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Angiotensin II induces superoxide anion production by mesangial cells

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Angiotensin II induces superoxide anion production by mesangial cells.

Background. The recognized role of angiotensin II (Ang II) in the pathogenesis of the progression of renal disease cannot be solely attributed to Ang II's hemodynamic effects. Indeed, growth stimulating signals driven by Ang II promote mesangial cell (MC) hypertrophy and extracellular matrix production, prominent features of progressive glomerular injury. Superoxide anion (O_2^-) avidly interacts with nitric oxide, an endogenous vasodilator that inhibits growth factor stimulated MC growth and matrix production. In addition, O_2^- acting as an intracellular signal is linked to growth related responses such as activation of mitogen activated protein (MAP) kinases. The studies reported herein were designed to investigate: (*a*) whether Ang II induces MC O_2^- production and (*b*) if increased O_2^- production elicits growth responses in MC.

Methods. MC were exposed to Ang II for 24 or 48 hours. In some experiments, in addition to Ang II, MC were exposed to: diphenylenieodonium (DPI), an inhibitor of the flavin containing NADH/NADPH oxidase; losartan (LOS), an Ang II type 1 (AT1) receptor blocker; PD 98059, a MAP kinases inhibitor; the protein kinase C inhibitors Calphostin C or H-7; and the tyrosine kinase inhibitors, herbymycin A or genistein.

Results. Ang II (10^{-5} M to 10^{-8} M) dose dependently increased MC O_2^- production up to 125% above control (ED 50 5×10^{-7} M). LOS as well as DPI, and the PKC inhibitors blocked Ang II stimulated MC O_2^-production. Ang II dose dependently increased MC ^{3}H-leucine incorporation, and MC protein content, two markers of MC hypertrophy, as well as ^{3}H-thymidine incorporation, a marker of MC hyperplasia. PD98059, a specific inhibitor of MAP kinases prevented Ang II induced MC hypertrophy. Moreover, LOS, DPI, and the PKC inhibitors each independently inhibited MC ^{3}H-leucine incorporation, thereby establishing the specificity of Ang II induced O_2^- in driving MC hypertrophy.

Conclusions. The current studies demonstrate a previously unrecognized link between Ang II and MC O_2^- production that may participate in the pathophysiology of progressive renal disease by concomitantly affecting the hemodynamics of the glomerular microcirculation as well as growth related responses of MC to injury.

The glomerular mesangium, which is composed of mesangial cells (MC) and extracellular matrix, occupies the centrolobular area of the glomerulus, and also extends outward a short distance into the capillary wall [1]. The endothelial cell lining of the glomerulus is strategically situated at the interface between the blood and the mesangium, without interposition of the glomerular basement membrane. In this location, endothelial cells serve as direct sensors and transducers of physical and biochemical signals within the microenvironment of the glomerulus [1]. Due to the direct juxtaposition of endothelial cells and MC, products synthesized by one of these cells can reach the other in high concentrations with minimal inactivation or dilution by circulating plasma [2]. In addition, the intrarenal concentration of Ang II is up to 1000-fold higher than in the serum [3]. MC contain actin-myosin filaments that alter their contractile state in response to vasoactive agents acting in an autocrine and/or paracrine fashion [4]. Thus, Ang II, eicosanoids, endothelin-1 and nitric oxide (NO) synthesized and released locally, modulate the glomerular microcirculation during physiologic and pathologic conditions by acting upon the mesangium as well as the afferent and efferent arterioles [5]. Moreover, similar to what occurs in systemic vascular beds, these vasoactive agents may also affect the response of glomerular cells to injury and promote architectural changes, such as mesangial hypertrophy and/or hyperplasia [6, 7], as well as increased MC matrix production [8]. In this context Ang II modulates MC synthesis of growth factors including platelet-derived growth factor (PDGF) [9] and transforming growth factor-β (TGF-β) [8], which have been incriminated in the pathological remodeling of the glomerulus in response to injury [10].

Upregulation of the local tissue renin angiotensin system (RAS) in response to injury has been shown in a variety of

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renal diseases [11]. Furthermore, in many patients with chronic renal failure, blockade of the RAS with angiotensin converting enzyme (ACE) inhibitors slows the decline of renal function [12–15]. These effects of inhibition of the RAS are in addition to, but independent of blood pressure control [13, 15]. These clinical studies as well as a large number of *in vivo* [16, 17] and *in vitro* [8, 18] experimental studies support the notion that the nonhemodynamic effects of Ang II are pathophysiologically important in progressive glomerular injury. Thus, further understanding of these mechanisms is necessary for the development of new therapeutic strategies.

MC are endowed with a NADH/NADPH oxidase, a flavin containing oxidase, whose components are antigenically related but not identical to that of phagocytes [19, 20]. Recent studies demonstrated that Ang II activates NADH/NADPH oxidase in vascular smooth muscle cells resulting in synthesis of superoxide anion (O_2^-) [21, 22]. The vascular smooth muscle cell NADH/NADPH oxidase is structurally different from that present in leukocytes; in addition it produces quantitatively less O_2^-, but for a much longer period of time [21]. O_2^- is a powerful intracellular signal for the activation of responses linked to mitogen activated protein (MAP) kinases-driven cell growth [23], particularly cell hypertrophy [21]. Increased O_2^- in the extracellular milieu, given its great affinity for NO, rapidly interacts with this molecule and results in NO inactivation and/or production of toxic peroxynitrite [24]. Within the glomerulus a decrease in NO bioactivity: (*a*) would curtail the capacity of NO to counteract Ang II actions on mesangial contraction and arteriolar tone, and (*b*) would reduce the homeostatic role of NO in antagonizing Ang II driven growth related processes affecting MC and extracellular matrix as well as preventing platelet and leukocyte adhesion to the endothelium [5]. Heretofore no data are available regarding whether Ang II can activate MC NADH/NADPH oxidase and promote O_2^- synthesis.

Given the potential pathophysiologic and therapeutic implications linked to increased MC O_2^- production within the glomerular microcirculation is that we designed studies aimed at elucidating whether Ang II activates MC NADH/NADPH oxidase, as well as the mechanism(s) involved and its relationship with MC hypertrophy and/or hyperplasia.

METHODS

Materials

Ang II, lucigenin, xanthine, xanthine oxidase, herbimycin A, genistein, H-7 and calphostin C were purchased form Sigma Chemical Co. (St. Louis, MO, USA); ^{3}H-leucine and ^{3}H-thymidine were purchased from Amersham Life Science (Little Chalfont, UK); diphenylenieodinium (DPI) was purchased from Toronto Research Chemicals (Toronto, Canada). LOS was kindly provided by Dupont Merck (Wilmington, DE, USA). PD98059 was purchased from Park Davis (Ann Arbor, MI, USA).

Rat mesangial cells culture

Primary MC cultures were prepared from male Sprague-Dawley rats as previously described [25]. Cells were grown using techniques that yield macrophage-free homogenous cultures, as previously characterized [26]. Cells were grown in RPMI-1640 media (Gibco Laboratories, Grand Island, NY, USA) supplemented with 17% heat-inactivated fetal calf serum (FCS; Hyclone Laboratories, Logan, UT, USA), 100 U ml penicillin, 100 μg/ml streptomycin, 15 mM HEPES, 10 ml/liter insulin-transferrin-selenium supplement (Sigma Chemical Co.). Cells were passed by trypsinization when confluent, and used between the third and ninth passages. Cells were grown in either six-well dishes or 75 cm^2 flasks (see below).

Measurement of O_2^- anion production

O_2^- production was determined by chemiluminescence of lucigenin, an acridylium dinitrate compound that emits light on reduction and interaction with O_2^- [27]. Briefly, MC grown in 75 cm^2 flasks were made quiescent in RPMI 1640 with 0.5% FCS for 72 hours and exposed to Ang II (10^{-5} to 10^{-8} M) for five hours. At the end of the incubation period, MC were trypsinized, pelleted by centrifugation, resuspended in 2 ml Krebs buffer and kept on ice until use. A 50 μl aliquot was saved for protein determination. To measure superoxide production cells suspensions were incubated at room temperature for three minutes and then added to a scintillation vial containing dark adapted lucigenin (250 μM) in 2 ml 100 mM phosphate buffer (pH 7.4). Photon emission was measured every minute for 10 minutes in a scintillation counter in the out-of-coincidence mode. A buffer blank was subtracted from each reading and superoxide production calculated by comparison with a standard curve generated using xanthine/xanthine oxidase as described by Ohara et al [27]. Protein content was determined by the Bio-Rad method (Hercules, CA, USA).

^{3}H leucine incorporation

MC protein synthesis measured by ^{3}H-leucine incorporation was used as an index of MC hypertrophy. MC cells were grown in six well dishes, made quiescent in RPMI 1640 with 0.5% FCS for 72 hours. MC were then incubated for 24 or 48 hours in the presence or absence of Ang II (10^{-8} to 10^{-5} M). Six hours before harvesting MC were pulsed with ^{3}H-leucine 2 μCi/ml. At the end of this incubation period, cells were washed three times with phosphate buffered saline (PBS) and solubilized overnight with 1.5 ml sodium dodecyl sulfate (SDS) 0.1%. The content of two wells was pooled and transferred to a plastic tube containing 60 μl 10% bovine serum albumin. Proteins were precipitated with 300 μl 20% trichloroacetic acid (TCA) and left overnight at 4°C. Samples were then spun at

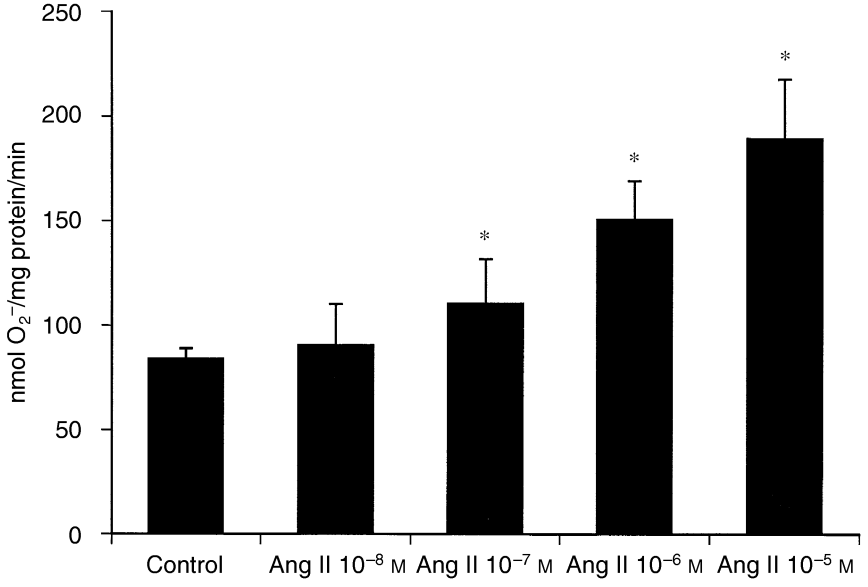

Fig. 1. Angiotensin II (Ang II) induces superoxide (O_2^-) production in a dose dependent manner. Mesangial cells (MC) were exposed to Ang II in the indicated concentrations for five hours. MC O_2^- production was estimated 10 minutes after exposure to lucigenin. Data represent the mean ± SEM of 3 to 6 experiments by duplicate. *$P < 0.05$ versus control.

2,000 g × 30 minutes, the supernatant discarded and pellet resuspended in 0.5 N sodium hydroxide (NaOH). Duplicate aliquots (0.5 ml) were removed and counted in a scintillation counter. Results were expressed as cpm/million cells.

Mesangial cell protein content

Mesangial cell total protein content was used as additional index of MC hypertrophy. MC were grown in six well dishes, made quiescent in RPMI 1640 with 0.5% FCS for 72 hours. MC were then incubated for 48 hours in the presence or abscence of Ang II (10^{-5} M). At the end of the incubation period MC were trypsinized, counted, and MC total protein content determined by Bio-Rad (Hercules, CA, USA). Results were expressed as μg protein/million cells.

^{3}H-thymidine incorporation

^{3}H-thymidine incorporation by MC was used as an index of MC proliferation. MC were grown in six well dishes, and made quiescent in RPMI 1640–0.5% FCS for 72 hours. MC were then incubated for 24 or 48 hours in the presence or absence of Ang II (10^{-5} M). Four hours before harvesting, cells were pulsed with ^{3}H-thymidine 1 μCi/ml. At the end of this incubation period, cells were washed three times with PBS. Protein precipitated with 1 ml TCA 10% for five minutes and solubilized in 1 ml 0.5 N NaOH/0.1% SDS. Duplicate aliquots (0.5 ml) were removed, neutralized with 50 μl 6 N hydrochloric acid and counted in a liquid scintillation counter. Results were expressed as counts/million cells.

Statistical analysis

Data are expressed as mean ± SEM. For statistical comparison involving two groups, an unpaired Student's *t*-test was used, while for comparison involving more than two groups, ANOVA using the Statview 521 statistical program was employed (Abacus Concepts, Inc., Berkeley, CA, USA). Significance was considered present when $P < 0.05$.

RESULTS

Angiotensin II induces superoxide production by MC

Mesangial cells exposed to Ang II (10^{-8} M to 10^{-5} M) for five hours significantly increased (O_2^-) production in a dose dependent manner (Fig. 1). This increased O_2^- was fully blocked with losartan (LOS) 10^{-6} M, suggesting that the effects of Ang II on superoxide production are mediated through the AT1 receptor: Control, 84.01 ± 5 nmol O_2^-/mg protein/min; Ang II 10^{-5} M, 189.66 ± 28.4 nmol O_2^-/mg protein/min; Ang II 10-5 M + LOS 10^{-6} M, 80.71 ± 24.24 nmol O_2^-/mg protein/min; $P < 0.05$, Ang II versus control and Ang II+LOS (Fig. 2).

To determine whether mesangial NADH/NADPH oxidase activation was responsible for the Ang II-induced superoxide production, MC were exposed for one hour to the flavin containing oxidase inhibitor diphenyleneiodonium (DPI) 10^{-5} M, prior to Ang II stimulation. DPI inhibited Ang II-induced superoxide production by MC (Ang II 10^{-5} M, 189.66 ± 28.4 nmol O_2^-/mg protein/min vs. Ang II 10^{-5} M + DPI 10^{-5} M, 31.8 ± 17.07 nmol O_2^-/mg protein/min; $P < 0.05$). As shown in Figure 2, DPI also inhibited basal O_2^- production in unstimulated MC. Thus, Ang II in a dose dependent manner induced O_2^- production by MC, the process involved NADH/NADPH oxidase activation, and was mediated through AT1 receptor activation (Fig. 2).

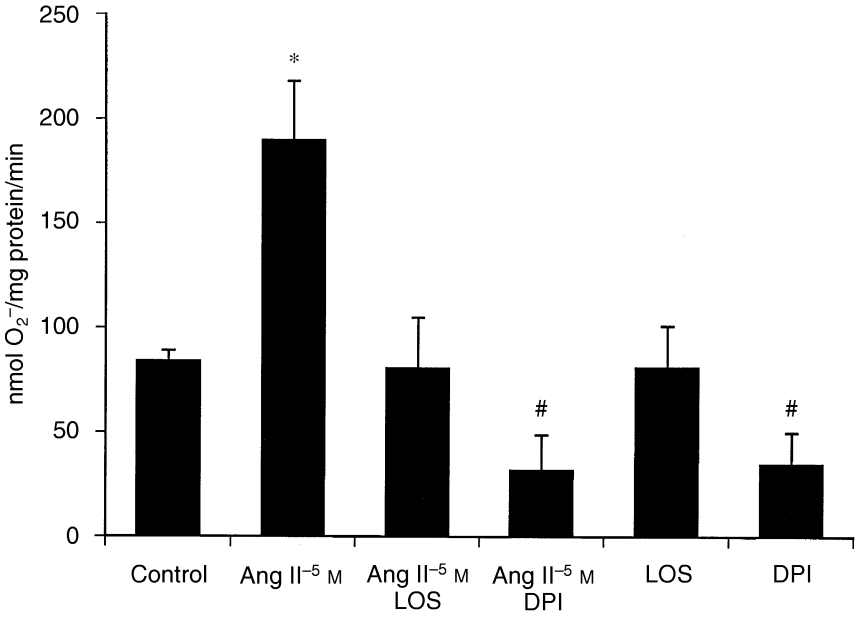

Fig. 2. Effect of diphenylenieodonium (DPI) and losartan (LOS) on antiotensin II (Ang II) induced superoxide (O_2^-) generation by mesangial cells (MC). MC were exposed to the inhibitor of flavin containing oxidases diphenyleneiodonium (DPI) or the AT1 receptor blocker losartan (LOS), prior to Ang II (10^{-5} M). DPI, as well as LOS abolished MC O_2^- production induced by Ang II. DPI also inhibited O_2^- production in unstimulated MC. Data represent the mean ± SEM of 3 experiments by duplicate. *$P < 0.05$ versus all other conditions; #$P < 0.05$ versus control.

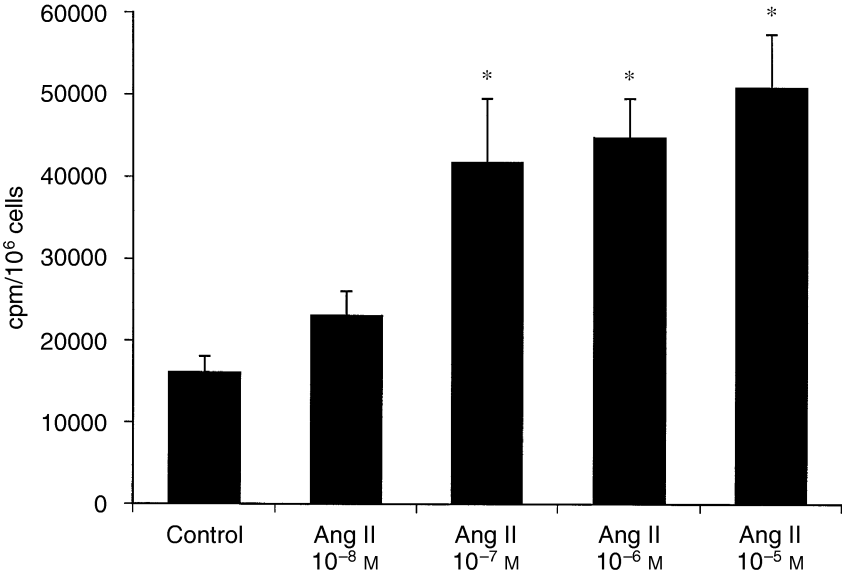

Fig. 3. Ang II induces MC hypertrophy in a dose dependent manner. MC were exposed to the indicated Ang II concentrations for 48 hours. MC ^{3}H leucine incorporation was used as an index of MC hypertrophy. Data represent the mean ± SEM of 3 experiments by duplicate. *$P < 0.05$ versus control.

Angiotensin II induces MC hypertrophy

Mesangial cells exposed to Ang II (10^{-5} to 10^{-8} M) for 48 hours increased protein synthesis in a dose dependent manner as measured by ^{3}H-leucine incorporation (Fig. 3). Furthermore, Ang II (10^{-5} M) increased total MC protein content for 48 hours (Control, 221 ± 26.52 μg protein/10^6 cells vs. Ang II 10^{-5} M, 312 ± 36.9 μg/10^6 cells; $N = 3$, $P < 0.05$). Treatment with LOS was not accompanied by increased cell lethality as assessed by trypan blue exclusion (trypan blue exclusion > 90%). LOS completely blocked the effects of Ang II on ^{3}H-leucine incorporation, therefore suggesting that these effects were mediated by activation of the AT1 receptor (Ang II 10^{-5} M, 50871 ± 1854 cpm/10^6 cells versus Ang II 10^{-5} M + LOS 10^{-6} M, 17849 ± 526 cpm/10^6 cells, $P < 0.05$; Fig. 4). Ang II (10^{-5} M) exposure for 24 hours, however, did not significantly increase MC ^{3}H-leucine incorporation (Control, 27839.44 ± 1486 cpm/10^6 cells versus Ang II 10^{-5} M, 30813.99 ± 1198 cpm/10^6 cells; $N = 3$, P = NS).

NADH/NADPH oxidase activation, superoxide production and MC hypertrophy

To determine whether NADH/NADPH oxidase activation can mediate MC hypertrophy in response to Ang II, MC were exposed to the NADH/NADPH oxidase inhibitor DPI (10^{-5} M), for one hour prior to Ang II. Treatment with

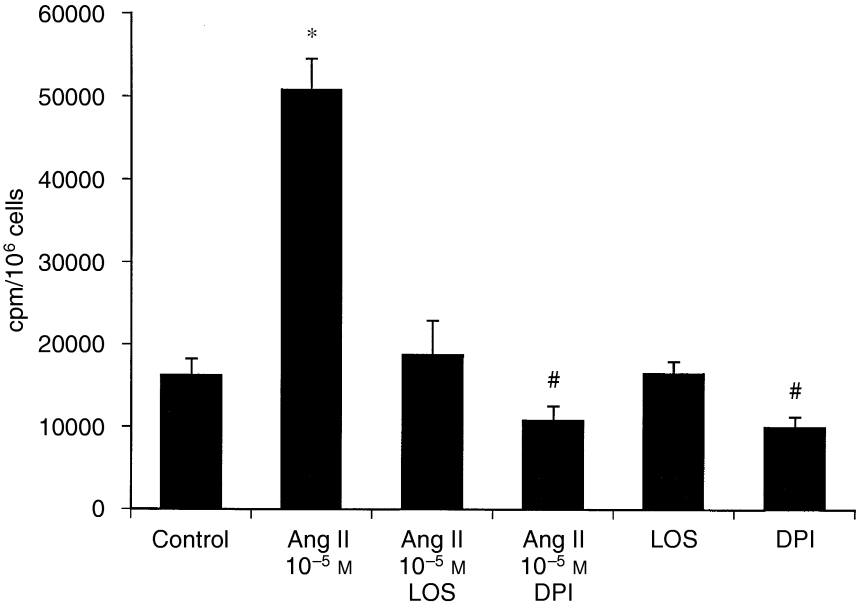

Fig. 4. LOS and DPI prevents MC ^{3}H leucine incorporation induced by Ang II. MC were exposed for 48 hours to Ang II 10^{-5} M in the presence of LOS or DPI. MC ^{3}H leucine incorporation was used as an index of MC hypertrophy. Data represent the mean ± SEM of 4 experiments by duplicate. $*P < 0.05$ versus all other conditions; $\#P < 0.05$ versus control.

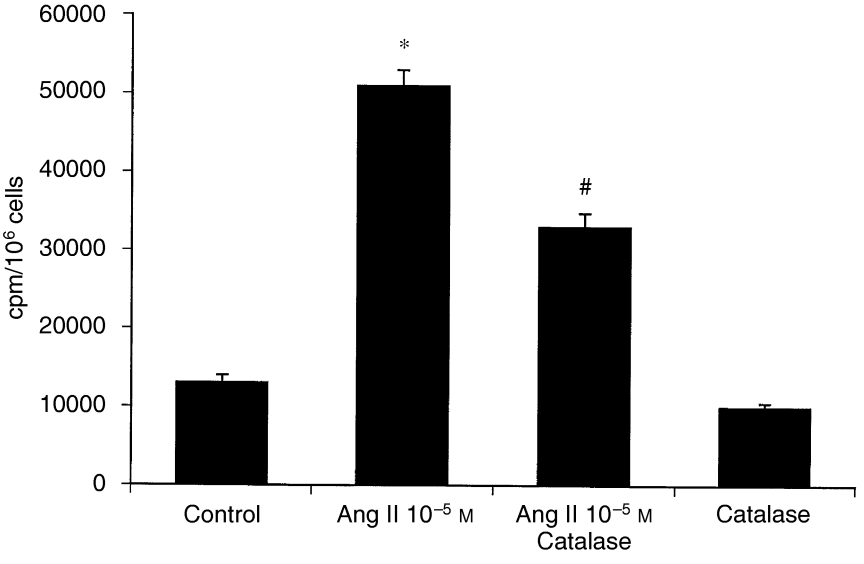

Fig. 5. Catalase prevents MC ^{3}H leucine incorporation induced by Ang II. MC were exposed for 48 hours to Ang II 10^{-5} M in the presence of catalase (800 U/ml). MC ^{3}H leucine incorporation was used as an index of MC hypertrophy. Data represent the mean ± SEM of 3 experiments by duplicate. $*P < 0.05$ versus all other conditions; $\#P < 0.05$ versus control and catalase.

DPI was not accompanied by increased cell lethality as assessed by trypan blue exclusion (trypan blue exclusion > 90%). DPI completely abolished MC increased protein synthesis in response to Ang II, suggesting that the hypertrophic response to Ang II in MC is linked to NADH/NADPH oxidase activation (Fig. 4). DPI also significantly inhibited basal O_2^- production and ^{3}H-leucine incorporation in unstimulated MC (Figs. 2 and 4). To determine whether ROS scavengers can inhibit Ang II induced MC hypertrophy, MC were exposed to the O_2^- scavenger superoxide dismutase (SOD; 150 U/ml) prior to Ang II. SOD, which does not penetrate cell membranes [28], did not inhibit Ang II induced MC protein synthesis (Ang II 10^{-5} M, 36077.8 ± 5515.6 cpm/10^6 cells versus Ang II 10^{-5} M + SOD 150 U/ml, 38193.8 ± 7243 cpm/10^6 cells; $N = 3$, P = NS). O_2^- can dismutate either spontaneously or through SOD to H_2O_2 [29]. We therefore exposed MC to the H_2O_2 scavenger catalase (800 U/ml), an enzyme that penetrates cell membranes [30], prior to Ang II. Catalase blunted Ang II induced MC protein synthesis, therefore suggesting that H_2O_2 probably generated by O_2^- dismutation contributes to the Ang II-induced MC hypertrophy (Fig. 5).

Reactive oxygen species (ROS), including O_2^-, have been shown to induce MAP kinases activation [30–33], a process that is associated with cell growth but does not affect O_2^- production [18, 34]. MC were treated with the specific MAP kinase kinase (MEK) inhibitor PD98059 (50

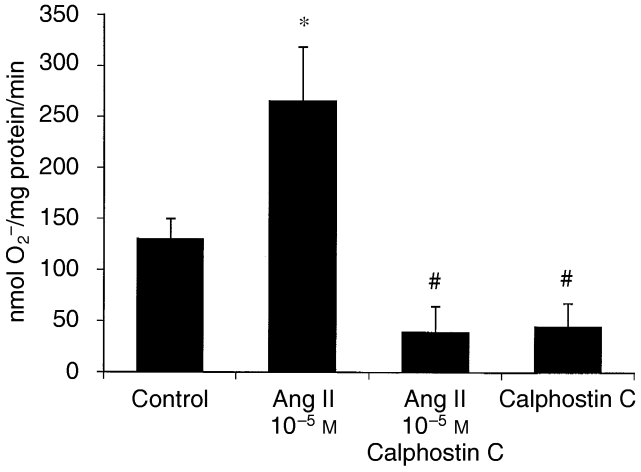

Fig. 6. Calphostin C inhibits Ang II induced MC O_2^- production. MC were exposed to the PKC inhibitor calphostin C (1 μM) prior to Ang II 10^{-5} M. Data represent the mean ± SEM of 3 experiments by duplicate.*$P < 0.05$ versus all other conditions; #$P < 0.05$ versus control.

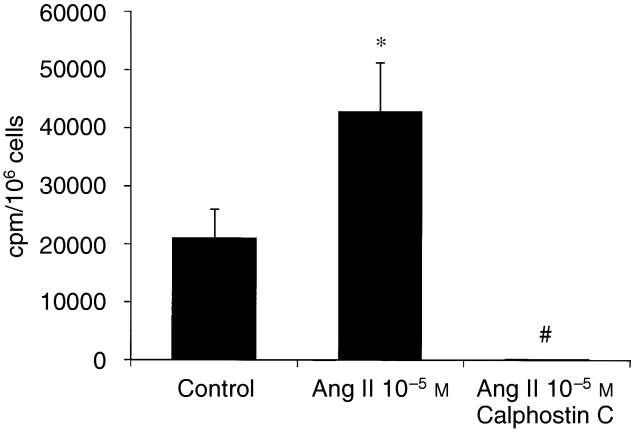

Fig. 7. Calphostin C prevents MC ^{3}H leucine incorporation induced by Ang II. MC were exposed for 48 hours to Ang II 10^{-5} M in the presence of the PKC inhibitor calphostin C (1 μM). MC ^{3}H leucine incorporation was used as an index of MC hypertrophy. Data represent the mean ± SEM of 3 experiments by duplicate. *$P < 0.05$ versus all other conditions; #$P < 0.05$ versus control.

μM) one hour prior to Ang II stimulation. Treatment with PD98059 was not accompanied by increased cell lethality as assessed by trypan blue exclusion (trypan blue exclusion > 90%). MAP kinases inhibition completely prevented MC ^{3}H-leucine uptake in response to Ang II (Ang II 10^{-5} M, 50440.1 ± 4125 cpm/10^6 cell vs. Ang II 10^{-5} M + PD98059, 9031.78 ± 1782.2 cpm/10^6 cells; $N = 4$, $P < 0.05$), suggesting that MAP kinases were involved in the hypertrophic response to Ang II that was initiated by increased O_2^- production. Similar to what we observed with DPI, PD98059 also inhibited basal MC ^{3}H-leucine uptake in unstimulated MC (Control, 11099.55 ± 231 cpm/10^6 cells versus PD98059, 4759.3 ± 499 cpm/10^6 cells; $N = 3$, $P < 0.05$).

Angiotensin II induces MC O_2^- production and hypertrophy by a PKC-mediated mechanism

Angiotensin II binding to the AT1 receptor induces several transduction signals that lead ultimately to increased intracellular Ca^{2+} concentration [34] and protein kinase C (PKC) activation [35]. PKC is involved in NADPH activation in polymorphonuclear cells [36] and macrophages [37], however, its role in vascular or MC NADH/NADPH oxidase activation has not been previously elucidated. To determine whether PKC activation is involved in Ang II induced O_2^- generation, MC were treated with the highly specific PKC inhibitor calphostin C (10^{-6} M) prior to Ang II stimulation. Calphostin C significantly inhibited Ang II induced O_2^- generation, suggesting that Ang II induced O_2^- generation by MC involves a PKC mediated mechanism (Ang II 10^{-5} M, 265.9 ± 52.8 nmol O_2^-/mg protein/min vs. Ang II 10^{-5} M + calphostin C, 39.41 ± 25.18 nmol O_2^-/min/mg; $N = 3$, $P < 0.05$; Fig. 6). Furthermore, the less specific PKC inhibitor H-7 (10^{-5} M) also inhibited Ang II stimulated MC O_2^- production (data not shown). Hence, PKC activation is necessary for Ang II induced O_2^- generation in MC.

Accompanying the inhibitory effects on O_2^- production, calphostin C (10^{-6} M), completely inhibited ^{3}H-leucine incorporation in response to Ang II (Ang II 10^{-5} M, 42807 ± 8419 cpm/10^6 cells versus Ang II 10^{-5} M + calphostin C 10^{-6} M, 65.97 ± 12 cpm/10^6 cells; $N = 3$ $P < 0.05$; Fig. 7). These results suggest that PKC activation is necessary to induce O_2^- generation and the subsequent MC hypertrophy in response to Ang II. Treatment with calphostin C was not accompanied by increased cell lethality as assessed by trypan blue exclusion (trypan blue exclusion > 90%); however, the extent of ^{3}H-leucine incorporation inhibition obtained with calphostin C was substantially larger compared with DPI or PD98059. This suggested that in addition to MC NADH/NADPH oxidase, calphostin C may be inhibiting other PKC dependent cellular processes involved in cell growth. In addition, in separate experiments calphostin C also inhibited basal ^{3}H-leucine incorporation in unstimulated MC (Control, 11099.5 ± 230.5 cpm/10^6 cells vs. Control + calphostin C, 1339.2 ± 160.9 cpm/10^6 cells; $N = 3$, $P < 0.05$). Furthermore, the less specific PKC inhibitor H-7 (10^{-5} M) also significantly inhibited Ang II stimulated ^{3}H-leucine incorporation (Ang II 10^{-5} M, 36078.5 ± 5515.5 cpm/10^6 cells vs. Ang II + H-7, 7573.2 ± 1914.8 cpm/10^6 cells; $N = 3$, $P < 0.05$).

It has been suggested that tyrosine kinase phosphorylation partially mediates the growth responses to Ang II in VSMC [38]. However, in our studies, treatment with the tyrosine kinase inhibitors herbimycin A (10^{-6} M) for 18 hours or genistein (10^{-5} M) for one hour prior to Ang II (10^{-5} M) did not inhibit Ang II-mediated O_2^-generation by MC, therefore indicating that tyrosine kinases activation is not necessary for Ang II-mediated O_2^- generation by MC

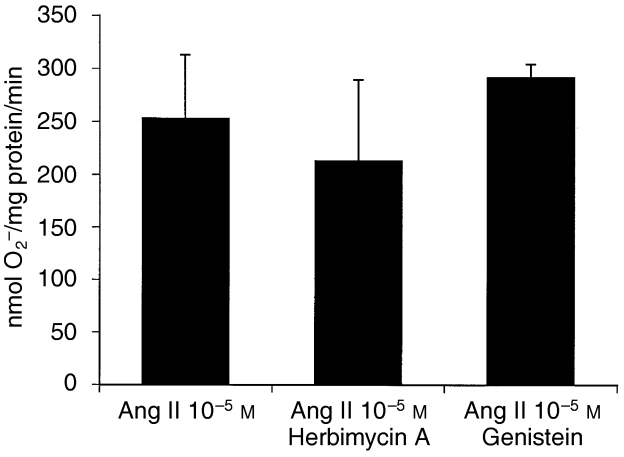

Fig. 8. Tyrosine kinase inhibition does not inhibit Ang II induced MC O_2^- production. MC were exposed to the tyrosine kinase inhibitors herbimycin (1 μM) or genistein (10 μM) prior to Ang II 10^{-5} M. Neither herbimycin nor genistein had a significant effect on Ang II induced MC O_2^- production. Data represent the mean ± SEM of 3 to 6 experiments by duplicate. P = NS.

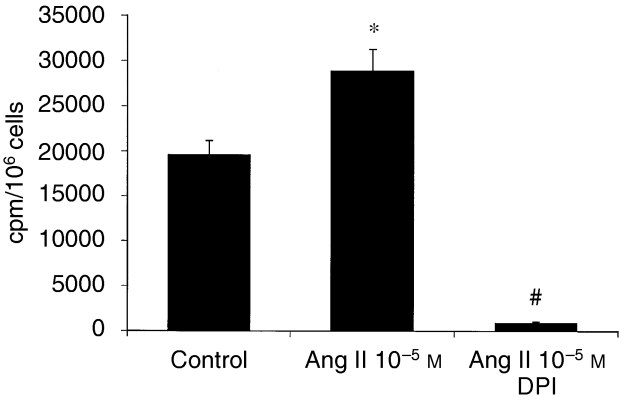

Fig. 9. DPI prevents MC ^{3}H thymidine incorporation induced by Ang II. MC were exposed for 48 hours to Ang II 10^{-5} M in the presence of DPI. MC ^{3}H thymidine incorporation was used as an index of MC hyperplasia. Data represent the mean ± SEM of 3 experiments by duplicate. *$P < 0.05$ versus all other conditions; #$P < 0.05$ versus control.

[Ang II 10^{-5} M, 253.18 ± 60 nmol O_2^-/mg protein/min (N = 6); Ang II + herbimycin A, 213 ± 77 nmol O_2^-/mg protein/min (N = 4); Ang II + genistein, 292 ± 13 nmol O_2^-/mg protein/min (N = 3); P = NS; Fig. 8).

Superoxide generation mediates angiotensin II induced MC hyperplasia

Mesangial cells exposed to Ang II (10^{-5} M) for 48 hours increased MC proliferation measured by ^{3}H-thymidine incorporation (Control, 19579.2 ± 1596.2 cpm/10^6 cells vs. Ang II 10^{-5} M, 28905.95 ± 2410. 87 cpm/10^6 cells; N = 3, $P < 0.05$; Fig. 9). To determine whether NADH/NADPH oxidase activation participates in MC hyperplasia in response to Ang II, MC were exposed to the NADH/NADPH oxidase inhibitor DPI (10^{-5} M), for one hour prior to Ang II. Treatment with DPI was not accompanied by increased cell lethality as assessed by trypan blue exclusion (trypan blue exclusion > 90%). DPI completely abolished Ang II stimulated MC ^{3}H-thymidine incorporation, suggesting that the hyperplastic response to Ang II in MC is linked to NADH/NADPH oxidase activation (Ang II 10^{-5} M, 28,906 ± 2411 cpm/10^6 cells vs. Ang II 10^{-5} M + DPI, 909 ± 152; N = 3, $P < 0.05$; Fig. 9). MC exposure to Ang II (10^{-5} M) for 24 hours, however, did not increase ^{3}H-thymidine incorporation (Control, 13,069 ± 1380 cpm/10^6 cells vs. Ang II 10^{-5} M, 14,894 ± 1920 cpm/10^6 cells; N = 3, P = NS).

DISCUSSION

These studies demonstrate that, to the best of our knowledge for the first time, Ang II induces NADH/NADPH oxidase-dependent O_2^- production by MC. The effects of Ang II were blocked by the selective AT1 receptor blocker LOS as well as the NADH/NADPH oxidase inhibitor, DPI. Current evidence suggests that NADH/NADPH oxidase systems are an important source of O_2^- in endothelial cells [27], vascular smooth muscle cells (VSMC) [21] and MC [19]. Studies by Griendling and Minieri have shown that Ang II activates NADH/NADPH oxidase system in VSMC [21]. Our studies demonstrate a similar effect to be initiated by Ang II in MC. Although all the mechanisms that lead to NADH/NADPH activation by Ang II in VSMC are not completely elucidated [21], it is clear that specific binding to the AT1 receptor is necessary. Binding of Ang II to the AT1 receptor is known to initiate intracellular signaling responses that include G proteins, phospholipase C, and adenylate cyclase, resulting in an increase in 1,4,5-inositol triphosphate and diacylglycerol [39], which ultimately results in increased intracellular calcium concentration [34] as well as PKC activation [35]. PKC is involved in NADPH oxidase activation in inflammatory cells such as polymorphonuclear cells [36] and macrophages [37], however, its role in MC NADH/NADPH oxidase activation has not been previously studied. In the current studies, Ang II induced O_2^- generation and MC hypertrophy were inhibited by PKC inhibitors, thereby suggesting that in MC, NADH/NADPH oxidase activation by Ang II is a PKC dependent process.

Ang II not only increases intracellular calcium and activates PKC, but also stimulates some of the signal transduction events activated by growth factors, including protein-tyrosine phosphorylation [40], stimulation of c-*fos* [41], and activation of mitogen-activated protein (MAP) kinases [42, 43]. ERK1 and ERK2, the most well studied MAP kinases, require phosphorylation of MEK to be activated [44] and are involved in the early response to a

variety of stimuli involved in cellular growth [45], transformation and differentiation [46]. Studies from several laboratories have shown that reactive oxygen species (ROS), including O_2^- and hydrogen peroxide, are an important intracellular signal for the activation of ERK1 and ERK2 [23], synthesis of growth factors [47, 48], and synthesis of vasoactive agents [49]. Our results show that the specific MEK inhibitor PD98059 inhibited MC hypertrophy induced by Ang II, thereby suggesting that ERK1 and ERK2 activation is at a distal point of convergence along the pathway triggered by the ROS generated by NADH/NADPH oxidase, and which ultimately leads to MC hypertrophy. Whether similar intracellular signaling mechanisms mediate Ang II-induced MC hyperplasia has not been explored and it is beyond the scope of the current studies.

The tyrosine kinase inhibitors herbimycin A and genistein did not inhibit Ang II induced O_2^- generation by MC, therefore suggesting that in MC, Ang II activation of NADH/NADPH oxidase is not tyrosine kinase dependent [38].

Functional, biochemical and molecular biological studies clearly support the existence of a local tissue RAS [50]. Ang II concentration in several intrarenal compartments, including the glomerulus, is 1000 times higher than those found systemically [3], sustaining the notion that a local tissue RAS can operate in the kidney *in vivo* and affect renal function independently of systemic Ang II. In fact, in the above described studies intrarenal Ang II was found to be in concentrations similar to those used in our current *in vitro* studies. Upregulation of the local tissue RAS in response to injury has been demonstrated in a variety of renal diseases including diabetes [12, 17]. *In vivo* transfection of human ACE into intact rat carotid arteries induces vascular hypertrophy without modifying blood pressure or serum ACE levels [47], thus suggesting that tissue RAS can generate Ang II in concentrations sufficient to elicit VSMC growth responses independently of hemodynamic changes [50]. Of particular interest are recent studies that demonstrate that NO modulates tissue ACE and *in vivo* inhibition of NO synthesis increases vascular ACE content and activity, and promotes cardiac and vascular hypertrophy independently of systemic blood pressure [51].

The current studies show that Ang II stimulates MC to synthesize O_2^- in quantities similar to those reported and which are known to be sufficient for reducing the bioactivity of NO within the vascular wall *in vivo* and *in vitro* [22]. Within the glomerulus, the interaction of NO and Ang II plays a pivotal role in mesangial regulation of glomerular microcirculation [52]. Furthermore, it has become increasingly clear that NO and Ang II have the capacity to induce long-term changes in cell growth, with NO being a proapoptotic and antiproliferative agent and Ang II antiapoptotic [53] and stimulatory of cell growth via synthesis of O_2^- [21] and growth factors such as PDGF [9] and TGF-β [8]. Therefore, the antagonistic interaction between NO and Ang II may play an important role in the modulation of the structural changes that result as a consequence of the glomerular response to injury in diseases such as hypertension and diabetes.

Clinically, ACE inhibitors do not achieve complete and permanent suppression of plasma Ang II, which returns to almost normal levels despite maintenance of ACE inhibitor administration and effective control of systemic hypertension [54]. The mechanism(s) involved in the rebound of plasma Ang II during ACE inhibition remain unclear and may include Ang II synthesis via enzymes other than ACE [54]. It is therefore reasonable to assume that the rebound of Ang II levels would permit actions of Ang II at the tissue level to remain pathophysiologically active. This may explain, at least in part, why in all clinical studies performed thus far, blockade of the RAS with ACE inhibitors achieved only a 50% risk reduction for decline in renal failure [12–15].

In conclusion, the current studies demonstrate a previously unrecognized link between Ang II and MC superoxide production, due to NADH/NADPH oxidase activation. Superoxide may participate in the pathophysiology of progressive renal disease by affecting the hemodynamics of the glomerular microcirculation as well as growth related responses of MC to injury.

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APPENDIX

Abbreviations used in this article are: ACE, angiotensin converting enzyme; Ang II, angiotensin II; AT1, angiotensin II type 1 receptor; DPI, diphenyleneiodonium; H_2O_2, hydrogen peroxide; LOS, losartan; MC, mesangial cell; MAP, mitogen activated protein; MEK, mitogen-activated protein kinase kinase; NO, nitric oxide; O_2^-, superoxide anion; PDGF, platelet-derived growth factor; PKC, protein kinase C; RAS, renin-angiotensin system; TGF-β, transforming growth factor β; VSMC, vascular smooth muscle cell.

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