Angiotensin II Causes Formation of Platelet Activating Factor in Cultured Rat Mesangial Cells

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Angiotensin II may contribute to the progression of renal glomerular diseases. Beneficial effects of converting enzyme inhibition in models of renal disease are, however, not always explicable by hemodynamic consequences of angiotensin II inhibition. Angiotensin increases intracellular calcium in glomerular mesangial cells and activates phospholipase A₂, factors required for the formation of the lipid mediator of inflammation platelet activating factor (PAF). We therefore examined whether angiotensin II could stimulate PAF production in cultured rat mesangial cells. During a 15-minute incubation angiotensin II caused formation of PAF in a dose-dependent manner with a threshold around 10⁻⁶ M. In four experiments PAF formation in response to angiotensin II (10⁻⁴ M) occurred within 5 minutes and was 29±8 pmol PAF/mg protein. The amount of PAF detected then declined to 9±2 and 13±3 pmol after 15 and 30 minutes of incubation with angiotensin II. More than 90% of the PAF remained cell-associated. The PAF formation was confirmed by negative ion chemical ionization mode of mass spectrometry. A single species of PAF was detected and identified as hexadecyl PAF. We speculate that part of the detrimental effects of angiotensin II in progressive renal disease may relate to PAF formation. The PAF generated may in turn influence glomerular function, platelets, and eicosanoid synthesis, all factors implicated in renal disease. Furthermore, we speculate that angiotensin II-induced PAF formation may contribute to microvascular pathology in general. (Circulation Research 1989;64:1224–1229)

Angiotensin II may contribute to the progression of chronic renal disease. It has been proposed that this is because angiotensin increases intraglomerular capillary pressure, which in turn causes glomerular damage and sclerosis.¹–³ This hypothesis is supported by studies showing beneficial effects of converting enzyme inhibitors on the progression of various forms of experimental renal disease.¹–⁴ Surprisingly, however, in the puromycin aminonucleoside and adriamycin models, converting enzyme inhibition with lowering of angiotensin II did not lower intraglomerular pressure, yet slowed the development of renal glomerular disease.³ Therefore, alternative explanations for the beneficial effects of converting enzyme inhibitors may have to be considered. These include changes in eicosanoid production, in the immune system, or in vascular smooth muscle and mesangial cell proliferation and matrix production.²,³,⁵–⁶ These factors could be influenced by the reduction in angiotensin II or by the converting enzyme inhibitor itself. In the present study, we examined whether some of the detrimental effects of angiotensin II and the beneficial effects of converting enzyme inhibitors may also relate to the ability of angiotensin II to stimulate the production of platelet activating factor (PAF). Angiotensin II activates phospholipase A₂ in mesangial cells,⁷,⁸ and this in general is also the initial step in the formation of PAF.⁹ PAF is a lipid mediator that activates platelets and leukocytes and enhances production of eicosanoids, including thromboxane, in a number of cells, including glomerular mesangial cells.⁹,¹⁰ PAF can cause mesangial shape change consistent with contraction and can be generated in the kidney, specifically by mesangial cells.¹¹ PAF affects glomerular filtration and contributes to glomerular pathology in models of renal disease.⁹,¹⁰ We therefore examined the ability of angiotensin II to stimulate PAF production in cultured rat mesangial cells.
Materials and Methods

Culture of Glomerular Mesangial Cells

The culture of rat mesangial cells was carried out as previously described.11 After 3 weeks in primary culture, the mesangial cells were detached from the flask by adding a 0.25% trypsin-EDTA solution (GIBCO Laboratories, Grand Island, New York) and transferred to a plastic flask containing 5 ml growth medium including RPMI 1640 (GIBCO Lab), 10% fetal calf serum (GIBCO Lab), penicillin (50 units/ml), and streptomycin sulfate (50 μg/ml) (GIBCO Lab). Culture flasks were kept in 95% air-5% CO2 environment at 37°C. Within 7–10 days the mesangial cells grew to confluence. Subsequent subcultures were at 7–10 day intervals by the above method. Only third-passage cells were used for our experiments. The mesangial cells used represent an apparently uniform cell population as evaluated by the following criteria: 1) morphology, 2) uniform fluorescence with fluorescein-labeled phallacidin for F-actin, 3) uniformly positive immunofluorescence with the monoclonal antibody Thy 1.1, 4) pattern of binding for specific lectins, and 5) absence of immunofluorescence with factor VIII antibody, with antibodies for la, or common rat leucocyte antigen.12

Determination of PAF Generation

For PAF generation mesangial cells (third subcultures) were grown to confluence in six-well plates (Inter-Med NUNC, Roshide, Denmark). After removal of culture medium, the cells were washed three times with Krebs-Ringer buffer and left to equilibrate for 30 minutes in Krebs-Ringer buffer at 37°C. Angiotensin II or buffer was then added to wells at the concentrations indicated and incubations continued for the times indicated in “Results.” Incubations were terminated by removal of the buffer into cold methanol, the cells were washed once with cold buffer that was added to the buffer-methanol mixture. Two milliliters of cold methanol were added to the cells, and the cells were scraped with a rubber policeman and collected into a glass extraction tube. The wells were washed with an additional 2 ml methanol, which was added to the cell extract. Both incubation buffer and cell extract were spiked with 2,000 counts/min of [3H]PAF (179 mCi/mmol; Amersham, Arlington Heights, Illinois) for monitoring of recovery. Incubation buffer and cells were extracted, purified, and assayed separately for PAF.

Methods of Lipid Extraction

All samples (buffer or cell fractions) in methanol were homogenized in a Polytron (Brinkmann Instruments, Westbury, New York) on power output setting #6 for 30 seconds, then centrifuged at 1,300g for 5 minutes. The pellet was saved for protein determination by Biorad kit. The supernate samples were extracted by the methods of Bligh and Dyer13 as described previously in detail.11 Extracted samples were further purified on thin layer chromatography (TLC) plates (LK6D silica gel plates, Whatman, Inc, Clifton, New Jersey) that had previously been heated to 180°C for 1 hour, allowed to cool, and run in a system of chloroform:methanol:water (165:35:6). [3H]-labeled PAF, Lyso-PAF, and phosphatidylcholine standards were run in other lanes of the TLC plates. Positions of the [3H] standards were visualized by autoradiography and areas corresponding to the [3H]PAF standard were scraped off the sample lanes and extracted from the TLC silica by incubation with methanol:water (95:5).

Bioassay for PAF Activity

For PAF bioassay we used aggregation of rabbit platelets, as previously described.11,14 Platelet aggregation was determined in a four-channel aggregometer (Platelet Aggregation Profiler-4, Bio-Data, Hatboro, Pennsylvania) using 0.45 ml of the platelet suspension, and adding standards or samples resuspended in the Tris-Tyrodes buffer with calcium and magnesium. A standard curve of PAF bioactivity was obtained using this method, from 10^{-10} to 10^{-7} M PAF. In some experiments, half of the sample was subjected to alkaline hydrolysis (15 minutes in 0.5 M KOH at 45°C) followed by reextraction and bioassay, a treatment that destroys the platelet aggregating ability of standard PAF.9,10 Results derived from the PAF standard curve were expressed as picomoles PAF bioactivity generated per milligrams mesangial cell protein.

Mass Spectrometry for PAF

The lipid extracts of experiments that tested positive for PAF bioactivity were further analyzed as the pentafluorobenzoyl ester by negative ion chemical ionization mass spectrometry.14,15 By this method, the hexadecyl PAF species yields an ion peak at mass per charge (m/z) 552, the octadecyl PAF species at m/z 580 and the C18:1 species at m/z 578. Approximately, 8 ng deuterated PAF (D3) was added to each sample before analysis, as this gives an m/z ion at 555, clearly separated from the hexadecyl PAF, and allows standardization.14,15

Results

No bioactive PAF was detectable in either cells or incubation buffer under control conditions, as previously reported by us.11 Angiotensin II caused dose-dependent generation of bioactive PAF (Figure 1) with a threshold between 10^{-10} and 10^{-9} M angiotensin II. With 10^{-8} M angiotensin II, mesangial cells had generated a total of 9.6±2.3 pmol PAF/mg cell protein during a 15-minute incubation. This compares with 27±8 pmol PAF generated during incubations carried out in parallel with 10^{-3} M A23187 (mean±SEM of five experiments). As shown in Figure 1 the majority of the PAF generated in response to angiotensin II remained cell-associated with only a small percentage of the PAF detectable in the incubation buffer after stimulation with 10^{-8} M A23187.
angiotensin II. No bioactive PAF was detectable in the incubation buffer after exposure to lower (10^{-11}-10^{-9} M) concentrations of angiotensin II.

Next we examined the time course of the angiotensin II-induced PAF generation (Figure 2). A 5-minute exposure to angiotensin II (10^{-8} M) resulted in the production of 29 pmol PAF/mg cell protein (combined extracts of both cells and buffer). The amount of PAF detected after 15 minutes of stimulation with angiotensin II was less than after 5 minutes but remained stable after 30 minutes with angiotensin II (Figure 2). The distribution of intracellular to extracellular PAF appeared unchanged over this time course, with about 10% of the total PAF being detectable in the incubation buffer and 90% remaining cell-associated (Figure 2). Authenticity of the bioactive PAF material with PAF was established by loss of bioactivity of the material after alkaline hydrolysis9 and by mass spectrometry. For the latter determinations, lipids were extracted separately from both mesangial cells and the buffer after 5-minute incubations with angiotensin II (10^{-8} M). After purification by TLC and high-performance liquid chromatography, the samples were derivatized and analyzed as the pentafluorobenzoyl esters together with deuterated standards in the negative ion chemical ionization mode of mass spectrometry.14,15 This allows both quantitation of the amount of PAF present by comparison with the deuterated standard as well as identification of the molecular species of PAF present.14,15 Figure 3 shows the mass fragmentograms of PAF detected in cell and buffer extracts and of the deuterated standards. Extracts of cells and incubation buffer showed an anion with an m/z of 552, which corresponds to the hexadecyl PAF species. The corresponding deuterated standards have the m/z of 555. The doublet peaks for both the sample and the deuterated PAF standards are due to isomerization that occurs during the derivatization process. By comparison with the deuterated PAF standards, mesangial cells stimulated with angiotensin II for 5 minutes had generated 60 pmol PAF/mg cell protein of which only 2 pmol were found in the incubation buffer. These quantitative results essentially confirm those obtained by bioassay for PAF.

Discussion

Our present results demonstrate that angiotensin II causes production of PAF in mesangial cells. This occurs in a dose-dependent manner and with a rapid time course. The PAF generated is exclusively of the hexadecyl species, and more than 90% of the PAF generated remains cell-associated.

We have previously shown that angiotensin II activates both phospholipase C and A2 in mesangial
Figure 3. Mass fragmentograms of platelet activating factor (PAF) synthesized by mesangial cells during incubation with angiotensin II (10⁻⁸ M) for 5 minutes. PAF was extracted separately from the incubation buffer and the cells, purified, derivatized, and analyzed by gas chromatography-negative ion chemical ionization mass spectrometry as described in "Materials and Methods." The mass per charge (m/z) ions represent the molecular anion of the PAF species present, i.e., m/z 552 corresponding to the hexadecyl PAF species found and m/z 555 to the deuterated standard added (8 ng). There are two peaks in each ion channel because of isomerization of the diglycerides occurring during derivatization. Please note the different scales for the relative responses in the four ion channels recorded on the ordinate that are due to the different sensitivities adjusted to the amounts of PAF in the samples.
cells. The former results in the generation of inositol trisphosphate and diacylglycerol, which in turn increase intracellular calcium and activate protein kinase C. Activation of phospholipase A\textsubscript{2} appears to be responsible for the release of arachidonic acid and prostaglandin formation. Activation of phospholipase A\textsubscript{2} is also considered to be the initial step in the formation of PAF, yielding the inactive precursor lyso-PAF from 1-alkyl-2-acetylglucero phosphatidylycholine. Formation of PAF from lyso-PAF requires the additional calcium-dependent activation of a specific acetyltransferase. As angiotensin II causes activation of phospholipase A\textsubscript{2} and increases intracellular calcium, we speculated that angiotensin II might also result in PAF formation, a speculation substantiated by our findings. These results are also consistent with findings in other systems showing a close correlation between arachidonic acid metabolism and PAF formation. In this context, it is of interest to note the close similarity in the dose response and time course of angiotensin II-induced prostaglandin formation observed in our previous studies and the present data on PAF formation. The rapid time course of PAF generation in response to angiotensin II would correspond to the initial increase in intracellular calcium and phospholipase A\textsubscript{2} activation. The subsequent moderate decline in the amount of PAF found after 15 and 30 minutes of incubation with angiotensin II may represent a combination of tachyphylaxis to angiotensin II and metabolism of PAF. Intracellularly PAF would be reconverted via lyso-PAF to 1-alkyl-2-acetylglucero phosphatidylycholine whereas extracellular PAF would be rapidly inactivated by a specific ectoenzymatic acetylhydrolase.

The PAF generated in response to angiotensin II was identified by negative ion chemical ionization mode of mass spectrometry as being exclusively of the hexadecyl species, that is, containing a 16 carbon-saturated alkyl group in position one. This finding implies a high degree of specificity of PAF formation in mesangial cells and confirms our previous findings of PAF formation in response to the calcium ionophore A23187 and to immune complexes. The use of mass spectrometry in the negative ion chemical ionization mode not only allowed identification of the PAF but also its quantitation. Quantification by this method yielded values for PAF that were in the same range though slightly exceeding those obtained by the platelet aggregation bioassay, probably reflecting the limits of the bioassay. Evaluation of the distribution of PAF released into the incubation buffer versus the PAF that remained cell-associated also yielded comparable results by either mass spectrometry or bioassay methods. Over 90% of the PAF remained intracellular, but up to 10% was found in the incubation buffer. These findings are similar to those reported for PAF generated by endothelial cells and to our previous observations of PAF formation by mesangial cells exposed to immune complexes. At present, the physiological significance of intracellular PAF remains unclear. It has been proposed that PAF could be incorporated into the cellular lipid membrane resulting in surface changes that could cause adherence of leukocytes and perhaps platelets. Irrespective of potential effects of intracellular PAF, the PAF released by mesangial cells could influence both the cells themselves and the adjacent endothelium and cells circulating through the glomerular capillary. Intrarenal PAF administration has been shown to reduce glomerular filtration rate and alter the glomerular ultrafiltration and permeability characteristics.

The fact that angiotensin II can cause PAF generation by mesangial cells invites a number of speculations. For example, not all beneficial effects of inhibition of angiotensin II formation with converting enzyme inhibitors on progression of renal disease can be attributed to hemodynamic effects of angiotensin II. Perhaps some of the unexplained salutary responses of converting enzyme inhibition may relate to concomitant decline of PAF generation as angiotensin II formation is reduced. Hypothetically PAF could even establish a link between the detrimental influences of angiotensin II on the one hand and platelets and thromboxane on the other hand in the progression of renal disease. Extrapolation of the 20 pmol PAF generated by 1 mg cellular protein of cultured rat mesangial cells to an in vivo situation could indicate a potential PAF concentration of about 20 pmol/10 mg wet tissue wt or a range of 10^{-9} M PAF. Even if only 10% of the PAF is released from the cells, this would be sufficient to activate, for example, human platelets with a threshold of 10^{-9} M. PAF activates platelets resulting amongst others in the generation of thromboxane and release of platelet-derived growth factor, both of which have been implicated in the development of glomerular sclerosis. Furthermore, these speculations may apply not only to angiotensin II and the glomerulus, but to angiotensin II-associated microvascular pathology in general, as the mesangial cell represents a modified vascular smooth muscle cell. The availability of a number of potent PAF antagonists should allow experimental evaluation of these hypotheses.

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References


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