were suspected for Alzheimer's disease (51). *In vitro* we showed that in some cell lines even the dopamine concentration of 6.25 µM can lead to significant DNA damage. It still needs to be determined whether and at which concentration of dopamine there is a threshold for our observed mechanisms.

In conclusion, the present results show that dopamine can cause genotoxicity *in vitro*. There are also indications of oxidative lesions in PD patients under L-Dopa therapy. The uptake of dopamine via DAT is the key component of dopamine-induced genotoxicity. Once inside the cells, dopamine undergoes deamination, mainly by MAO, which leads to the production of ROS as by-products. These ROS are a major cause of the observed DNA damage in our system. D2R signaling is involved in this action via G-protein mediated activation of PI3K and ERK which ultimately results in higher activation and cell surface expression of DAT and hence elevated dopamine uptake. According to these findings, the proposed model of dopamine-induced genotoxicity is illustrated in Figure 59.

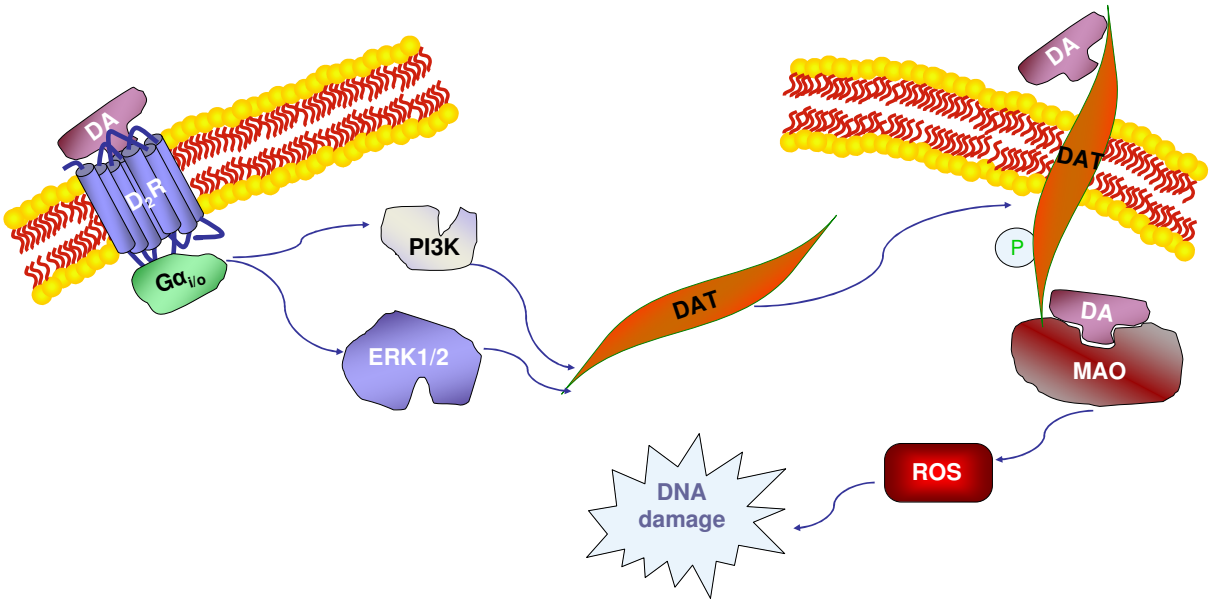

Figure 59 Model of signaling in dopamine-induced DNA damage. DA = dopamine, D2R = dopamine type 2-like receptor, $G_{\alpha i/o}$ = G-protein subunits $G_{\alpha i/o}$, PI3K = Phosphoinositide 3-kinase , ERK1/2 = extracellular signal-regulated kinases, DAT = dopamine transporter, MAO = monoamine oxidase, ROS = reactive oxygen species.

It is known from the study of several hormones and cytokines that endogenous compounds are able to produce ROS and in some cases DNA damage (101). In the current work, we focused on two compounds which mostly exert their effect in the body via GPCR signaling. In the case of Ang II, the genotoxic effect is completely receptor dependent and the pathway which starts with the stimulation of the AT1R eventually leads to the formation of DNA damage. For dopamine, although the stimulation of the receptor is not enough to induce genomic damage, the receptor signaling enhances the genotoxicity of dopamine by affecting the localization of DAT and dopamine uptake. Therefore, we claim that our study provides evidence for receptor-mediated genotoxicity of two compounds with different mechanism of actions. Due to the involvement of the GPCR signaling in several pathways of physiological as well as pathological processes, it still has to be investigated whether receptor-mediated oxidative stress and subsequent DNA damage can trigger new complications which adds to the conditions that patients already suffer from.

6 Abstract

Reactive oxygen species (ROS) are continuously generated in cells and are involved in physiological processes including signal transduction but also their damaging effects on biological molecules have been well described. A number of reports in the literature implicate excessive oxidative stress and/or inadequate antioxidant defense in the pathogenesis of cancer, atherosclerosis, chronic and age related disorders.

Several studies have indicated that activation of the renin-angiotensin-aldosterone-system can lead to the formation of ROS. Epidemiological studies have revealed higher renal cell cancer incidences and also higher cancer mortalities in hypertensive individuals. Recently, our group has shown that perfusion of the isolated mouse kidney with Ang II or treatment of several cell lines with Ang II leads to formation of DNA damage and oxidative base modifications. Here, we tried to scrutinize the pathway involved in genotoxicity of Ang II.

We confirmed the genotoxicity of Ang II in two kidney cell lines of human origin. Ang II treatment led to the production of superoxide anions which we could hinder when we used the membrane permeable superoxide dismutase (SOD) mimetic TEMPOL. One of the enzymes which is activated in the cells after Ang II treatment and is able to produce ROS is NADPH oxidase. Phosphorylation of p47 subunit of NADPH oxidase after Ang II treatment was enhanced. Using two inhibitors we showed that NADPH oxidase inhibition completely prevents DNA damage by Ang II treatment. To differentiate between Nox2 and Nox4 isoforms of NADPH oxidase subunits in the genotoxicity of Ang II, we performed siRNA inhibition and found a role only for Nox4, while Nox2 was not involved.

Next, we investigated PKC as a potential activator of NADPH oxidase. We showed that PKC becomes phosphorylated after Ang II treatment and also that inhibition of PKC hinders Ang II from damaging the cells. Our results from using several inhibitors of different parts of the pathway revealed that PKC activation in this pathway is dependent on the action of PLC on membrane phospholipids and production of IP3. IP3 binds to its receptor at endoplasmic reticulum (ER), opening a channel which allows calcium efflux into the cytoplasm. In this manner, both ER calcium stores and extracellular calcium cooperate so that Ang II can exert its genotoxic effect. PLC is activated by AT1R stimulation. We could also show that the genotoxicity of Ang II is mediated via AT1R signaling using the AT1R antagonist candesartan.

In conclusion, here we have shown that Ang II is able to damage genomic damage in cell lines of kidney origin. The observed damage is associated with production of ROS. A decrease in Ang II-induced DNA damage was observed after inhibition of G-proteins, PLC, PKC and NADPH oxidase and interfering with intra- as well as extracellular calcium signaling. This leads to the following preliminary model of signaling in Ang II-induced DNA damage: binding of Ang II to the AT1 receptor activates PLC via stimulation of G-proteins, resulting in the activation of PKC in a calcium dependent manner which in turn, activates NADPH oxidase. NADPH oxidase with involvement of its Nox4 subunit then produces reactive oxygen species which cause DNA damage.

Dopamine content and metabolism in the peripheral lymphocytes of PD patients are influenced by L-Dopa administration. The PD patients receiving a high dose of L-Dopa show a significantly higher content of dopamine in their lymphocytes compared to PD patients who received a low dose of L-Dopa or the healthy control. Central to many of the processes involved in oxidative stress and oxidative damage in PD are the actions of monoamine oxidase (MAO), the enzyme which is responsible for the enzymatic oxidation of dopamine leading to production of H_2O_2 as a by-product. We investigated whether dopamine oxidation can cause genotoxicity in lymphocytes of PD patents who were under high dose L-Dopa therapy and afterward questioned the occurrence of DNA damage after dopamine treatment *in vitro* and tried to reveal the mechanism by which dopamine exerts its genotoxic effect. The frequency of micronuclei in peripheral blood lymphocytes of the PD patients was not elevated compared to healthy age-matched individuals, although the formation of micronuclei revealed a positive correlation with the daily dose of L-Dopa administration in patients who received L-Dopa therapy together with dopamine receptor agonists.
In vitro, we describe an induction of genomic damage detected as micronucleus formation by low micromolar concentrations in cell lines of different tissue origins. The genotoxic effect of dopamine was reduced by addition of the antioxidants TEMPOL and dimethylthiourea which proved the involvement of ROS production in dopamine-induced DNA damage. To determine whether oxidation of dopamine by MAO is relevant in its genotoxicity, we inhibited MAO with two inhibitors, trans-2-

phenylcyclopropylamine hydrochloride (PCPA) and Ro 16-6491 which both reduced the formation of micronuclei in PC12 cells.

We also studied the role of the dopamine transporter (DAT) and dopamine type 2 receptor (D2R) signaling in the genotoxicity of dopamine. Inhibitors of the DAT, GBR-12909 and nomifensine, hindered dopamine-induced genotoxicity. These results were confirmed by treatment of MDCK and MDCK-DAT cells, the latter containing the human DAT gene, with dopamine. Only MDCK-DAT cells showed elevated chromosomal damage and dopamine uptake. Although stimulation of D2R with quinpirole in the absence of dopamine did not induce genotoxicity in PC12 cells, interference with D2R signaling using D2R antagonist and inhibition of G-proteins, phosphoinositide 3 kinase and extracellular signal-regulated kinases reduced dopamine-induced genotoxicity and affected the ability of DAT to take up dopamine. Furthermore, the D2R antagonist sulpiride inhibited the dopamine-induced migration of DAT from cytosol to cell membrane.

Overall, the neurotransmitter dopamine causes DNA damage and oxidative stress *in vitro*. There are also indications that high dose L-Dopa therapy might lead to oxidative stress. Dopamine exerts its genotoxicity *in vitro* upon transport into the cells and oxidation by MAO. Transport of dopamine by DAT has the central role in this process. D2R signaling is involved in the genotoxicity of dopamine by affecting activation and cell surface expression of DAT and hence modulating dopamine uptake.

We provided evidence for receptor-mediated genotoxicity of two compounds with different mechanism of actions. The involvement of these receptors in many human complications urges more investigations to reveal whether abnormalities in the endogenous compounds-mediated signaling can play a role in the initiation of new conditions like carcinogenesis.

7 Zusammenfassung

Reaktive Sauerstoffspezies (ROS) werden kontinuierlich in Zellen generiert und sind an physiologischen Prozessen wie der Signaltransduktion beteiligt. Aber auch ihre schädigenden Auswirkungen auf biologische Moleküle sind seit langem bekannt. Eine Reihe von Literaturberichten sieht einen Zusammenhang zwischen übermäßigem oxidativen Stress oder einer unzureichenden antioxidativen Verteidigung und Krebs, Atherosklerose und chronischen bzw. altersbedingten Erkrankungen. Mehrere Studien haben belegt, dass die Aktivierung des Renin-Angiotensin-Aldosteron-Systems zur Bildung von ROS führen kann. Epidemiologische Studien haben gezeigt, dass Nierenkarzinom-Inzidenzen und Mortalitäten bei Hypertonikern erhöht sind. Vor kurzem konnte unsere Gruppe zeigen, dass die Perfusion von isolierten Mäusenieren und die Behandlung mehrerer Zelllinien mit Angiotensin II (Ang II) zur Bildung von DNA-Schäden und oxidativen Basenmodifikationen führt. Ziel der vorliegenden Arbeit war es, die Signalwege der Genotoxizität von Ang II zu bestimmen. Die Genotoxiziät von Ang II in zwei Nieren-Zelllinien humaner Herkunft konnte bestätigt werden. Wir zeigten, dass Ang II-Behandlung zur Produktion von Superoxid-Anionen führt, die durch das membrangängige Superoxid-Dismutase-Mimetikum TEMPOL verhindert werden kann. Eines der Enzyme, das in den Zellen nach Ang II-Behandlung aktiviert wird und ROS produzieren kann, ist die NADPH-Oxidase. Die mittels RT-PCR gemessene Hochregulierung von p47 beweist die Aktivierung der NADPH-Oxidase nach Ang II-Behandlung. Auch die Phosphorylierung von p47 nach Ang II-Behandlung wurde gesteigert. Mittels zweier Inhibitoren zeigten wir, dass NADPH-Oxidase-Hemmung DNA-Schäden durch Ang II-Behandlung vollständig verhindert. Wir versuchten, die Rolle der Nox2- und Nox4-Isoformen der NADPH-Oxidase-Untereinheiten bei der Genotoxizität von Ang II zu differenzieren. Hemmung mittels siRNA bestätigte nur eine Beteiligung der Nox4. Anschließend überprüften wir die Rolle der PKC als potentiellem Aktivator der NADPH-Oxidase. Wir zeigten, dass die PKC nach Ang II-Behandlung phosphoryliert wird und durch die Hemmung der PKC Ang II-induzierte Schäden verhindert werden. Die Verwendung mehrerer Inhibitoren der verschiedenen Teile des Signalweges zeigte, dass die PKC-Aktivierung von der Reaktion der PLC mit Membranphospholipiden und der Produktion von IP3 und DAG abhängig ist. IP3 bindet an seinen Rezeptor am Endoplasmatischen Retikulum (ER). Die in der Folge auftretende Öffnung eines Kanals ermöglicht einen Calcium-

Ausstrom in das Cytoplasma. Auf diese Weise sind sowohl ER-Calcium als auch extrazelluläres Calcium an der Ang II-induzierten genotoxische Wirkung beteiligt. PLC wird durch AT1R-Stimulation aktiviert. Wir konnten mit Hilfe des AT1R-Antagonisten Candesartan auch zeigen, dass die Genotoxizität von Ang II über AT1R-Signaltransduktion vermittelt wird.
Zusammenfassend haben wir gezeigt, dass Ang II genomische Schäden in humanen Nieren-Zelllinien verursacht. Die Schäden sind mit der Produktion von ROS verbunden. Eine Reduktion der Ang II-induzierten DNA-Schäden wurde nach Hemmung von G-Proteinen, der PLC, PKC und NADPH-Oxidase und Beeinflussung intra- sowie extrazellulärer Calium-Signalgebung gezeigt. Dies führt zu folgendem vorläufigen Modell der Signaltransduktion der von Ang II-induzierten DNA-Schäden: Die Bindung von Ang II an AT1R aktiviert die PLC durch Stimulierung der G-Proteine und die PKC in Calcium-abhängiger Weise, dies wiederum aktiviert die NADPH-Oxidase. Die NADPH Oxidase unter Beteiligung ihrer Nox4-Untereinheit erzeugt dann reaktive Sauerstoffspezies, die DNA-Schäden verursachen.

Dopamingehalt und -stoffwechsel in peripheren Lymphozyten von Parkinson-Patienten werden durch L-Dopa-Gabe beeinflusst. Die Patienten, die eine hohe Dosis L-Dopa erhalten, zeigen einen signifikant höheren Gehalt an Dopamin in den Lymphozyten im Vergleich zu Patienten, die eine niedrige Dosis L-Dopa erhalten oder der gesunden Kontrollgruppe. Im Mittelpunkt vieler Prozesse bei der Entstehung von oxidativem Stress und oxidativer Schäden bei Parkinson-Patienten steht die Monoaminoxidase (MAO), die für die enzymatische Oxidation von Dopamin und in der Folge für die Entstehung von H_2O_2 verantwortlich ist. Wir untersuchten, ob die Oxidation von Dopamin genotoxische Wirkung in Lymphozyten von Parkinson-Patienten mit hochdosierter L-Dopa-Therapie induzieren kann. Danach überprüften wir, ob die Behandlung mit Dopamin *in vitro* DNA-Schäden induzieren kann und versuchten aufzuzeigen, durch welchen Mechanismus Dopamin seine genotoxische Wirkung entfaltet.
Die Häufigkeit von Mikrokernen in peripheren Lymphozyten der Parkinson-Patienten war nicht erhöht im Vergleich zur gesunden Kontrollgruppe, allerdings zeigte die Mikrokernfrequenz eine positive Korrelation mit der täglichen L-Dopa-Dosis bei Patienten, die eine L-Dopa-Therapie zusammen mit einem Dopamin-Rezeptor-Agonisten erhielten.

In vitro beobachteten wir bei niedrigen mikromolaren Konzentrationen eine Induktion des genomischen Schadens in Zelllinien, die aus verschiedenen Geweben stammten. Die genotoxische Wirkung von Dopamin wurde durch Zugabe der Antioxidantien TEMPOL und DMTU reduziert, wodurch die Beteiligung von ROS gezeigt werden konnte. Um festzustellen, ob die Oxidation von Dopamin durch MAO für die Genotoxizität relevant ist, hemmten wir MAO mit zwei Inhibitoren, trans-2-Phenylcyclopropylamin-Hydrochlorid (PCPA) und Ro 16-6491, die beide die Bildung von Mikrokernen in PC-12-Zellen reduzieren konnten. Wir untersuchten auch die Rolle des Dopamin-Transporters (DAT) und Dopamin-Typ-2-Rezeptor (D2R)-assoziierter Signalwege in der Genotoxizität von Dopamin. Die Inhibitoren des DAT, GBR-12909 und Nomifensin verhinderten die Dopamin-induzierte Genotoxizität. Diese Ergebnisse wurden durch Behandlung von MDCK- und MDCK-DAT- Zellen (die das humane DAT-Gen besitzen) mit Dopamin bestätigt. Nur MDCK-DAT-Zellen zeigten erhöhte chromosomale Schäden und Dopaminaufnahme. Obwohl die Stimulation mit dem D2R-Rezeptor-Agonisten Quinpirol in Abwesenheit von Dopamin keine Genotoxizität in PC-12-Zellen induzierte, reduzierten sowohl ein D2R-Antagonist, wie auch Inhibitoren des in der Signalkaskade involvierten G-Proteins, der Phosphoinositol-3-Kinase und der extrazellulären signalregulierten Kinasen die Aufnahme von Dopamin mittels DAT und die Dopamin-vermittelte Genotoxizität. Der D2R-Antagonist Sulpirid hemmte die Dopamin-induzierte Migration von DAT aus dem Cytosol zur Zellmembran.

Insgesamt verursacht der Neurotransmitter Dopamin DNA-Schäden und oxidativen Stress *in vitro*. Es gibt Hinweise, dass eine hochdosierte L-Dopa-Therapie zu oxidativem Stress führt. *In vitro* führt Dopamin zu Genotoxizität durch Transport in die Zellen und Oxidation durch MAO. Der Transport von Dopamin durch DAT spielt eine zentrale Rolle in diesem Prozess. Die D2R-Signalwege sind an der Genotoxizität von Dopamin durch Auswirkung auf die Aktivierung und Membranexpression von DAT und damit der Dopaminaufnahme beteiligt.

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10 Curriculum Vitae

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Publications

Papers

- **Fazeli G**, Oli RG, Schupp N, Stopper H. 2010. The Role of the Dopamine Transporter in Dopamine-Induced DNA Damage. Brain Pathology Epub, ahead of print.

- Queisser N, **Fazeli G**, Schupp N. 2010. Superoxide anion and hydrogen peroxide-induced signaling and damage in angiotensin II and aldosterone action. Biological chemistry. 391(11): 1265-1279.

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Abstracts

- *"Signaling in angiotensin II-induced DNA damage"* **Gholamreza Fazeli**, Helga Stopper, Nicole Schupp. 5th international symposium organized by the students of the Graduate School of Life Sciences, October 2010, Wuerzburg, Germany,

- *"The role of calcium and NADPH oxidase in angiotensin II-induced DNA damage"* **Gholamreza Fazeli**, Helga Stopper, Nicole Schupp. The Society for Free Radical Research-Europe meeting, September 2010, Oslo, Norway

- *"Signaling in angiotensin II-induced DNA damage"* **Gholamreza Fazeli**, Helga Stopper, Reinhard Schinzel, Nicole Schupp. 33rd Scientific congress Hypertonie, November 2009, Luebeck, Germany

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11 Affidavit

I hereby declare that my thesis entitled “Signaling in the induction of genomic damage by endogenous compounds“ is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.
Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg 30,11,2010

Gholamreza Fazeli