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Angiotensin II Stimulates Proliferation of Primary Human Keratinocytes via a Non-AT_1, Non-AT_2 Angiotensin Receptor

U. Muscha Steckelings, Metin Artuc, Martin Paul,* Monika Stoll,† and Beate M. Henz

*Department of Dermatology, Virchow Klinikum, Humboldt Universität, Augustenburger Platz 1, 13353 Berlin, Germany; *Department of Clinical Pharmacology, University Hospital Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany; and †Department of Pharmacology, Christian-Albrecht-Universität, Hospitalstr.4, 24105 Kiel, Germany*

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Angiotensin II is a hormone with long known cardiovascular actions. Recent studies revealed an additional role for angiotensin II in the regulation of cell proliferation. This study was performed to clarify whether skin is a target organ for these novel angiotensin actions. Radioligand binding studies identified a high-affinity angiotensin receptor on human primary keratinocytes *in vitro* with a Kd of 4.5 nM and a B_{max} of 0.12 nM. Competition experiments with losartan and PD 123177 revealed that this receptor was not of the AT_1- nor the AT_2-subtype. Stimulation of human keratinocytes with angiotensin II (10^{-10} to 10^{-5} M) led to a dose dependent increase in ^{3}H-thymidine incorporation, indicating that the keratinocyte receptor mediates a mitogenic effect. This effect is comparable at 10^{-9} M to stimulation of keratinocytes by EGF (50ng/ml) and FGF (50ng/ml). These results demonstrate for the first time a possible physiological role for angiotensin II in human skin involving the regulation of keratinocyte proliferation.

For many years, the renin-angiotensin-system has been regarded as solely responsible for the regulation of blood-pressure and volume- and electrolyte-homeostasis (1). In the eighties, however, it became clear that angiotensin II (ANG II) additionally had mitogenic properties very similar to a growth factor. Following the characterization of two distinct angiotensin receptor subtypes termed AT_1 and AT_2 (2,3), the growth promoting effect of ANG II could be attributed to the AT_1 subtype (4). AT_2-receptors, on the other hand, were recently shown to mediate antiproliferation (5,6). In tissues expressing both angiotensin receptor subtypes, stimulation of the AT_2-receptor counteracts the mitogenic effect mediated via the AT_1-receptor as well as mitogenic stimuli by growth factors (5,6). Therefore, ANG II may be regarded as a regulatory factor of proliferation.

The epidermal layer of the skin is a strongly proliferating tissue with a physiological generation time of keratinocytes of about 30 days. Modulation of proliferation is needed in case of wound healing and is disturbed in different kinds of skin diseases such as psoriasis or skin tumors. Although several factors have been identified which are involved in the regulation of proliferation, it has not been clarified so far whether ANG II might play a role as a regulator of proliferation in skin.

The present study was aimed at elucidating whether human keratinocytes express ANG II receptors and—in case of a positive result—whether these receptors mediate biologic effects such as stimulation or inhibition of keratinocyte proliferation.

MATERIALS AND METHODS

Cell culture. Samples of normal human skin were obtained during routine surgical procedures such as face lifting or circumcision. After removal of subcutaneous tissue, samples were incubated in 0.25% trypsin at 4°C over night. The epidermal layer was manually separated from the dermal layer and keratinocytes were gently detached from the tissue, filtered and plated onto collagen coated culture dishes. Cells were grown in standardized keratinocyte growth

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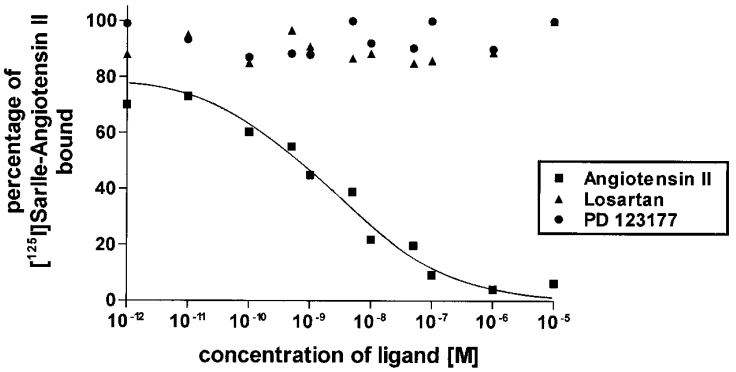

FIG. 1. Competition of angiotensin II and the selective AT_1 and AT_2 receptor ligands losartan and PD 123177 (each in concentrations of 10^{-5} to 10^{-12} M) for [^{125}I]Sar_1Ile_8 -angiotensin II (0.25 nM) on human primary keratinocytes. Only angiotensin II and not the specific receptor subtype ligands was able to compete for radioligand binding indicating the presence of a non-AT_1, non- AT_2 receptor subtype.

medium (Clonetics) at 37°C under a humified atmosphere of 5% CO_2. Cells for all experiments were harvested at their second or third passage with trypsin/EDTA 0.02/0.05%.

Receptor binding studies. Cells were seeded onto collagen-coated 24-well plates at a density of 50 000 cells per well and grown to subconfluency. 24 hours prior to the experiment, cells were cultured in keratinocyte basal medium (Clonetics) without any supplements.

0.25 nM[^{125}I]-Sar_1Ile_8-angiotensin II (2200 Ci/mmol; Amersham) was added to the cells as a radioligand which was competed for by ANG II, the AT_1-receptor specific ligand losartan or the AT_2-receptor specific ligand PD 123177, respectively, at concentrations from 10^{-12} to 10^{-5} M. After a 1 hour incubation at room temperature, cells were washed 3 times in ice cold PBS containing 1% BSA in order to remove unbound radioligand and subsequently lysed in 2M NaOH. Lysates were counted in an automated γ-counter.

Non-specific binding was defined as radioligand binding after competition with 10^{-5} M ANG II. The binding data were analysed with the computer program LIGAND to determine the affinity (K_d), number of sites (B_{max}).

[^{3}*H*]*Thymidine incorporation.* For determination of nucleic acid incorporation as a marker of mitogenic activity, cells were seeded onto 24-well plates at a density of 50 000 cells per well. After 12 hours, growth medium was displaced by basal medium and the cells cultured to quiescence over a period of 24 hours. Cells were then stimulated with ANG II (10^{-10} to 10^{-5} M) for 24 hours. For comparison cells exposed to EGF or FGF at 50 μg/ml were included in some experiments. Stimulation was followed by addition of [^{3}H]-thymidine and another 12 hour incubation period. Cells were subsequently washed 3 times with PBS in order to remove unbound radioactivity, once with 5% TCA and once with 100% ethanol. Afterwards, cells were lysed in 1 M NaOH, and 200 μl aliquots of the lysate counted in a β-counter.

Statistics. Data are reported as means of sixfold determination ± SEM. Statistical analysis was performed by Students t test for comparison between groups. A probability of 0.05 or less was considered significant.

RESULTS

Radioligand binding studies were performed on cultured human primary keratinocytes (passage 2 or 3). Figure 1 shows a typical displacement curve where [^{125}I]-Sar_1Ile_8-angiotensin II was competed for by either cold ANG II (10^{-12} to 10^{-5} M) or by the AT_1- and AT_2-receptor specific ligands losartan or PD 123177 (10^{-12} to 10^{-5} M). Since the AT_1- and AT_2-receptor specific ligands were not able to displace the radioligand, keratinocytes seem to express an ANG II receptor subtype distinct from AT_1- and AT_2-receptors. Scatchard analysis revealed a Kd of 4.5 nM and a B_{max} of 0.12 nM for this so far uncharacterized receptor subtype which binds ANG II with high affinity.

Searching for a putative physiological role of ANG II in keratinocytes we tested for ANG II induced keratinocyte proliferation since among the known actions of ANG II, stimulation of proliferation seemed to be most likely. We found indeed a dose-dependent stimulation of keratinocyte proliferation by ANG II with a maximal effect at a concentration of 10^{-6} M

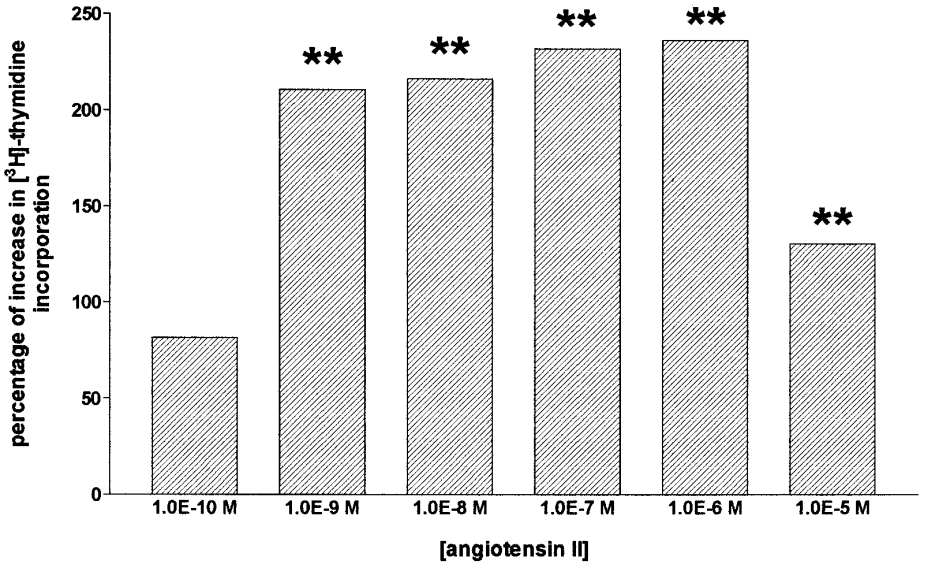

FIG. 2. Stimulatory effect of angiotensin II on the DNA synthesis of cultured human keratinocytes. Keratinocytes were incubated with increasing concentrations of angiotensin II (10^{-5} to 10^{-12} M) for 36 hours. [^{3}H]-thymidine was added during the last 12 hours of incubation in order to estimate the amount of DNA synthesis. Data are given as percentage of increase in [^{3}H]-thymidine incorporation in treated cells compared to untreated cells (0%). ** $p < 0.001$ in comparison to non-treated cells (calculated from original raw data).

(Fig.2). At this concentration, the increase in ^{3}H-thymidine incorporation amounted to 236%. The proliferative stimulus to the cells by ANG II is comparable in strength to that of well-known epidermal growth factors such as EGF (50 ng/ml) or FGF (50 ng/ml) (Fig.3).

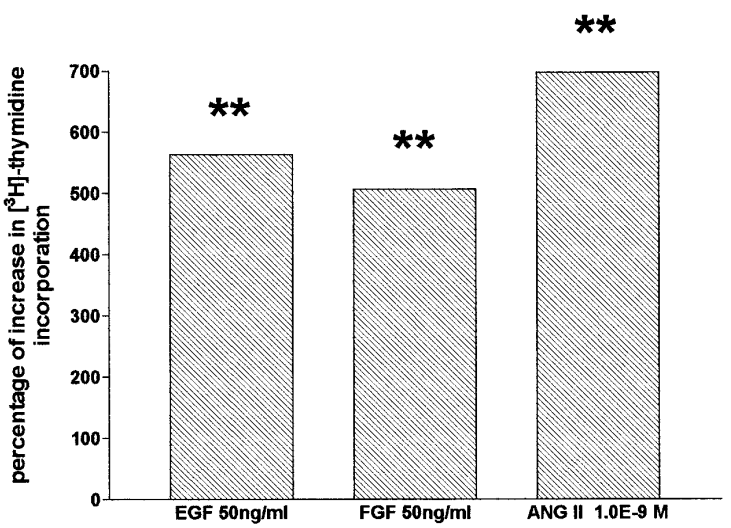

FIG. 3. Comparison of the stimulatory effects of angiotensin II (10^{-9} M) versus the ''classical'' growth factors EGF (50ng/ml) and FGF (50ng/ml) on DNA synthesis of cultured human keratinocytes. Data are given as percentage of increase in [^{3}H]-thymidine incorporation in treated cells compared to untreated cells (0%). For experimental conditions, see legend to Fig.2 or text. ** $p < 0.001$ in comparison to non-treated cells (calculated from original raw data).

DISCUSSION

Apart from its cardiovascular effects, proliferation control seems to be one of the major actions of angiotensin II (ANG II). A stimulatory effect of ANG II can be found in a variety of cell types including vascular endothelial cells, 3T3 fibroblasts and renal glomerulosa fasciculata cells (4,5,7). Inhibition of proliferation is one of the very few actions of ANG II which could be attributed to AT_2-receptor stimulation (5,6). In *in vitro* experiments, it was demonstrated that AT_2-receptor-mediated antiproliferation counteracts the AT_1-receptor-mediated mitogenic effect (5,6). Therefore, ANG II can be considered to be part of the regulatory mechanisms in proliferating tissues.

Skin is one of the most rapidly proliferating tissues. Thus, we wondered whether ANG II might also be involved in proliferation control of human skin (8). Experiments using rat cutaneous biopsies had already brought some evidence for the existence of ANG II receptors in skin (9,10,11). It was not determined, however, which specific cell types in rat skin express ANG II receptors, although the localisation of ANG II-binding in autoradiography experiments suggested skin fibroblasts as the primary target cells for ANG II (10). To our knowledge, no data are available so far about ANG II receptor expression in human skin.

Our experiments revealed that human keratinocytes express a high-affinity, non-AT_1-, non-AT_2-angiotensin receptor. The specific AT_1- and AT_2-receptor antagonists losartan and PD 123177 were not able to compete for Sar_1Ile_8-ANG II binding. Non-AT_1-, non-AT_2-angiotensin receptors have recently also been reported for other human tissues, namely cardiac fibroblasts and endometrium (12,13). The new ANG II receptor expressed on human cardiac fibroblasts may represent the same subtype as the receptor found in our experiments since it has comparable binding characteristics (K_d: 0.6 nM; B_{max}: 1.5 fmol/mg protein) and mediates proliferation as well (12).

Theoretically, the renin-angiotensin-system (RAS) may influence the proliferation rate of keratinocytes by two mechanisms: either by up- and downregulation of receptor expression or by generating locally different concentrations of ANG II. A local RAS has been described for various tissues such as the vascular wall or brain (14). There is evidence for the existence of a local RAS in rat and porcine skin (15,16). Furthermore, enzymatically active angiotensin-converting enzyme has been demonstrated in human skin indicating that ANG I can be converted into ANG II within the tissue itself (17). It remains to be clarified whether human skin possesses a complete local RAS and thereby the ability to also synthesize ANG II locally.

Based on our findings, it is highly likely that ANG II exerts a physiological role in skin, specifically involving the regulation of keratinocyte proliferation. It remains to be determined whether dysregulation of ANG II-receptor expression and/or dysregulation of a local synthesis of ANG II in skin may be involved in pathophysiological mechanisms underlying skin diseases associated with changes in keratinocyte proliferation such as benign (warts) or malignant (squamous cell carcinoma) tumors or psoriasis. This speculation is supported by the observation that ANG II-receptors (of unknown subtype) are markedly downregulated in human hepatocellular and hepatic metastatic colon cancer (18). Furthermore, ANG II may be involved in wound healing since in healing rat skin, the ANG II-AT_2-receptor has been shown to be upregulated (10).

The identification of functional ANG II receptors on human keratinocytes offers new insights into keratinocyte physiology. The nature of these receptors, their additional functional activities as well as their regulation need to be further explored in order to assess the role of ANG II in cutaneous pathophysiology.

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