Angiotensin II Stimulates the Release of Interleukin-6 and Interleukin-8 From Cultured Human Adipocytes by Activation of NF-κB

Thomas Skurk, Vanessa van Harmelen, Hans Hauner

Objective—Several proinflammatory cytokines including IL-6 and IL-8 are produced by human adipocytes, but it is still unclear how this process is regulated. Angiotensin (Ang) II, which is also produced by adipocytes, might play a role as a regulator. In the present study, we investigated the effect of Ang II on the production of IL-6 and IL-8 in in vitro differentiated human adipocytes.

Methods and Results—Isolation of preadipocytes and differentiation of these cells into adipocytes, Real-time quantitative reverse-transcriptase polymerase chain reaction, Western-blot, enzyme-linked immunosorbent assay, and electromobility shift assay. Ang II-stimulated IL-6 and IL-8 mRNA expression and protein release in a time- and concentration-dependent way. This action of Ang II was completely blocked by the NF-κB–blocker Bay 117082 and the AT1 blocker candesartan, but only partially by the AT2-blocker PD 123 319. Incubation of adipocytes with Ang II resulted in an increased phosphorylation of the p65 subunit of NF-κB and an increased translocation of NF-κB to the nucleus.

Conclusion—Ang II stimulates IL-6 and IL-8 production and release from human adipocytes by a NF-κB–dependent pathway. This proinflammatory action of Ang II seems to be mediated by the AT1 and less by the AT2 receptor subtype.

Key Words: cytokines ■ adipocytes ■ angiotensin ■ AT1, receptor ■ NF-κB

Human adipocytes are known to produce a variety of proteins, which are released into the circulation. For instance, adipocytes secrete proinflammatory cytokines like interleukin (IL)-1β, IL-6, and IL-8. Recently, plasma levels of IL-6 and other cytokines have been shown to be correlated with body mass index (BMI) and body fat mass; therefore, it has been suggested that these cytokines may represent the state of chronic inflammation observed in obesity. It is well-established that the expression and release of these cytokines in human adipocytes are subject to a complex hormonal regulation.

Adipocytes are also a major source of products of the renin-angiotensin-system (RAS), particularly of its active component angiotensin (Ang) II. It has recently been discovered that Ang II exerts transcriptional activity via an Ang II-responsive element in the IL-6 promoter in rodent cardiomyocytes. Moreover, Ang II can activate the proinflammatory transcription factor NF-κB in various cell types. Ang II has been shown to cause release of IL-6 from cultured vascular smooth muscle cells via an NF-κB–dependent pathway. As described by Ruiz-Ortega et al, activation of NF-κB may be attributed to both functional receptor subtypes AT1 and AT2. The possible modification of the inflammatory process by Ang II may have clinical implications. Recent studies have shown a successful treatment of patients with atherosclerotic complications with angiotensin-converting enzyme (ACE) inhibitors and AT1 receptor antagonists.

The aim of the present study was to examine whether Ang II is involved in the release of proinflammatory cytokines in human adipocytes, particularly in the release of IL-6 and IL-8. In addition, we were interested to investigate whether a possible effect of Ang II on cytokine release is mediated by NF-κB–dependent pathway. Moreover, we used selective Ang II receptor blockers to unravel the role of the AT1 and AT2 receptor subtypes in this context.

Methods

Subjects
Adipose tissue for the isolation of pre-adipocytes was obtained from healthy and normal-weight women (BMI ≤26 kg/m², age range 18 to 50 years) undergoing elective mammary reduction. All subjects were white and did not have acute infection, malignancies, or any other consuming disease. Informed consent was obtained from all subjects. The study was approved by the ethical committee of the Heinrich-Heine University in Düsseldorf.

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Pre-Adipocyte Differentiation
Adipocyte precursor cells were isolated and differentiated as described previously.17 To enhance differentiation, the adipogenic medium was supplemented with 1 μg/mL troglitazone and 0.5 mM/L isobutyl-methylxanthine (IBMX) for the first 3 days. All experiments were performed after 16 days in culture when at least 50% of the cells had developed the adipocyte phenotype.

Incubation of Adipocytes
The newly developed fat cells were incubated in the presence and absence of the following agents: Ang II (Sigma, Munich, Germany), candesartan (AT1 receptor blocker; AstraZeneca, Wedel), PD 123 319 (AT2 receptor blocker; Sigma, Munich), and Bay 117602 (NF-κB blocker; Biomol, Hamburg). The cells were pre-incubated for 1 hour with the respective blocker before Ang II was added. The blockers were not cytotoxic at the concentrations used as tested in an MTS (data not shown).

RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction
Total RNA was isolated and reverse-transcribed as described previously.18 The specific mRNAs were determined by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using light-cycler technology (Roche Diagnostics). The primer sequences were as follows: IL-6: (accession: AF372214) 5'-TAG CCG CCC CAC ACA GAC AG and 3'-GGC TGG CAT TTG TGG TTG; GG product length 407 bp, annealing temperature 57°C, melting temperature 84.5°C; IL-8: (accession: AF385628) 5'-GGC TGG CAT TTG TGG TTG and 3'-TAG CCG GTA CCC CAC TTT GCT CT; product length 231 bp, annealing temperature 57°C, melting temperature 86.0°C. Sp1 was used as a reference gene.

Measurement of Proteins
For quantification of IL-6 and IL-8 protein in the culture medium, a commercially available enzyme-linked immunosorbent assay (ELISA) was used (HIS technologies, Freiburg, Germany). The interassay and intraassay coefficients of variation were ≤10% and 5%, respectively.

Western Blot Analysis
Immunological detection of the phosphorylated form of the p65 subunit of NF-κB was performed as described before.19

Electromobility Shift Assay and Supershift Assay
Nuclear extracts were prepared by lysing adipocytes in a buffer containing 20 mM/L HEPES, pH 7.9, 10 mM/L NaCl, 0.2 mM/L EDTA, 2 mM/L DTT, and Complete (Roche Diagnostics GmbH, Mannheim, Germany). The cells were centrifuged and the pellet was resuspended in a buffer containing 2 mM/L HEPES, pH 7.9, 0.75 mM/L spermidine, 0.15 mM/L spermin, 420 mM/L NaCl, 0.2 mM/L EDTA, 2 mM/L DTT, 25% glycerol, and Complete (Roche Diagnostics GmbH). Nuclear extracts were incubated for 30 minutes on ice and centrifuged; after that, the supernatant was resuspended in a buffer containing 2 mmol/L HEPES, pH 7.9, 0.25 mmol/L EDTA, 2 mmol/L DTT, and Complete (Roche Diagnostics GmbH). Nuclear extracts were incubated for 30 minutes on ice and centrifuged; after that, the supernatant was used for the EMSA or the supershift assay. The NF-κB probe had the following consensus sequence: 5'-AGT TGA GGG GAC TTC CCC AGG C (top strand only). The labeling of the probe and the binding reaction were performed using the DIG Gel shift kit (Roche Diagnostics GmbH). Samples were separated in a nondenaturing 6% PAGE gel, and bands were detected using a Lumi-imager device (Roche Diagnostics GmbH). For the supershift assay, antibodies specific for the subunits of NF-κB: p70, p65, and p50, respectively, were included in the NF-κB–binding reaction in a dilution of 1:109.

Statistical Analysis
Results are expressed as mean±SD. A paired t test was used for statistical comparisons.

Figure 1. Concentration-dependent (A) and time-dependent (B) effects of Ang II on IL-6 protein release into the culture medium from in vitro differentiated human adipocytes. Ang II was added on day 16 after cells had acquired the adipocyte phenotype for the time periods and concentrations indicated. The incubation time was 24 hours (A), the Ang II concentration was 10⁻⁵ M (B). C, Cells were preincubated for 1 hour with or without the blockers indicated, followed by an incubation with Ang II with or without the blockers for 24 hours. Results represent mean±SD of 4 independent experiments in duplicate. A, B, *P<0.05 versus controls. C, *P<0.05 versus Ang II-stimulated cells.

Results
Effect of Ang II on IL-6 and IL-8 Release From Human Adipose Cells
Incubation of the cells was performed on day 16 when at least 50% of the stromal cells had acquired adipocyte morphology. Cells were incubated for 24 hours with increasing concentrations of Ang II. Under these conditions, there was a concentration-dependent increase in IL-6 and IL-8 release into the culture medium. The concentrations at which Ang II significantly enhanced IL-6 and IL-8 release were 10⁻⁶ M and 10⁻⁵ M, respectively (Figures 1A and 2A). At 10⁻⁵ M Ang II, the release of both IL-6 and IL-8 protein was increased 3- and 4-fold, respectively. In addition, there was a time-dependent increase in IL-6 and IL-8 release on exposure to 10⁻⁵ M Ang II. The stimulatory effect was observed after an incubation of 6 hours and remained stable for the whole incubation period (Figures 1B and 2B).

Effect of Ang II on mRNA Levels of IL-6 and IL-8
To address the question whether the stimulatory effect of Ang II is associated with changes at the transcriptional level, we measured mRNA levels of IL-6 and IL-8 in the absence and presence of Ang II. The peptide induced a time- and concentration-dependent increase in IL-6 and IL-8 mRNA compared with control cultures. The stimulatory effect was
maximal after 6 hours of incubation (corresponding to a 2- to 3-fold increase of IL-6 and IL-8, respectively) (P<0.05) (Table 1). The minimum stimulatory concentration of Ang II was 10^{-7} M and the maximum stimulatory concentration was 10^{-5} M (Table 1).

Effect of Candesartan, PD 123 319, and Bay 117082 on IL-6 and IL-8 Protein Release
To investigate whether the AT_{1} and or the AT_{2} receptor subtype is involved in the effect of Ang II on IL-6 and IL-8 release, adipocytes were incubated with 10^{-5} M Ang II in the absence and presence of the specific AT_{1} receptor blocker candesartan or the specific AT_{2} receptor blocker PD 123 319. Candesartan significantly counteracted the Ang II-stimulated IL-6 and IL-8 release, although this inhibition was not complete (paired t test Ang II versus Ang II plus candesartan, P<0.05) (Figures 1C and 2C). PD 123 319 showed a tendency to inhibit Ang II-stimulated IL-6 and IL-8 release, but this effect did not reach statistical significance (Figures 1C and 2C).

To test whether Ang II induced IL-6 and IL-8 production via a signaling pathway involving NF-κB, adipocytes were incubated with 10^{-5} M Ang II in the absence and presence of the specific NF-κB blocker Bay 117082 (10^{-5} M). Bay 117082 prevented the stimulation of IL-6 and IL-8 release on Ang II exposure (paired t test Ang II versus Ang II plus Bay 117082; P<0.05) (Figures 1C and 2C).

Effect of Ang II on NF-κB Activation and Translocation
We next investigated whether Ang II is able to stimulate NF-κB phosphorylation in adipocytes by using a Western blot analysis.

Cells were incubated with Ang II (10^{-5} M) for 0, 1, 2, 5, 10, 30, and 60 minutes, respectively. After an incubation of 5 minutes, a significant 2-fold increase of phosphorylation of the p65 subunit of NF-κB was found (P<0.05) (Figure 3).

To confirm the action of Ang II on NF-κB translocation, an EMSA was performed on nuclear extracts from cells treated with or without 10^{-5} mol/L Ang II for 3 hours. It was clearly observed that Ang II increases NF-κB translocation to the nucleus in adipocytes (Figure 4). Additionally, to confirm that the observed bands in the EMSA were specific for NF-κB, we performed a supershift assay that included NFκB subunit antibodies in the binding reaction of Ang II-treated nuclear extracts. A clear reduction in the intensity of the original NF-κB probe complex was observed with the p50 and p65 antibody, but not with the p70 antibody (Figure I, available online at http://atvb.ahajournals.org). Therefore, the observed band in the EMSA seemed to be specific for the p50/p65 heterodimer.

Ang II Stimulates NF-κB Translocation via AT_{1} Receptors
To study whether the effect of Ang II on NF-κB is mediated via the AT_{1} and or the AT_{2} receptor, the effect of candesartan and PD 123 319 on Ang II-stimulated NF-κB translocation was investigated. Candesartan abolished Ang II-induced NF-κB translocation significantly (Figure 4, P<0.05), whereas Bay 117082 showed a tendency to reduce the stimulatory effect of Ang II on NF-κB activation.

The Effect of Repetitive Addition of Ang II on IL-6 Release in Adipocytes
In most of the experiments, Ang II was used at a supraphysiological concentration of 10^{-5} M. The reason for using this concentration was that Ang II is rapidly degraded under culture conditions. To show that Ang II also stimulates IL-6 secretion at lower concentrations, we performed additional experiments in which we added Ang II at a concentration of 10^{-9} M every 6 hours for 24 hours. The stimulation of IL-6 protein release was similar as compared with 10^{-5} M Ang II administered once (Table 2). Moreover, Ang II at 10^{-9} M stimulated NF-κB translocation to the nucleus in adipocytes (data not shown).
Discussion

It is well-established that adipocytes release substantial amounts of the cytokines IL-6 and IL-8, and it is apparent that this release is subject to complex regulation. In the current study, we show for the first time to our knowledge that Ang II stimulates gene expression and protein release of IL-6 and IL-8 from human adipocytes in a time- and concentration-dependent fashion. The stimulation of IL-6 release by Ang II has been shown previously for other cell types in rodents. The production of IL-6 by adipocytes may be of special importance because up to one-third of circulating IL-6 is secreted by adipose tissue. In addition, the circulating levels of IL-8 are positively correlated with BMI, indirectly suggesting that adipose tissue may also release IL-8 into the blood.

It is known from recent studies that the effect of Ang II on inflammation involves an activation of the NF-κB signaling pathway. In the current study, we found that Ang II stimulates phosphorylation of the p65 subunit of NF-κB, using Western blot analysis. In addition, an EMSA revealed that Ang II induces translocation of NF-κB to the nucleus in human adipocytes. Further experiments demonstrated that blockade of NF-κB by Bay 117082 abolished the Ang II-mediated stimulation of IL-6 and IL-8 release. Taken together, these results strongly suggest that Ang II is mediating its effects on IL-6 and IL-8 via NF-κB activation.

In adipose tissue, most effects of Ang II are mediated via the AT$_1$ receptor, although there is evidence that the AT$_2$ receptor subtype is also expressed. The receptor subtypes may share common signaling pathways leading to subsequent activation of NF-κB. By using candesartan, a specific antagonist to the AT$_1$-receptor subtype, we found that the Ang II-mediated effects on NF-κB signaling and subsequent cytokine release were nearly completely abolished. When studying the effect of the AT$_2$ receptor antagonist PD 123 319 in our model, a small but not significant inhibition was observed. These results indicate that signal transduction via the AT$_1$ receptor is the predominant pathway for Ang II-stimulated NF-κB activation and cytokine release in newly differentiated human adipocytes.

One has to keep in mind that degradation of Ang II released by human adipocytes in primary culture occurs very rapidly, which makes it difficult to determine the true concentrations of Ang II required to stimulate cytokine production. Because of the high degradation rate of Ang II, we decided to use Ang II at a supraphysiological concentration of $10^{-5}$ M in most of our experiments. However, when differentiated adipocytes were incubated with Ang II at a physiological concentration of $10^{-7}$ M, and when the Ang II was added every 6 hours, the stimulation of IL-6 protein release was similar as compared with the supraphysiological concentration of Ang II ($10^{-5}$ M) administered once. Moreover, Ang II at $10^{-9}$ M stimulated translocation of NF-κB in EMSA experiments. These results suggest that Ang II exerts its stimulating effects on cytokine production at near-physiological concentrations.

Table 2. Effect of Ang II on IL-6 Protein Secretion From In Vitro Differentiated Human Adipocytes

<table>
<thead>
<tr>
<th></th>
<th>Ang II</th>
<th>Ang II + Candesartan</th>
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<tr>
<td>Ang II</td>
<td>296±76</td>
<td>90±2</td>
</tr>
<tr>
<td>Ang II</td>
<td>226±20</td>
<td>78±7</td>
</tr>
</tbody>
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Ang II was added either at a concentration of $10^{-5}$ M or at a concentration of $10^{-7}$ M repetitively every 6 hours. The AT$_1$ blocker candesartan was used at a concentration of $10^{-9}$ M. IL-6 protein was determined in the culture medium after 24 hours of incubation using a specific ELISA. Basal (nonstimulated) IL-6 protein concentration was defined as 100%. Results are given as mean±SD.
Obesity is considered as a state of subclinical chronic inflammation. This view is based on the finding that plasma levels of cytokines are elevated in obesity. This is most evident for IL-6, in which a positive association between circulating levels and BMI has been reported.\(^6^,7\) At present, weight loss by dietary intervention or other methods have been found to be associated with decreased levels of IL-6 and other cytokines.\(^{26,27}\) It is rather unclear which factors are responsible for the upregulation of proinflammatory proteins in obesity. One possible mediator for this process might be Ang II. Angiotensinogen and all components of the renin-angiotensin system are produced by adipocytes\(^8\) and the expression of angiotensinogen is upregulated in obesity.\(^{28,29}\) Moreover, studies on the long-term use of ACE inhibitors and AT\(_1\) blockers in patients with atherosclerosis and diabetes mellitus have suggested that these drugs have antiinflammatory properties.\(^{30,31}\) Therefore, in view of our and former results, a role of Ang II in the pathophysiology of the proinflammatory state is rather likely.

In conclusion, the results of this experimental study indicate that Ang II stimulates production and release of IL-6 and IL-8 in in vitro differentiated human adipocytes. This stimulatory action seems to be mediated via the AT\(_1\) receptor and a NF-\(\kappa B\)-dependent signaling pathway.

### References

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Figure I

EMSA performed as described in figure 4 along with NF-κB subunit antibodies in the binding reaction. Incubation with p50 and p65 but not with p70 antibodies respectively, exhibited a reduction in the density signal of the investigated band for p50 and p65, but not with p70.
Figure I - online

NF-κB

control AngII AngII AngII AngII neg
+p70 +p50 +p65 control

aux units (%)

control AngII AngII+p70 AngII+p50 AngII+p65 neg control