Angiotensin II-induced genomic damage in renal cells can be prevented by angiotensin II type 1 receptor blockade or radical scavenging

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Schupp N. Schmid U. Rutkowski P. Lakner U. Kanase N. Heidland A. Stopper H. Angiotensin II-induced genomic damage in renal cells can be prevented by angiotensin II type 1 receptor blockade or radical scavenging. Am J Physiol Renal Physiol 292: F1427–F1434, 2007. First published January 16, 2007; doi:10.1152/ajprenal.00458.2006.—Hypertensive patients exhibit elevated cancer incidence, especially of cancers of the kidney. Elevated levels of ANG II, the active peptide of the renin-angiotensin system, are associated with renal vascular, hypertension. In particular, the renoprotection provided by AstraZeneca (Wedel, Germany). ANG II production, has been found in various organs, for example in the kidney, heart, and brain (2, 17).

The actions of ANG II are mediated by two receptor molecules, the ANG II type 1 receptor (AT1) and the ANG II type 2 receptor (AT2). AT1 receptors are expressed in many tissues, whereas AT2 receptors are highest in fetal tissues (25). All classic physiological effects of ANG II, such as vasoconstriction, aldosterone and vasopressin release, sodium and water retention, are mediated by the AT1 receptor. Via this receptor, ANG II is also involved in cell proliferation, nephrosclerosis, endothelial dysfunction, and processes leading to atherothrombosis. The AT2 receptor often functions as a counterregulatory receptor and is involved, for example, in cell differentiation and apoptosis (4, 13).

In proximal tubule cells, activation of the AT1 receptor by ANG II led to the induction of NAD(P)H-oxidase, a multi-enzyme complex which enhances intracellular synthesis of reactive oxygen species (ROS) (10). Increased formation of ROS, of various etiology, is associated with the induction of genomic damage (11).

The present study was undertaken to characterize the genotoxic effects of ANG II in vitro in the epithelial porcine kidney cell line LLC-PK1. We hypothesize these effects to be mediated by the AT1 receptor, which upon activation causes oxidative stress. To prove this, we modulated the genotoxicity of ANG II with an AT1 receptor antagonist and with antioxidants. Two standard genotoxicity assays were employed: the micronucleus frequency test, which detects a subset of chromosomal aberrations, inherited to the first generation of daughter cells after mitosis and the comet assay, which detects structural DNA damage, which may partially or completely be transient, then leading to repair before mitosis or to cell death.

METHODS

Materials. If not mentioned otherwise, all chemicals were purchased from Sigma (Taufkirchen, Germany). Candesartan was provided by AstraZeneca (Wedel, Germany).

Cell culture. LLC-PK1 cells, an epithelial porcine kidney cell line with proximal tubule properties, were obtained from ATCC and grown as

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Table 1. Sequences, annealing temperatures, and cycle times of used primer for the amplification of genes of the angiotensin II receptors type 1 and type 2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing Temperature, °C</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT₁ forward</td>
<td>5'-ATA GCA GAG CCT CCA GCT CA-3'</td>
<td>54</td>
<td>35</td>
</tr>
<tr>
<td>AT₁ reverse</td>
<td>5'-CCA GGG GTA TTC CAT AGC AG-3'</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>AT₂ forward</td>
<td>5'-ATC CCT GGC AAG CCT CTT AT-3'</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>AT₂ reverse</td>
<td>5'-GCT GAC CAT TGG GGA TAT TT-3'</td>
<td>50</td>
<td>40</td>
</tr>
</tbody>
</table>

The size of the amplified fragment is shown. AT₁, angiotensin II receptor type 1; AT₂, angiotensin II receptor type 2.

Table 2. Effect of 170 nM ANG II on the apoptosis and proliferation rate in human promyelocytic cells (HL-60), porcine kidney cells (LLC-PK₁), and rat kidney cells (NRK)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Fold Induction of Apoptoses (170 nM ANG II)</th>
<th>Fold Increase in Proliferation (170 nM ANG II)</th>
<th>MN/1,000 BN Control</th>
<th>MN/1,000 BN (170 nM ANG II)</th>
<th>Fold Induction of MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>0.99 n.s.</td>
<td>1.72 n.s.</td>
<td>2.84±0.92</td>
<td>11.73±0.77</td>
<td>4.16 P ≤ 0.01</td>
</tr>
<tr>
<td>LLC-PK₁</td>
<td>0.95 n.s.</td>
<td>1.57 n.s.</td>
<td>6.04±0.59</td>
<td>27.00±8.57</td>
<td>4.47 P ≤ 0.05</td>
</tr>
<tr>
<td>NRK</td>
<td>1.08 P ≤ 0.05</td>
<td>2.97 P ≤ 0.05</td>
<td>12.83±1.48</td>
<td>59.00±1.00</td>
<td>4.60 P ≤ 0.01</td>
</tr>
</tbody>
</table>

The values are means of 3 independent experiments. n. s., Not statistically significant. The number of micronuclei (MN) per 1,000 binucleated cells (BN) in untreated cells and in cells treated with 170 nM as well as the magnitude of micronuclei-induction are shown. The respective cell line was treated in the optimal way to form micronuclei with ANG II: 24-h incubation for HL-60, 48-h incubation for LLC-PK₁, and 2-h incubation for NRK.

Proliferation index. Furthermore, the slides prepared for the micronucleus frequency test were used to calculate the proliferation index of the cells treated with ANG II, using the following formula:

\[
PI = \frac{\text{mononucleated cells} \times 1 + \text{binucleated cells} \times 2}{\text{total cell number}}
\]

The result is the proliferation index (PI).
After incubation for 10 min on ice with 1 μg/ml propidium iodide.

Statistical analysis. If not mentioned otherwise, data from three independent experiments are shown ± SE. Statistical significance among multiple groups was tested with the nonparametric Kruskal-Wallis test. Individual groups were then tested using the Mann-Whitney U-test. A P value of ≤0.05 was considered significant. For calculations SPSS 13.0 was used. Analysis of flow cytometry histograms was done with the free software WinMDI ver. 2.8 (Scripps Research Institute Cytometry Software page at http://facs.scripps.edu/software.html).

RESULTS

The presence of the expression of the AT₁ and the AT₂ receptor in LLC-PK₁ cells was verified by RT-PCR. The obtained sequences were compared with the database GenBank, yielding 99% identity of the LLC-PK₁ AT₁ receptor to the database sequence of Sus sp. mRNA for AT₁ receptor (accession number D11340) and 100% identity of the LLC-PK₁ AT₂ receptor to the database sequence of Sus sp. mRNA for AT₂ receptor (accession number AF195509).

ANG II, in our experiments with LLC-PK₁, did not change cell proliferation (Table 2). Also, ANG II did not induce apoptosis, since the number of apoptotic cells observed under the microscope did not rise when increasing doses of ANG II were applied (Table 2). Of the two other cell lines HL-60 (human promyelocytic cells) and NRK (rat proximal tubule cells) also tested for apoptosis and proliferation after incubation with 24 h of treatment with 2 concentrations of ANG II with and without coincubation with either 6 mM N-acetylcysteine (NAC) or 5 μM candesartan (Cand). Control, no treatment. Six millimolar NAC or 5 μM candesartan alone had no effect on the DNA (1.04 ± 0.07 and 1.11 ± 0.35% DNA in tail, respectively). The positive control methyl methanesulfonate yielded a DNA damage of 62.30 ± 2.00% DNA in tail. *P ≤ 0.05 vs. control. °P ≤ 0.05 vs. ANG II treatment.

Fig. 1. A: representative images of a healthy (1) and a highly damaged (2) cell in the comet assay, stained with propidium iodide. A computer-aided analysis system determines the extent of migrated DNA. B: DNA damage in LLC-PK₁ cells as measured by the comet assay, after a 24-h treatment with various concentrations of ANG II. Control, no treatment; n.s., not statistically significant. The positive control methyl methanesulfonate yielded a DNA damage of 50.69 ± 5.30% DNA in tail. *P ≤ 0.05 vs. control. **P < 0.01 vs. control.

Fig. 2. DNA damage in LLC-PK₁ cells as measured by the comet assay, after 24-h treatment with 2 concentrations of ANG II with and without coincubation with either 6 mM N-acetylcysteine (NAC) or 5 μM candesartan (Cand). Control, no treatment. Six millimolar NAC or 5 μM candesartan alone had no effect on the DNA (1.04 ± 0.07 and 1.11 ± 0.35% DNA in tail, respectively). The positive control methyl methanesulfonate yielded a DNA damage of 62.30 ± 2.00% DNA in tail. *P ≤ 0.05 vs. control. °P ≤ 0.05 vs. ANG II treatment.

Fig. 3. A: representative micronuclei-containing binucleated cells, stained with acridine orange. B: micronuclei frequencies in binucleate LLC-PK₁ cells after treatment for 48 h with 2 ANG II concentrations with and without coincubation with either 6 mM NAC or 5 μM Cand. After 24 h, 5 μg/ml cytochalasin B was added to all samples to yield binucleated cells by inhibiting cytokinesis. Control, treatment only with cytochalasin B. The positive control methyl methanesulfonate yielded 34.13 ± 4.49 micronuclei per 1,000 binucleated cells (not shown). *P ≤ 0.05 vs. control. °P ≤ 0.05 vs. ANG II treatment.
tion with ANG II, only the NRK cells showed an induction of both apoptosis and proliferation (Table 2).

ANG II-induced DNA damage was first measured in LLC-PK<sub>1</sub> cells with the comet assay (Fig. 1). Upon treatment with ANG II, a dose-dependent increase in genomic damage was demonstrated (Fig. 1B). A statistically significant genomic damage appeared at 85 nM ANG II and increased with higher doses.

To analyze the role of the AT<sub>1</sub> receptor in ANG II-induced comet formation, the AT<sub>1</sub> receptor antagonist candesartan was added simultaneously with ANG II to the cells (Fig. 2). Candesartan prevented the ANG II-induced genomic damage completely.

ANG II is known to induce oxidative stress via its AT<sub>1</sub> receptor, which can lead to genomic damage. To demonstrate the participation of oxidative stress in ANG II-induced comet formation, the cells were coincubated with ANG II and the antioxidant NAC. NAC, like candesartan, prevented the ANG II-induced genomic damage (Fig. 2). Incubation with NAC alone did not lead to the formation of comets (data included in the figure legends).

As a second method to measure DNA damage, we chose the micronucleus frequency test. ANG II caused the induction of micronuclei in LLC-PK<sub>1</sub> cells (Fig. 3), which could also be reduced by NAC and candesartan. The number of micronuclei in cells treated with 170 nM ANG II combined with NAC and candesartan did not differ from the control cells. NAC was not able to prevent the induction of micronuclei caused by 340 nM ANG II completely; however, it did reduce them significantly.

Since it was reported that NAC inhibits the binding of ANG II to AT<sub>1</sub> receptor due to its ability to reduce disulfide bonds (28), the effect of a second antioxidant, which contains no free...
sulfhydryl groups, α-tocopherol, on ANG II-induced DNA damage was examined. As can be seen in Fig. 4A, comet formation due to ANG II incubation was prevented completely. Also, the formation of micronuclei by 170 nM ANG II was inhibited (Fig. 4B).

The application of the AT2 receptor antagonist PD123319 to ANG II-treated LLC-PK1 cells showed that this substance had no effect on the ANG II-induced comet formation (Fig. 5A). PD123319 lessened the ANG II-caused micronuclei number slightly but was not able to reduce them significantly (Fig. 5B).

The induction of micronuclei by ANG II was confirmed in two other cell lines, HL-60 and NRK, which also showed an increase in micronuclei-containing cells (Table 2). All cell lines (HL-60, LLC-PK1, and NRK) had a similar factor of micronuclei induction around 4 (Table 2).

Since we assume that the ANG II-induced genomic damage is caused by the formation of ROS upon AT1-mediated activation of the NAD(P)H oxidase, the generation of ROS was measured flow cytometrically. As shown in Fig. 6, ANG II led to a significant formation of ROS, which could be prevented either by candesartan, NAC, or α-tocopherol.

To exclude the possibility of candesartan being a radical scavenger itself, a coincubation of LLC-PK1 cells with hydrogen peroxide (H2O2) and candesartan was performed (Fig. 7,

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

Fig. 6. Flow cytometric analysis of ANG II-induced reactive oxygen species (ROS) production in LLC-PK1 cells. A: representative frequency histogram of the green fluorescence of H2DCF-positive cells of control cells (broken line), and cells after 4-h incubation with 170 nM ANG II without (fat solid line) and with coincubation with 6 mM NAC (light gray solid line). B: representative frequency histogram of the green fluorescence of H2DCF-positive cells of control cells (broken line), and cells after 4-h incubation with 170 nM ANG II without (fat solid line) and with coincubation with 5 μM CAND (dark gray solid line). C: quantification of the flow cytometry measurements by WinMDI 2.8. Relative fluorescence units are shown. Control, treatment with only H2DCF-DA. D: quantification of the flow cytometry measurements of cells incubated 4 h with 170 nM ANG II with and without 750 μM α-TOC. Control, treatment with only H2DCF-DA. *P ≤ 0.05 vs. control. °P < 0.05 vs. ANG II treatment.
A–D). An H$_2$O$_2$ concentration was chosen, which led to a shift to the right of the curve, comparable to the shift caused by 170 nM ANG II (see Fig. 6), and which also caused DNA damage in the comet assay (Fig. 7E). The flow cytometric analysis of this experiment showed that candesartan could not reduce the H$_2$O$_2$-induced oxidative stress in the cells, in contrast to NAC, which could.

**DISCUSSION**

In LLC-PK$_1$ pig kidney cells, we detected a direct DNA damaging potential of ANG II by the comet assay. Thus our data confirm and extend similar observations in cells of the ascending limb of the loop of Henle (20) and in microvessel endothelial cells (18). For the first time, chromosomal mutations caused by ANG II as revealed by micronucleus formation were observed. Micronuclei were induced in three different cell lines, in LLC-PK$_1$, in rat kidney cells (NRK), and in human promyelocytic cells (HL-60).

Depending on the cell type, the stimulation of the ANG II receptor AT$_1$ leads to cellular contraction, hypertrophy, proliferation, and/or apoptosis (31). In LLC-PK$_1$, ANG II had no impact on the proliferation or the apoptosis rate in contrast to observations by Hannken et al. (10), who detected a cell cycle arrest in LLC-PK$_1$. This difference might be due to the pres-

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**Fig. 7.** Flow cytometric analysis of a potential antioxidative effect of Cand. **A:** representative frequency histogram of the green fluorescence of H$_2$DCF-positive cells of control cells (broken line), and cells after 30-min incubation with 1.25 μM hydrogen peroxide (H$_2$O$_2$) without (fat solid line) and with co-incubation with 6 mM NAC (light gray solid line). **B:** quantification of the flow cytometry measurements by WinMDI 2.8. Relative fluorescence units are shown. *Control, treatment with only H$_2$DCF-DA. C:** representative frequency histogram of the green fluorescence of H$_2$DCF-positive cells of control cells (broken line), and cells after 30-min incubation with 1.25 μM H$_2$O$_2$ without (fat solid line) and with 5 μM Cand (dark gray solid line). **D:** quantification of the flow cytometry measurements by WinMDI 2.8. Relative fluorescence units are shown. Control, treatment with only H$_2$DCF-DA. *P ≤ 0.05 vs. control. **E:** DNA damage in LLC-PK$_1$ cells as measured by the comet assay, after 30-min treatment with 1.25 μM H$_2$O$_2$. Control, no treatment.
ence of serum in our experiments, while Hannken et al. incubated serum free. Of the other two cell lines we analyzed for micronuclei induction with ANG II, only the rat kidney cells showed enhanced proliferation and apoptosis.

Coincubation with the AT1 receptor antagonist candesartan prevented the appearance of genomic damage detected with the comet assay and the micronucleus frequency test, while the application of the AT2 receptor antagonist PD123319 had no effect.

It is well-known that ANG II binding to the AT1 receptor induces superoxide anions (7). This was also already demonstrated for LLC-PK1 cells (10). Here, it is shown for the first time that the genotoxic action of ANG II in the comet assay and its micronuclei-inducing effect could be prevented by the antioxidants NAC and α-tocopherol, linking the formation of ROS to the genotoxic effects.

DNA damage caused by ROS involves single- or double-strand DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links (29). DNA repair, which starts after the oxidative attack on the chromosomes, often transforms the above-mentioned modifications to additional strand breaks. The comet assay detects such structural DNA damage (1), which, as we have shown, was also induced by incubation with ANG II. Micronuclei are formed for example after double-strand breaks which lead to chromosome fragments lagging behind at the anaphase during nuclear division (5). Hydrogen peroxide induces DNA double-strand breaks in a time- and dose-dependent manner, as revealed by an antibody specifically detecting double-strand breaks (15). Superoxide anions released after ANG II stimulation by NAD(P)H oxidase are rapidly converted to hydrogen peroxide (8), which can lead to the ANG II-induced micronuclei.

Our evidence for ANG II-induced genomic damage in renal tubular cells could be of significance with regard to the increased occurrence of kidney carcinomas under an activated renin-angiotensin-system (6, 19). In animal models, it was shown that the concentration of ANG II in the kidney is 25 to 1,000 times higher than in plasma (24, 30). Under pathological conditions, the ANG II concentration in the renal interstitial fluid of dogs can reach 800 nM (24) implying a physiological relevance of a genotoxic effect of 85 nM ANG II.

In the presence of hypertension, the incidence of renal cancer is enhanced (3). The potential involvement of a stimulated RAS in the kidney is underlined by the observation that long-term treatment with diuretics is associated with an increased risk of renal cancer (9). On the contrary, ANG I-converting enzyme inhibitors have been discussed as anticancer drugs (16), and ANG II type 1 receptor blockers have been tested in a pilot study with patients who suffer from advanced hormone-refractory prostate cancer (26, 27).

In conclusion, our in vitro results show that ANG II induces genomic damage in mammalian cells, most likely via oxidative mechanisms. This injury can be prevented by ANG II type 1 receptor blockade and by antioxidants.

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REFERENCES

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