AT_2 receptor stimulation induces generation of ceramides in PC12W cells

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Received 19 October 1998

Abstract The angiotensin AT_2 receptor has been implicated in both regeneration and apoptosis. To further investigate the molecular mechanisms leading to AT_2 receptor-induced programmed cell death in PC12W cells we studied the effects of angiotensin II (ANG II) on ceramide levels by HPTLC analysis. We could demonstrate that ANG II time- (1–10 h) and dose-dependently (10^{-8}–5×10^{-6} M) increased ceramide levels by maximally 175% but did not affect sphingomyelin degradation. The ANG II effects were mediated by AT_2 receptors since they were completely abolished by co-incubation with the AT_2 receptor antagonist, PD123177 (10^{-5} M), but not by the AT_1 receptor antagonist, losartan (10^{-5} M). These data suggest a novel signal transduction pathway to the AT_2 receptor leading to apoptosis in neuronal cells.

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Key words: Angiotensin II; AT_2 receptor; Ceramide; Apoptosis; PC12W cell

1. Introduction

The octapeptide angiotensin II (ANG II) inhibits, via its AT_2 receptor, cell growth [1–5] and promotes cell differentiation [2,6]. The AT_2 receptor-induced neurite extension in PC12W and NG108-15 cells is accompanied by differential regulation of several cytoskeletal proteins [6–8].

AT_2 receptors are highly expressed during the process of ontogenesis [9–11] in several tissues including the brain [12] but also after myocardial infarction [13] and axotomy of sciatic nerves [14]. We have recently demonstrated that these receptors can promote axonal regeneration of retinal ganglion cells in vitro and in vivo [15], providing the first direct evidence for a role of AT_2 receptors in neuroregenerative processes.

Under different experimental conditions, however, AT_2 receptor stimulation can also lead to apoptosis as has been shown, among others, in PC12W [16] and rat ovarian granulosa cells [17]. This AT_2 receptor-mediated apoptotic cell death is accompanied by activation of mitogen-activated protein kinase phosphatase-1 (MKP-1) and the subsequent dephosphorylation of Bcl-2 [18]. In human umbilical vein endothelial cells (HUVECs), AT_2 receptor stimulation leads to activation of caspase-3 (CPP-32) resulting in apoptosis [19].

The ubiquitous second messenger ceramide, one of the most hydrophobic molecules in mammalian cells, has been implicated as an important mediator of programmed cell death [20]. Ceramide can either be synthesized de novo by condensation of serine and palmitoyl-CoA [21] or be generated by sphingomyelinases (SMases) hydrolyzing sphingomyelin [22]. Ceramides, which play a role in cellular differentiation, proliferation and also apoptosis in a variety of cell types [23], have already been shown to induce apoptosis in PC12W cells [24].

To test the hypothesis that the AT_2 receptor-mediated signal transduction leading to apoptotic cell death involves the generation of ceramides, we studied the effects of ANG II on ceramide levels in PC12W cells. Our results showing an AT_2 receptor-mediated time- and dose-dependent increase in C16/C18 ceramide levels after ANG II stimulation further elucidate the signal transduction pathway of AT_2 receptors leading to programmed cell death.

2. Materials and methods

2.1. Materials

Antibiotics and Dulbecco's modified Eagle's medium were purchased from Gibco BRL (Eggenstein, Germany). Fetal calf serum was obtained from Bio Whittaker Bioproducts (Walkersville, MD, USA) and horse serum was supplied by Bioconcept GmbH (Umkirch, Germany). Collagen A was purchased from Biochrom (Berlin, Germany). ANG II was obtained from Bachem (Bubendorf, Switzerland) and sphingomyelin was supplied by Sigma (Deisenhofen, Germany). HPTLC plates were purchased from Merck (Darmstadt, Germany) and the in situ cell death detection kit was obtained from Boehringer Mannheim (Mannheim, Germany). Losartan was a generous gift from Dr. R. Smith, DuPont Merck Pharmaceutical Company (Wilmington, DE, USA), and PD123177 was generously provided by Dr. D. Taylor, Parke Davis Pharmaceutical Research (Ann Arbor, MI, USA). Synthetic ceramide (*N*-palmitoylsphinganine) was a generous gift from Dr. Bernardo (Kiel, Germany) and was synthesized as described [25]. All other reagents used were of the highest commercially available grade.

2.2. Cell culture

PC12W cells, a substrain isolated from a rat adrenal chromaffin cell tumor, were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 10% horse serum and penicillin/streptomycin (100 U/100 μg/ml). Cells were exclusively used from passages 9–18 and were grown at 37°C in a humidified atmosphere of 95% air/5% CO_2. In previous studies, these cells have been demonstrated to express predominantly AT_2 receptors [2]. However, since some AT_1 receptor binding can be seen at higher passages, the AT_1 receptor antagonist, losartan, was used in the present study to exclude any effects mediated by AT_1 receptors.

In order to examine AT_2 receptor-mediated effects on ceramide generation, PC12W cells (10^5, 2.5×10^5, 5×10^5, 10^6, 5×10^6 cells/cm^2) were first grown on collagen A-precoated culture dishes in Dulbecco's modified Eagle's medium for 4 subsequent days containing 5% fetal calf serum, 10% horse serum, penicillin/streptomycin (100 U/100 μg/ml) and 10 ng/ml nerve growth factor (NGF). Being cultivated this way, the cells, which initially do not require NGF for survival, differentiate and become dependent on NGF.

Following this procedure, cells were cultured in serum-free Dulbecco's modified Eagle's medium containing penicillin/streptomycin (100U/100 μg/ml) and a small amount of NGF (1 ng/ml) to prevent the onset of apoptosis.

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PII: S0014-5793(98)01675-5

To determine ANG II effects, cells were stimulated with ANG II (10^{-8}–5×10^{-6} M) for 1–10 h. In further experiments, cells were additionally treated with the AT_1 receptor antagonist, losartan (10^{-5} M) or the AT_2 receptor antagonist, PD123177 (10^{-5} M). Vehicle-treated cells served as negative controls.

2.3. Effects of ANG II on apoptosis in PC12W cells (in situ cell death detection kit)

To detect apoptotic cell death in PC12W cells, an in situ cell death detection kit was used according to the manufacturer's recommendations. Briefly, PC12W cells were cultured as described above and treated for 48 h with ANG II (10^{-7} M) in the presence or absence of the respective receptor antagonists (10^{-5} M). The air-dried cells were fixed with paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at room temperature. Cells were incubated for 30 min with blocking solution (0.3% H_2O_2 in methanol) followed by incubation in permeabilization solution for 2 min on ice (0.1% Triton X-100 in 0.1% sodium citrate). PC12W cells were rinsed with PBS and TUNEL reaction mixture was added. After incubation in a humidified chamber (60 min at 37°C) converter POD was added for 30 min at 37°C. Finally, cells were incubated with DAB substrate solution, rinsed and analyzed under a light microscope.

2.4. Extraction and separation of neutral lipids

After treatment, PC12W cells were harvested, centrifuged (for 5 min at $1000\times g$, 4°C) and cell pellets were stored at −70°C until lipid extraction was performed as previously described [26]. Briefly, the samples were resuspended in methanol (1 ml), transferred to glass tubes containing water (1 ml) and methanol (1.5 ml) and sonicated for 5 min. After addition of chloroform (1.25 ml), the samples were centrifuged for 10 min at $6000\times g$ and the supernatants were saved in new glass vials. The pellets were resuspended in a mixture of water (1 ml), methanol (2.5 ml) and chloroform (1.25 ml) and were centrifuged for phase separation (5 min, $4000\times g$). Water (2.5 ml) and chloroform (2.5 ml) were added to the combined supernatants. After centrifugation (5 min, $4000\times g$) the lower organic phase was transferred to new vials and the procedure was repeated once. The combined chloroform phases were dried down under nitrogen and the pellets were dissolved in chloroform/methanol (ratio 9:1).

2.5. Thin-layer chromatography (HPTLC) for ceramide detection and charring densitometry

After pre-running of HPTLC plates in a solvent system composed of chloroform/methanol (ratio 1:1), the resuspended samples were spotted onto the completely dried plates. The TLC plates were placed in an equilibrated chamber (solvent system for ceramides: dichloromethane/methanol/acetic glacial acid (100:2:5); for sphingomyelin: chloroform/methanol/acetic glacial acid/water (100:60:20:5)) and the plates were allowed to develop at room temperature.

The TLC separation was followed by visualization and quantification of phospholipids by charring of the plates with cupric reagent [26]. The plates were dried for 10 min at 180°C, cooled down to room temperature and exposed to a solution of 10% copper(II) sulfate in 8% aqueous phosphoric acid for 15 s. After drying (2 min, 110°C), charring was performed at 175°C for approx. 10 min. For quantification, the charred TLC plates were scanned by 2D laser scanner densitometry (Molecular Dynamics personal densitometer).

2.6. Statistics

Statistical comparison of data was performed using one-way analysis of variance (ANOVA) followed by an appropriate post-hoc test (Bonferroni). A probability of $P<0.05$ or less was considered significant. Further details of statistical analysis are given in the legends to the figures.

3. Results

3.1. AT_2 receptor-mediated induction of apoptosis

To establish our experimental model, the effects of ANG II on apoptotic cell death in PC12W cells (10^6 cells/cm^2) were investigated by TUNEL staining using the in situ cell death detection kit. PC12W cells cultivated in the presence of 1 ng/ml NGF served as negative control and did not undergo apoptosis (Fig. 1a). Treatment of these cells with ANG II (10^{-7} M) induced apoptosis as depicted in Fig. 1b. The ANG II-induced effects were mediated by AT_2 receptors since they were completely suppressed by pretreatment with the AT_2 receptor antagonist, PD123177 (10^{-5} M), but not affected by the AT_1 receptor antagonist, losartan (10^{-5} M) (data not shown).

3.2. Correlation of ceramide levels and PC12W cell density

PC12W cells were plated at different cell densities (10^5, 2.5×10^5, 5×10^5, 10^6, 5×10^6 cells/cm^2) in order to determine the optimal experimental cell number. An increase in ceramide levels was detected in parallel with an increase in cell density (data not shown).

Since saturation was to be avoided to allow detection of alterations in ceramide levels, a cell density of 10^6 cells/cm^2 was chosen for the investigation.

3.3. Time-dependent ANG II effects on ceramide levels

To determine the time dependence of ANG II effects on ceramide levels, PC12W cells were stimulated with ANG II (5×10^{-6} M) for 1, 2, 4, 6, 8 and 10 h. The basal ceramide content was 4.2 nmol/10^{-6} cells/cm^2. Compared to untreated cells, significantly enhanced ceramide levels were observed after 8 and 10 h (175% and 188%, respectively) whereas no changes could be detected after 1–6 h (Fig. 2).

According to these observations, all subsequent experiments were performed based on an 8 h treatment with ANG II in the absence or presence of the respective receptor antagonists.

3.4. Dose-dependent ANG II effects on ceramide levels

PC12W cells were stimulated for 8 h with increasing ANG II concentrations (10^{-8}–5×10^{-6} M) resulting in a dose-dependent increase of ceramide levels (Fig. 3). Significant ANG II effects were observed starting with a concentration of 10^{-7} M (120%). Co-incubation with ANG II at higher concentrations (10^{-6} M and 5×10^{-6} M) resulted in further increases of ceramide (142% and 175%, respectively).

Based on these studies, the following experiments were carried out for 8 h with an ANG II concentration of 10^{-6} M.

3.5. Determination of the involved angiotensin receptor subtype

In lower passages, PC12W cells have previously been demonstrated to express AT_2 but not AT_1 receptors. To confirm that the observed ANG II effects were AT_2 receptor-mediated, the cells were pretreated with either the selective AT_1 receptor antagonist, losartan (10^{-5} M), or the selective AT_2 receptor antagonist, PD123177 (10^{-5} M).

As depicted in Fig. 4, the ANG II-induced (10^{-6} M) ceramide generation (142%) was completely abolished by co-incubation with the selective AT_2 receptor antagonist PD123177 in the absence (109%) or presence of the AT_1 receptor antagonist, losartan (102%). The AT_1 receptor antagonist, losartan, also did not affect these ANG II effects by itself (152.5%) indicating that the ANG II-induced ceramide generation is due to an AT_2 receptor activation.

3.6. Sphingomyelin determination

To investigate whether the observed generation of ceramide is due to degradation of sphingomyelin by means of sphingomyelinases or de novo synthesis, PC12W cells were harvested

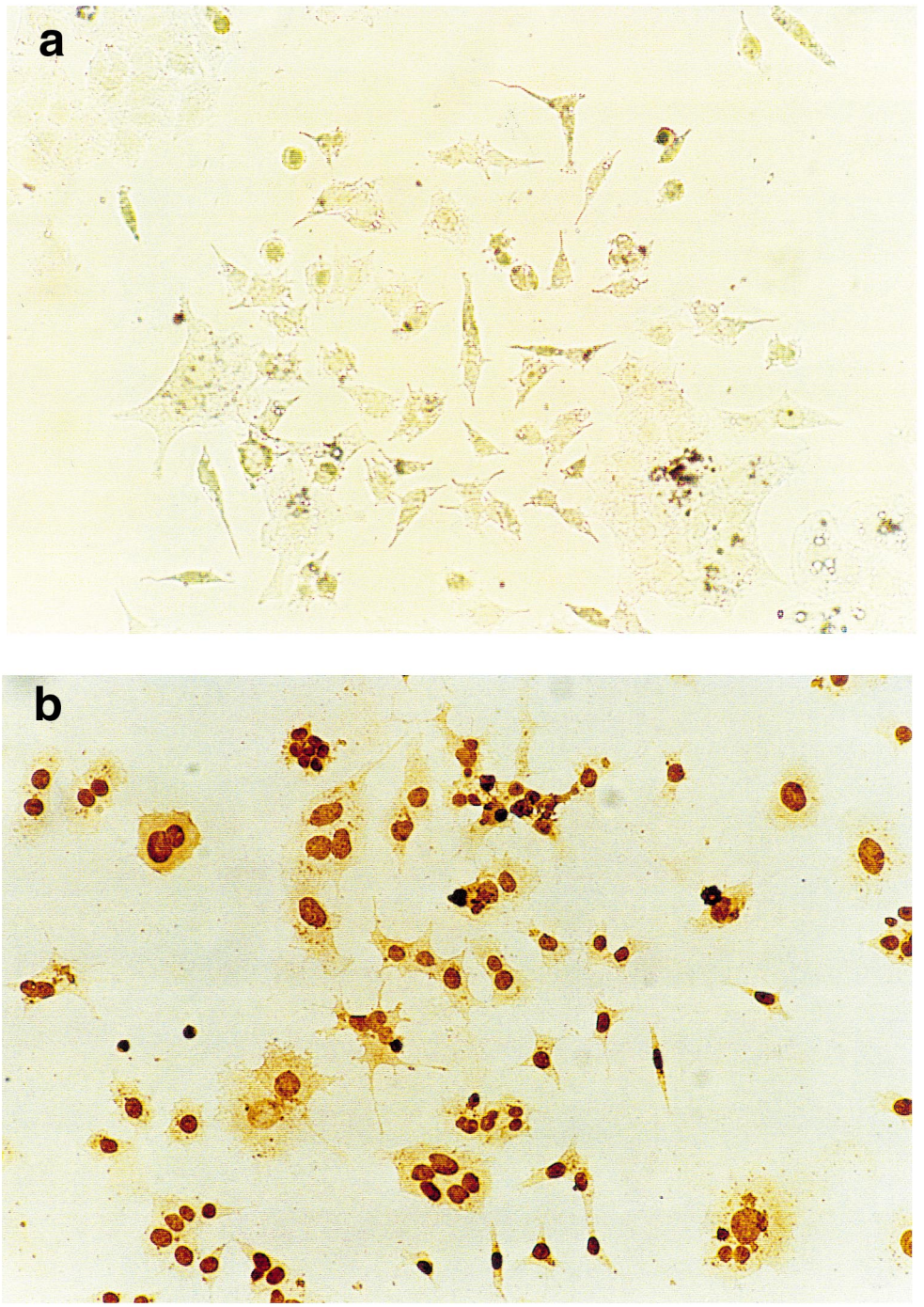

Fig. 1. AT_2 receptor stimulation induced apoptosis in PC12W cells as evidenced by internucleosomal DNA strand breaks and detected by TUNEL staining. Whereas no apoptosis was observed in cells cultivated in the presence of NGF (1 ng/ml) (a), treatment with ANG II (10^{-7} M) for 48 h (b) resulted in programmed cell death. These ANG II-induced effects were completely abolished by co-incubation with the selective AT_2 receptor antagonist, PD 123177 (10^{-5} M), but not affected by the AT_1 receptor antagonist, losartan (10^{-5} M).

after treatment and divided into two fractions. This procedure allowed the determination of both ceramides and sphingomyelin, from the same sample by using two different solvents for HPTLC separation (see Section 2). The sphingomyelin concentration did not change after an 8 h stimulation with ANG II at different doses (10^{-8}–5×10^{-6} M) compared to untreated control cells (data not shown).

4. Discussion

Previous studies have demonstrated that ANG II is capable of inducing apoptosis via AT_2 receptors. The mechanism of action involves e.g. activation of MKP-1 and subsequent dephosphorylation of Bcl-2 in PC12W cells [18] but also stimulation of CPP-32 in HUVECs [19]. Since the second messen-

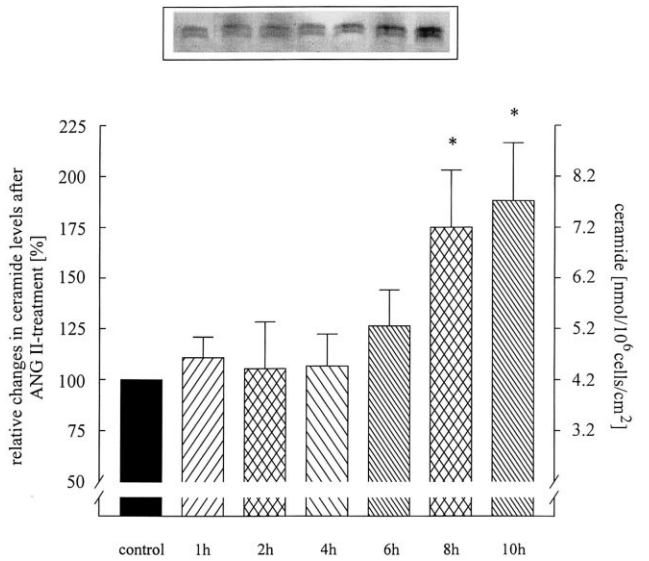

Fig. 2. Stimulation of PC12W cells with ANG II (5×10^{-6} M) evoked time-dependent increases of ceramide levels. Significant effects were observed after 8 and 10 h whereas no changes were detected between 1 and 6 h ($n=4$, mean ± S.E.M.). *$P<0.05$ compared to respective untreated cells.

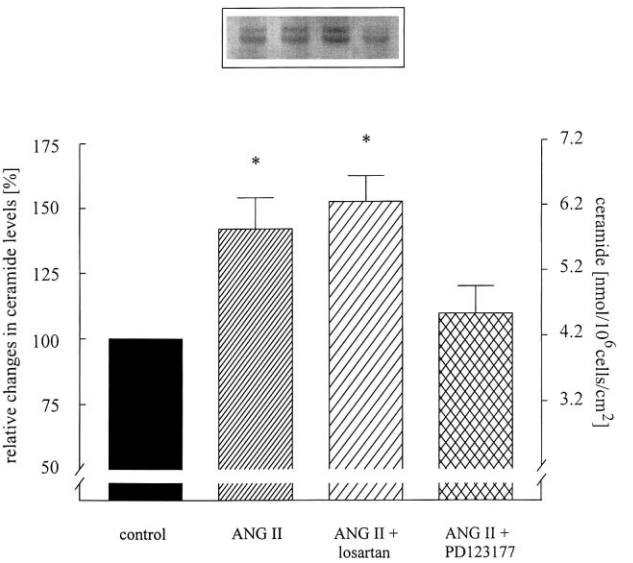

Fig. 4. Co-stimulation of PC12W cells with ANG II (10^{-6} M) and the selective AT_2 receptor antagonist, PD 123177 (10^{-5} M), entirely abolished the ANG II-induced ceramide synthesis whereas it was not affected by the AT_1 receptor antagonist, losartan (10^{-5} M) ($n=4$, mean ± S.E.M.). *$P<0.05$ compared to respective untreated cells.

ger, ceramide, represents a possible activator of CPP-32 we were prompted to investigate possible AT_2 receptor-mediated effects on ceramide levels.

To confirm previous studies and to establish our experimental model, the apoptotic effects of ANG II in PC12W cells were investigated by TUNEL staining, and, under the conditions described (see Section 2), it could be demonstrated that AT_2 receptor stimulation mediates apoptosis in these cells (Fig. 1).

The present study now demonstrates that under identical experimental conditions, AT_2 receptor stimulation induces generation of the lipid second messenger ceramide ascribing a novel signal transduction pathway to these receptors. The ANG II effects were dose- and time-dependent and were entirely suppressed by co-incubation with the selective AT_2 receptor antagonist, PD123177, but not with the AT_1 receptor antagonist, losartan. The ANG II effects did not include alterations in sphingomyelin levels suggesting a lack of activation of SMases and, thus, pointing to an AT_2 receptor-mediated de novo synthesis of ceramide.

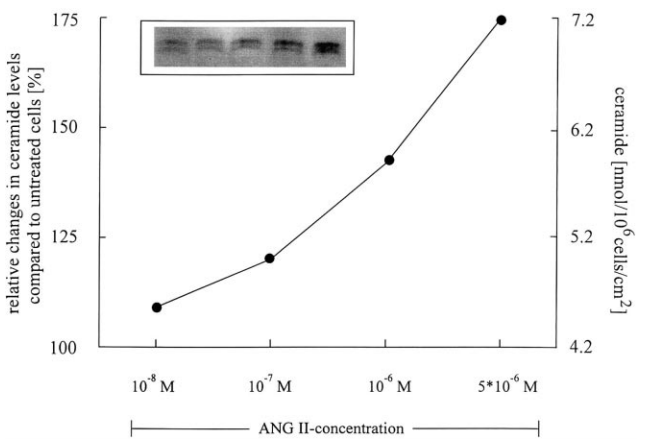

Fig. 3. Treatment of PC12W cells with increasing ANG II concentrations (10^{-8}–5×10^{-6} M) resulted in dose-dependent increases in ceramide generation (data are from single experiments representative of four independent experiments, mean ± S.E.M.). *$P<0.05$ compared to respective untreated cells.

A number of observations support the view that ceramide plays an important role in mediating apoptosis. It has for instance been shown that environmental stresses such as UV radiation or oxidative stress induce ceramide generation [27,28]. Ceramide not only activates the SAPK/JNK cascade but also acts on mitochondrial permeability leading to activation of caspases and, finally, to apoptosis (for review see [23]). Although numerous questions remain to be answered regarding the precise role of ceramide in apoptotic signalling, the finding of an AT_2 receptor-mediated ceramide generation in PC12W cells connects this receptor to important apoptotic pathways which have to be elucidated in the future.

Although it was not investigated in the present study whether ANG II leads to activation of SMases or ceramide synthase, the time course of ceramide generation suggests an ANG II-induced de novo synthesis. In contrast to SMases which are activated within minutes, activation of ceramide synthase requires hours. In addition, the lack of AT_2 receptor-mediated sphingomyelin degradation in PC12W cells supports a role of ceramide synthase since SMase activity involves sphingomyelin breakdown. However, it should be noted that sphingomyelin levels are high compared to ceramide levels and that minor changes in the sphingomyelin content might not be detected by the assay used. Further investigations will, thus, be necessary to unambiguously answer this question.

Taken together, we could show for the first time that the AT_2 receptor-mediated apoptosis in PC12W cells involves ceramide generation, thus attributing a novel signal transduction pathway to these receptors. Future investigations have to elucidate whether ANG II actions involve de novo synthesis of ceramide or sphingomyelin breakdown.

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