Angiotensin II stimulation of VEGF mRNA translation requires production of reactive oxygen species

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Angiotensin II stimulation of VEGF mRNA translation requires production of reactive oxygen species. Am J Physiol Renal Physiol 290: F927–F936, 2006. First published October 25, 2005; doi:10.1152/ajprenal.00331.2005.—ANG II, a mediator of renal injury in diabetic renal disease, promotes vascular endothelial growth factor (VEGF) mRNA translation in proximal tubular epithelial (MCT) cells (Feliers D, Duraisamy S, Barnes IL, Ghosh-Choudhury G, and Kasinath BS. Am J Physiol Renal Physiol 288: F521–F529, 2005). The mechanism by which ANG II elicits this effect is not known. ANG II is known to induce oxidative stress and the rapidity of the effect suggested a role for reactive oxygen species (ROS). The aim of this study is to test the hypothesis that ANG II regulates VEGF mRNA translation in MCT cells through ROS production. In MCT cells exposed to 1 nM ANG II, ROS production was increased in a time-dependent manner. Inhibition of ROS production by N-acetylcysteine (NAC), a precursor of glutathione, and diphenylidenoiodonium (DPI), an inhibitor of flavoproteins that include NAD(P)H oxidase, prevented ANG II-stimulated VEGF protein expression. NAC and DPI also inhibited phosphorylation of 4E-BP1 on Thr46 and association of eIF4G with eIF4E, steps that are important in the initiation phase of mRNA translation. NAC and DPI also blocked Akt activation which is required for 4E-BP1 phosphorylation. LY-294002, a selective phosphatidylinositol (PI 3-kinase) inhibitor, did not prevent ROS accumulation in response to ANG II, whereas DPI blocked ANG II activation of PI 3-kinase, demonstrating that ROS production is upstream of the PI 3-kinase signaling pathway. Preincubation with catalase abolished ANG II stimulation of VEGF expression and mRNA translation, suggesting involvement of hydrogen peroxide (H2O2). H2O2 reproduced the effects of ANG II on VEGF expression and aforementioned parameters of mRNA translation. Finally, neither preincubation of MCT cells with specific inhibitors of the mitochondrial respiratory chain nor inactivation of the mitochondrial respiratory chain in MCT cells prevented ANG II stimulation of VEGF expression. Inhibition of nitric oxide synthase by L-NAME had no effect on ANG II stimulation of VEGF expression. These data show that ROS, generated probably through activation of an NAD(P)H oxidase, mediate ANG II stimulation of VEGF mRNA translation.

Vascular endothelial growth factor; diabetic microvascular injury

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) is among growth factors involved in diabetic microvascular injury. VEGF expression in renal cortex is increased in mice with either type 1 or type 2 diabetes, coinciding with hypertrophy and onset of matrix accumulation (48). Administration of a neutralizing antibody against VEGF has been shown to reduce proteinuria and renal hypertrophy in animal models of type 1 (8) or type 2 diabetes (11). These observations suggest a role for VEGF in a wide spectrum of changes present in the diabetic kidney. ANG II is acknowledged as a master regulator of kidney injury in diabetes, mediating the harmful effects of high plasma glucose (44). Its pathogenic importance is confirmed by the ameliorative effects of ANG II receptor blockade on the course of renal disease in diabetes (3). In addition to direct effects, ANG II recruits growth factors to regulate activity of renal cells. ANG II has been shown to increase VEGF production in the kidney via two independent mechanisms, a sustained stimulation of transcription of the vegf gene (13, 45, 55, 57) and a rapid and transient stimulation of translation of its mRNA (9). The rapidity of the latter response suggests that signaling pathways mediating this response have to be recruited within a short time. Among the early signaling pathways, reactive oxygen species (ROS) are important (43). ANG II has been shown to promote production of ROS which, in turn, mediate several effects of ANG II, notably protein synthesis and cell hypertrophy (17, 18, 23, 33, 51). In the present study, we tested the hypothesis that ANG II regulates VEGF mRNA translation in MCT cells through ROS production.

EXPERIMENTAL METHODS

Cell culture. SV40-immortalized murine proximal tubular epithelial cells (MCT cells) (kindly provided by Dr. E. Neilson, Vanderbilt University, Nashville, TN) were grown in DMEM containing 10% FBS, 5 mM glucose, and no insulin, as recently described (47, 49). Confluent monolayers of cells were serum deprived in DMEM for 18 h before treatment. MCT cells in culture express in vivo characteristics of proximal tubular epithelial cells (24). MCT-0 cells were generated by culturing MCT cells in regular DMEM medium containing ethidium bromide (50 ng/ml), uridine (100 μg/ml), and sodium pyruvate (1 mM) as described (29). The cells started to lose functional mitochondria (as assessed by immunoblot of subunit I of complex IV) after 10 days of treatment and were maintained for three passages before experiments were performed.

Immunoblot analysis. Immunoblots were performed as described (9, 10). Cells were washed twice with phosphate-buffered saline and lysed in RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM potassium chloride, 1 mM DTT, 1 mM EDTA, 50 mM glycerophosphate, pH 7.5, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM EGTA, 2 mM benzamidline, 1 mM PMFS, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Protein concentration was measured using the Bio-Rad protein reagent (Bio-Rad, Hercules, CA). Indicated amounts of lysates were separated on SDS-PAGE, transferred to nitrocellulose membranes, and probed with various primary antibodies at the indicated dilutions, and fluorochrome-coupled antibodies were used for detection using Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

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**4E-BP1 phosphorylation.** Phosphorylation status of 4E-BP1 was assessed by immunoblotting as described in (9, 10), using an antibody that specifically detects 4E-BP1 phosphorylated on Thr46 (New England Biolabs, Beverley, MA). Content of 4E-BP1 was assessed by immunoblotting with an antibody against 4E-BP1 (New England Biolabs).

**Polysome assay.** Polysome preparation was performed as recently described (9). Briefly, after treatment MCT cells were washed in PBS and pelleted by centrifugation. Pellets were lysed in 0.4 ml of resuspension buffer containing: 10 mM Tris (pH 7.5), 250 mM KCl, 2 mM MgCl₂. A 10% Tween 80, 5% (wt/vol) deoxycholate mix was added to the lysate, which was centrifuged for 10 min at 14,000 rpm. The cytosolic supernatants were laid on top of 15–40% sucrose gradient and centrifuged for 90 min at 200,000 g. After ultracentrifugation, the gradients were separated into four fractions, and RNA was extracted from each fraction using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Semi-quantitative RT-PCR amplification of VEGF or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was performed in polyribosomal fractions using the Superscript One-Step RT-PCR kit from Invitrogen and employing the following specific primers: VEGF sense (5'-ACATCTTCAAGCCGTCTGTGTC-3'), VEGF antisense (5'-AAATGGCAGATCCCACTCCAGGAG-3'); GAPDH sense (5'-CGATGCTGGGCGGTAGAC-3'), and, GAPDH antisense (5'-CTCAGCTAGGGATGACC-3'). PCR amplification was performed employing the following conditions: denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s for a total of 32 cycles. Fifteen-microliter samples from each 25-µl PCR product were removed and analyzed by electrophoresis on 1% agarose gel.

**Measurement of ROS production.** The peroxide-sensitive fluorescent probe 2,7'-dichlorodihydrofluorescein diacetate (Molecular Probes) was used to assess the generation of intracellular ROS as described previously (17). This compound is converted by intracellular esterases to 2,7'-dichlorodihydrofluorescein, which is then oxidized by H₂O₂ to the highly fluorescent 2,7'-dichlorodihydrofluorescein (DCF). DCF fluorescence was measured with an excitation wavelength of 488-nm light, and its emission was detected using a 510- to 550-nm band-pass filter.

**In-cell Western blot analysis.** MCT cells were plated in a 96-well plate and grown to 80% confluence, at which time they were incubated in serum-free medium for 20 h before being treated as indicated (8a). Immediately after treatment, cells were fixed in 4% formaldehyde in PBS for 20 min at room temperature. Cells were then permeabilized by five washes of 5 min each in PBS containing 0.1% Triton X-100. After a 90-min incubation in Li-Cor Odyssey blocking buffer (Li-Cor Biosciences) at room temperature, primary antibodies were added at a concentration of 1:500 and incubated overnight at 4°C with gentle shaking. Cells were then washed 5 × 5 min each in PBS containing 0.1% Tween 20. The secondary antibodies (IRDye800-conjugated or Alexa fluor-conjugated) were added at a concentration of 1:500 and incubated for 1 h at room temperature with gentle shaking, after which cells were washed 5 × 5 min in PBS containing 0.1% Tween 20. The plate was then scanned using the Odyssey Infrared Imaging System (169-µm resolution, 2-mm offset, intensity setting of 5 for both channels). Label intensity was measured by densitometric analysis of the wells.

**Immunofluorescence assay.** We previously showed that ANG II stimulates PI 3-kinase in MCT cells (49). Phosphatidylinositol triphosphate (PtdInsP₃) was detected by immunofluorescence as described (31). After a PBS wash, cell layers were fixed with cold methanol. Cell layers were then incubated with 10% normal donkey serum in 2% BSA/PBS for 15 min at room temperature. Incubations with a primary antibody to PtdInsP₃ (Echelon, Salt Lake City, UT) were performed with 1:100 dilution in blocking solution containing 0.1% Triton X-100 for 30 min. After five washes with PBS, slides were incubated with a secondary antibody conjugated to immunofluorescent dyes (Alexa Fluor 680, Molecular Probes, Eugene, OR) at 1:100 dilution for 30 min at room temperature. The stained layers were then washed, mounted using a Prolong Antifade kit, and examined with a fluorescent microscope.

**Statistics.** Data from a minimum of three experiments were expressed as means ± SE and analyzed by ANOVA for comparison among multiple groups using Bonferroni posttest analysis (Graphpad Prizm); P values of <0.05 were considered significant.

**RESULTS**

**ANG II stimulates ROS production in MCT cells.** We previously showed that 1 nM ANG II increased VEGF protein expression in MCT cells through increased cap-dependent translation of its mRNA. It was rapid, starting after 5 min of stimulation and lasted 30–60 min (9). The rapid time course of the stimulation of VEGF expression led us to hypothesize that it could be mediated by ROS. We examined the effect of ANG II on the generation of ROS in MCT cells, employing a fluorescence-based assay using peroxide-sensitive fluorophore DCF. Incubation with 1 nM ANG II resulted in a steady and time-dependent increase in DCF fluorescence, becoming significant after 2 min of treatment (Fig. 1A). ANG II-induced ROS production was completely blocked by preincubation for 30 min with 25 µM NAC, a nonspecific antioxidant (Fig. 1A). NAC is a generic antioxidant that does not permit identification of the sources or the individual types of ROS produced in cells. ANG II has been shown to activate Akt in a ROS-dependent manner in both vascular smooth muscle cells and mesangial cells following activation of an NAD(P)H oxidase (17–19). To assess whether a NAD(P)H oxidase system could be activated by ANG II in MCT cells, we used DPI, an inhibitor of flavoprotein-containing enzymes such as the NAD(P)H oxidase system. Figure 1A shows that both NAC and DPI abrogated ANG II stimulation of ROS production in MCT cells at all time points tested. These results show that the time course of intracellular ROS generation in response to ANG II is consistent with a potential role for ROS in downstream signaling events leading to increased VEGF expression. Furthermore, our data with DPI suggest that ROS production in cells treated with ANG II could be due to a NAD(P)H-oxidase.

**ANG II stimulation of VEGF expression is inhibited by antioxidants.** We explored whether ROS regulated VEGF translation in response to ANG II. Figure 1B shows that ANG II stimulation of VEGF expression (P < 0.01 ANG II vs. control) was completely prevented in MCT cells preincubated with 25 µM NAC (P < 0.01 ANG II vs. NAC + ANG II). Similar results were obtained after pretreatment of MCT cells with 10 µM DPI (P < 0.01 ANG II vs. DPI + ANG II). These results show that production of ROS is required for ANG II stimulation of VEGF protein expression and that this may occur through activation of a flavoprotein, such as NAD(P)H oxidase.

**NAC and DPI block ANG II-induced segregation of VEGF mRNA into polysomes.** We examined the initiation phase of translation of VEGF mRNA, which we previously showed to be the mechanism underlying rapid synthesis of VEGF in response to ANG II (9). Briefly, several ribosomes associate with mRNA that is targeted for increased translation, leading to the formation of polysomes. On a sucrose gradient, ribosomes are distributed according to their density with more aggregated ribosomes (polysomes) being distributed to progressively heavier fractions. Total RNA was extracted from cytosolic...
fractions separated on a sucrose gradient. RT-PCR was employed to detect VEGF and GAPDH mRNAs, with the latter serving as control. In control cells, distribution of VEGF mRNA was minimally increased in the heavy ribosomal fractions compared with the light ribosomal fractions. At 30 min of stimulation with ANG II, the VEGF mRNA was distributed exclusively into heavy polysomal fractions; preincubation with DPI prevented that shift of the VEGF mRNA into the heavy polysomal fractions (Fig. 1C). In contrast, there was no change in the preferential distribution of the GAPDH mRNA into heavier fractions at any time of ANG II treatment. These results show that ANG II stimulation of a flavin-containing oxidase is required for selective segregation of VEGF mRNA into polysomal fractions, a prerequisite for increased translation.

Dependence of VEGF mRNA translation initiation on ROS. Modulation of VEGF mRNA distribution to polysomes by ROS suggests that they regulate the initiation phase of mRNA translation. As VEGF mRNA translation is dependent on 4E-BP1 phosphorylation (9), we examined ROS regulation of 4E-BP1 phosphorylation, an important early event in the initiation phase. Quiescent MCT cells were treated with 1 nM ANG II stimulation of VEGF mRNA translation. ANG II increases reactive oxygen species (ROS) production and vascular endothelial growth factor (VEGF) synthesis in a ROS-dependent manner in MCT cells. A: quiescent MCT cells were loaded with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCF) for 1 h before treatment with 1 nM ANG II for up to 60 min without (●) or with pretreatment with 10 μM DPI (▲) or 25 μM NAC (●) for 30 min. DCF fluorescence was measured with an excitation wavelength of 488-nm light, and its emission was detected using a 510- to 550-nm band-pass filter. ROS production was expressed as fluorescence intensity (**P < 0.01 vs. control by ANOVA). B: quiescent MCT cells were pretreated with 25 μM NAC for 30 min before treatment with 1 nM ANG II for 15 min. VEGF protein expression was measured in cell lysates by immunoblot. Bottom: densitometric analysis of VEGF/actin ratio from 3 independent experiments, expressed as percent of control (***P < 0.01 ANG II vs. control; #P < 0.01 ANG II vs. DPI + ANG II; @@P < 0.01 ANG II vs. NAC + ANG II by ANOVA). C: polysomal analysis of VEGF and GAPDH mRNA was performed on MCT cells preincubated with 10 μM DPI before treatment with 1 nM ANG II for 30 min. RT-PCR was performed on an aliquot of RNA extracted from the polysomal fractions as described in EXPERIMENTAL METHODS. A polysome assay representative from 3 independent experiments is shown.

Fig. 1. ANG II increases reactive oxygen species (ROS) production and vascular endothelial growth factor (VEGF) synthesis in a ROS-dependent manner in MCT cells. A: quiescent MCT cells were loaded with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCF) for 1 h before treatment with 1 nM ANG II for up to 60 min without (●) or with pretreatment with 10 μM DPI (▲) or 25 μM NAC (●) for 30 min. DCF fluorescence was measured with an excitation wavelength of 488-nm light, and its emission was detected using a 510- to 550-nm band-pass filter. ROS production was expressed as fluorescence intensity (**P < 0.01 vs. control by ANOVA). B: quiescent MCT cells were pretreated with 25 μM NAC for 30 min before treatment with 1 nM ANG II for 15 min. VEGF protein expression was measured in cell lysates by immunoblot. Bottom: densitometric analysis of VEGF/actin ratio from 3 independent experiments, expressed as percent of control (***P < 0.01 ANG II vs. control; #P < 0.01 ANG II vs. DPI + ANG II; @@P < 0.01 ANG II vs. NAC + ANG II by ANOVA). C: polysomal analysis of VEGF and GAPDH mRNA was performed on MCT cells preincubated with 10 μM DPI before treatment with 1 nM ANG II for 30 min. RT-PCR was performed on an aliquot of RNA extracted from the polysomal fractions as described in EXPERIMENTAL METHODS. A polysome assay representative from 3 independent experiments is shown.

Fig. 2. ANG II-induced 4E-BP1 phosphorylation, eIF4E/eIF4B association, and Akt activation are ROS dependent. A: quiescent MCT cells were pretreated with 25 μM NAC or 10 μM DPI for 30 min before treatment with 1 nM ANG II for 15 min. 4E-BP1 phosphorylation on Thr46 was measured by immunoblot using a phospho-specific antibody. Loading control was assessed by immunoblot with an antibody directed against 4E-BP1. Bottom: combined data from 4 experiments in a graphic form (**P < 0.01 ANG II vs. control; #P < 0.01 ANG II vs. DPI + ANG II; @@P < 0.01 ANG II vs. NAC + ANG II by ANOVA). B: formation of eIF4E/eIF4G complexes was assessed by immunoprecipitation of eIF4G followed by immunoblot of eIF4E in the same lysates as in A.
ANG II for 15 min with or without preincubation with NAC or DPI, and 4E-BP1 phosphorylation on Thr46 was assessed by immunoblot of whole cell lysates using a phospho-specific antibody. Figure 2A shows that ANG II induced 4E-BP1 phosphorylation on Thr46 (P < 0.01 ANG II vs. control). This was prevented by preincubation with NAC (P < 0.01 ANG II vs. NAC + ANG II) or with DPI (P < 0.01 ANG II vs. DPI + ANG II), indicating that a flavoprotein-generating ROS is involved in ANG II-stimulated 4E-BP1 phosphorylation.

4E-BP1 phosphorylation is accompanied by dissociation of eIF4E/4E-BP1 complex and subsequent formation of eIF4F complex, comprising eIF4E, eIF4A, and eIF4G. eIF4F complexes facilitate ribosomal binding to the mRNA cap, and its scanning of the 5'-untranslated region (UTR) for the translation start site (14). eIF4G, a 180-kDa scaffold protein, contains binding sites for eIF4E and eIF3, the latter serves as a link between eIF4G and the ribosome. Because 4E-BP1 and eIF4G compete for the same site on eIF4E (35), binding of eIF4G to eIF4E can occur only after release of 4E-BP1 from eIF4E, after phosphorylation of the former. We previously showed that ANG II promotes dissolution of eIF4E-4E-BP1 complex in MCT cells (49). We next examined the effect of antioxidants on the binding of eIF4E to eIF4G. For these experiments, eIF4G was immunoprecipitated from lysates of MCT cells treated with 1 nM ANG II for 15 min, with or without preincubation with NAC or DPI, and presence of eIF4E in eIF4G immunoprecipitates was assessed by immunoblotting. Presence of eIF4G was also measured in the immunoprecipitates to assess loading. Figure 2B shows that ANG II treatment stimulated binding of eIF4E to eIF4G at 15 min; it was blocked by preincubation with either NAC or DPI. Together, these data demonstrate that ANG II-induced 4E-BP1 phosphorylation and association of eIF4E with eIF4G are dependent on ROS production.

ROS regulation of signaling events involved in ANG II stimulation of VEGF mRNA translation. Phosphorylation of 4E-BP1 on Thr46 is under the control of Akt, which stimulates mammalian target of rapamycin (mTOR), the direct kinase for 4E-BP1 (37, 52). We examined whether ANG II activation of Akt was dependent on ROS production. Akt is activated by phosphorylation on two serine residues, Ser308 and Ser473 (56). Figure 3A shows that ANG II stimulated phosphorylation of Akt on Ser473 at 15 min (P < 0.01 ANG II vs. control), which was prevented by preincubation with NAC (P < 0.01 ANG II vs. NAC + ANG II), or DPI (P < 0.01 ANG II vs. DPI + ANG II). These data show that activation of Akt by ANG II is dependent on ROS production.

ANG II stimulation of VEGF mRNA translation requires PI 3-kinase activation (9). Our present study showed that it is also dependent on ROS production. We next investigated whether...
ROS production is upstream of PI 3-kinase in this context. MCT cells were pretreated with LY-294002, a selective inhibitor of PI 3-kinase that prevents ANG II-stimulated VEGF mRNA translation (9), before incubation with 1 nM ANG II. ROS production was measured using the DCF fluorescence technique, as previously described. Figure 3B shows that ANG II-stimulated ROS production in MCT cells pretreated with the PI 3-kinase inhibitor was not significantly different from cells stimulated with ANG II alone (P < 0.01 ANG II vs. control by ANOVA; P < 0.01 LY-294002 + ANG II vs. control by ANOVA), indicating that ROS production is upstream of PI 3-kinase activation in ANG II-treated MCT cells. To confirm this finding, we performed the reverse experiment and studied the effect of DPI on ANG II-stimulated PI 3-kinase activity. PI 3-kinase activity was assessed by detection of its product, PtdInsP3, by immunofluorescence (31). Figure 3C shows that some of the quiescent MCT cells were positive for PtdInsP3 staining and that a 10-min incubation with 1 nM ANG II dramatically increased the number of positive-staining cells, showing that ANG II activated PI 3-kinase in these cells. Preincubation with DPI reduced the number of positive-staining cells to the level of control, showing that preincubation with the antioxidant prevented ANG II stimulation of PI 3-kinase activity in MCT cells. Together, these data show that ROS production is upstream of the PI 3-kinase/Akt signaling pathway in ANG II-stimulated MCT cells.

H2O2 stimulates VEGF mRNA translation. DPI inhibition of VEGF mRNA translation suggested that a flavoprotein such as NAD(P)H oxidase is involved in that process. Others have shown that NAD(P)H oxidase is a major component of ANG II signal transduction (18, 33). We thus tested the hypothesis that a NAD(P)H oxidase was involved in ANG II stimulation of VEGF mRNA translation. NAD(P)H-oxidase activation results in production of superoxide anion, which has a very short half-life and is rapidly dismutated into hydrogen peroxide (H2O2). H2O2 has a longer half-life and functions as a second messenger (43). H2O2 is metabolized to water by catalase (43). We preincubated MCT cells with 100 or 500 U/ml of catalase for 45 min before treatment with ANG II. Incubation with either concentration of catalase prevented ANG II stimulation of VEGF protein expression (P < 0.05 ANG II vs. control, P < 0.05 catalase + ANG II vs. ANG II; Fig. 4A). ANG II stimulation of 4E-BP1 phosphorylation on Thr46 (P < 0.01 ANG II vs. control) was also blocked by both concentrations of catalase (P < 0.01 catalase + ANG II vs. ANG II; Fig. 4B). Figure 4C shows that Akt activation, assessed by Ser473 phosphorylation, stimulated by ANG II (P < 0.01 ANG II vs. control) was prevented by catalase in a dose-dependent manner.

Fig. 4. ANG II activation of pathways leading to increased VEGF expression is blocked by catalase. A: VEGF expression was measured by immunoblot of lysates from MCT cells preincubated with 100 or 500 U/ml catalase and treated with 1 nM ANG II for 15 min. Actin expression was detected by immunoblot to assess loading. Bottom: densitometric analysis of VEGF to actin ratio from 3 independent experiments, expressed as percent of control [*P < 0.05 ANG II vs. control; #P < 0.05 ANG II vs. catalase (100 or 500 U/ml) + ANG II by ANOVA]. B: 4E-BP1 phosphorylation was examined by immunoblot as described in Fig. 2A. Loading control was assessed by immunoblot with an antibody directed against 4E-BP1. Bottom: combined data from 4 experiments in a graphic form [**P < 0.01 ANG II vs. control; ##P < 0.01 ANG II vs. catalase (100 or 500 U/ml) + ANG II by ANOVA]. C: Akt activation was assessed by immunoblot using a phospho-Ser 473-specific antibody as previously described. Bottom: densitometric analysis of 3 independent experiments, expressed as percent of control [**P < 0.01 ANG II vs. control; ##P < 0.01 ANG II vs. catalase (100 or 500 U/ml) + ANG II by ANOVA].
(P < 0.01 catalase + ANG II vs. ANG II). Inhibitory effect of catalase on ANG II stimulation of VEGF synthesis suggests requirement of H₂O₂.

We directly examined the potential regulation of VEGF mRNA translation by H₂O₂. Figure 5A shows that H₂O₂ significantly stimulated VEGF protein expression on a time course comparable to that of ANG II. Maximum stimulation (P < 0.01 by ANOVA) was observed at 5 min and was maintained for 60 min. Maximum stimulation of VEGF expression by H₂O₂ was 70 ± 16% of control, which was lower than stimulation of 95 ± 18% over control induced by ANG II; however, the difference did not reach statistical significance. Formation of polysomes on VEGF mRNA was also stimulated by H₂O₂ (Fig. 5B). After 15 min of treatment, the vast majority of VEGF mRNA was detected in the heaviest polysomal fractions, indicative of an active translation of this mRNA, whereas the proportion of GAPDH mRNA in the different polysomal fractions was not affected by H₂O₂ treatment. H₂O₂ also increased phosphorylation of 4E-BP1 on Thr46 (Fig. 5C), which started at 5 min and was maximal at 30 min (P < 0.01 H₂O₂ vs. control). Activation of Akt, an important upstream regulator of 4E-BP1 phosphorylation, was assessed by phosphorylation on Ser473 (Fig. 5D); it was increased at 5 min and reached a maximum after 15 min of H₂O₂ treatment (P < 0.01 H₂O₂ vs. control).

We next examined the requirement of 4E-BP1 phosphorylation for H₂O₂-stimulation of VEGF synthesis. We employed MCT cells overexpressing a Thr37,46→Ala37,46 phosphorylation-mutant of 4E-BP1 (MCT-mut.BP1) in which ANG II fails to stimulate VEGF mRNA translation (9). Figure 6 shows that H₂O₂ increased VEGF protein expression twofold at 15 min in MCT cells transfected with empty vector (MCT-vec cells) (P < 0.01 H₂O₂ vs. control), but not in MCT-mut.BP1 cells. Taken together, these data show that H₂O₂ directly stimulates VEGF mRNA translation and protein expression, a process that requires a functional 4E-BP1.

ROS derived from mitochondrial respiratory chain are not involved in ANG II regulation of VEGF synthesis. Our data using DPI suggest that NAD(P)H oxidase is involved in the production of ROS by ANG II. But NAD(P)H oxidase is not
the only source of ROS in cells that is inhibitable by DPI. Mitochondrial respiratory chain is an important source of ROS, which has been implicated in hyperglycemia-induced injury to target tissues including the kidney in diabetes; it is also sensitive to DPI (38). To investigate the role of the mitochondrial respiratory chain, we preincubated MCT cells with specific inhibitors: rotenone for complex I, antimycin A for complex III, KCN for complex IV and oligomycin for complex V, before treatment with 1 nM ANG II for up to 60 min. VEGF protein expression was detected by immunoblot to assess equal loading. Figure 7A shows that none of the inhibitors significantly affected ANG II stimulation of VEGF protein expression. These data suggest lack of involvement of complexes I, III, IV, and V of the mitochondrial respiratory chain in ANG II stimulation of VEGF expression. To confirm this finding, we generated MCT cells depleted of mitochondrial DNA (mtDNA) by long-term exposure to uridine and ethidium bromide, as described (29). These cells (MCTp0) are dependent on pyruvate for growth because of the absence of a functional respiratory chain. After 10 days of treatment, the surviving cells were growing at slightly slower pace than their wild-type counterpart (MCTwt). Three passages after selection, we measured expression of the subunit I of cytochrome c oxidase (complex IV), which is encoded by the mitochondrial genome. Figure 7B shows that there was a 70% decrease in this subunit expression in MCTp0 compared with MCTwt (P < 0.05 MCTp0 vs. MCTwt), indicating that these cells were indeed deficient in their mitochondrial respiratory chain. Figure 7C shows that in MCTp0 cells, ANG II increased VEGF protein expression with a time course similar to MCTwt, despite severe impairment in functional respiratory chain. Another source of ROS is the nitric oxide synthase/NO system which has been implicated in renal failure (16) and diabetic nephropathy (41). We preincubated MCT cells with 1 mM N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, for 30 min before treatment with 1 nM ANG II for 15 min. Figure 7D shows that L-NAME had no effect on ANG II stimulation of VEGF protein expression, making nitric oxide synthase an unlikely source of ROS.

**DISCUSSION**

We demonstrated that ANG II-induced rapid-phase VEGF mRNA translation is dependent on ROS production in MCT cells. Our data support the conclusion that NAD(P)H oxidase, but not mitochondrial respiratory chain or nitric oxide synthase, is the source of ROS that mediate ANG II effect. Our observations suggest ROS as a mediator of ANG II effect on VEGF mRNA translation.

ANG II is a master molecule of renal injury in diabetes and is involved in hemodynamic changes, hypertrophy, and matrix accumulation (44). ANG II-induced mesangial cell hypertrophy is ROS dependent (17). ROS have been suggested as mediators of target organ injury in diabetes (4). Nishikawa and associates (38) advocated a role for mitochondria as the source of ROS involved in this process based on observations in endothelial cells that were treated with inhibitors of mitochondrial respiratory chain complexes. Although their data may relate to diabetes-induced endothelial cell injury, they do not exclude other sources of ROS, particularly in diabetes-induced injury in nonendothelial cells. Induction of NAD(P)H oxidase activity and upregulation of NAD(P)H oxidase subunits by high glucose in vascular and renal cells (25, 26, 34, 42) and by diabetes in experimental animals (12, 21, 27, 30, 39) provide evidence that NAD(P)H oxidase-like enzymes are essential for increased oxidative stress in diabetes. The precise steps in the pathogenesis of diabetes-induced renal injury where NAD(P)H oxidase plays a role need better understanding.

In this context, our data suggest that NAD(P)H oxidase serves as the source of ROS in ANG II regulation of VEGF synthesis. This is supported by the following observations. First, ANG II-induced ROS production was inhibited by DPI, an inhibitor of flavin proteins which include NAD(P)H oxidase. Second, DPI inhibited ANG II-induced enrichment of VEGF mRNA transcripts in the polysomal fraction, 4E-BP1 phosphorylation, and association of eIF4E with eIF4G, steps that are necessary for initiation of VEGF mRNA translation. Third, H2O\textsubscript{2}, a product of NAD(P)H oxidase subsequent to the dismutation of superoxide dismutase, faithfully reproduced, and, catalase, an enzyme that promotes dismutation of H2O\textsubscript{2} to water, inhibited, the aforementioned steps involved in ANG II stimulation of VEGF translation. Finally, depletion of mitochondrial respiratory chain did not prevent ANG II induction of VEGF synthesis. An important attribute of the ROS-producing NAD(P)H oxidases present in nonphagocytic cells is their responsiveness to ANG II (18, 32). ANG II increases ROS production via NAD(P)H oxidase activation in various cells, including renal cells (18, 20, 23, 32). Impairment of NAD(P)H oxidase function in renal tubular epithelial cells or in mesangial...
cells inhibits ANG II-induced cell hypertrophy (18, 23, 32, 53, 54). Our data extend these observations showing that VEGF mRNA translation induced by ANG II is also mediated by activation of NAD(P)H oxidase (Fig. 8).

It is important to consider whether ROS generated in mitochondria also participate in ANG II signaling. Indeed, it has been recently reported that mitochondria-derived ROS play a key role in ANG II-induced JNK and p38MAPK activation in cardiomyocytes (2, 28). Moreover, H₂O₂-induced activation of Akt and JNK required generation of ROS by mitochondria in endothelial cells and fibroblasts (5). Our data show that mitochondria-dependent ROS generation is not part of the redox pathways engaged by ANG II for VEGF translation in MCT cells. Thus we conclude that membrane-derived ROS have an important role in ANG II regulation of VEGF synthesis in MCT cells. We also speculate that in diabetes, it is likely that both mitochondria and NAD(P)H oxidase contribute to generation of ROS, perhaps, in a cell- and phenotype-selective manner.

Our data provide evidence for the first time that ANG II-induced Akt activation is redox dependent in proximal

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**Fig. 7.** Mitochondrial respiratory chain is not the source of ROS involved in ANG II regulation of VEGF synthesis. A: quiescent MCT cells were treated with ANG II (1 nM, *) for 0 to 60 min, with or without a 30-min preincubation with rotenone (5 μM, ▲), antimycin A (10 μM, ▲), potassium cyanide (KCN; 1 mM, ◼), or oligomycin (10 μg/ml, □). VEGF protein expression was measured by in-cell Western analysis using a specific antibody as described in EXPERIMENTAL METHODS. Quantification was performed by densitometric analysis of individual wells and was normalized for cell number in each well by actin immunoreactivity. VEGF protein expression is expressed as percentage of control (4 experiments; **P < 0.01, *P < 0.05 compared with control by ANOVA). B: MCTp0 and MCTwt cells from corresponding passages were homogenized in lysis buffer, and lysates were used for immunoblot of subunit 1 of complex IV (cytochrome c oxidase) of the mitochondrial respiratory chain. The blots were then stripped and blotted again with an anti-actin antibody to assess equal loading. Top: representative immunoblot of 3 independent experiments. Bottom: result of densitometric analysis of these experiments, expressed as complex IV (subunit 1)/actin ratio (*P < 0.05 MCTp0 vs. MCTwt). C: MCTp0 and MCTwt cells from corresponding passages were made quiescent by overnight incubation in serum-free DMEM and treated with 1 nM ANG II for up to 60 min. VEGF protein expression was measured by immunoblot, and actin expression was measured to assess equal loading. D: VEGF expression was measured by immunoblot of lysates from MCT cells preincubated with or without 1 mM L-NAME and treated with 1 nM ANG II for 15 min. Actin expression was detected by immunoblot to assess loading. Top: densitometric analysis of VEGF-to-actin ratio from 4 independent experiments, expressed as percentage of control (**P < 0.01 ANG II vs. control; NS, ANG II vs. L-NAME + ANG II by ANOVA).
tubular epithelial cells, which is consistent with findings in other cell types, such as mesangial cells (17). Our data also show that ROS generation is upstream of PI 3-kinase activation in MCT cells. However, this is not the case in all cell types. In vascular smooth muscle cells (50), PI 3-kinase activation has been found to be upstream of NAD(P)H oxidase-dependent ROS generation in growth factor signaling (1, 40). In insulin signaling, ROS generation has been shown to be independent of PI 3-kinase signaling (15, 36), again demonstrating the cell-specific and agonist-specific nature of interaction between ROS and PI 3-kinase activation.

NAD(P)H oxidases produce superoxide anion on the outer side of the membrane into which it is inserted (6). Our data in MCT cells suggest a scenario in which superoxide generated by NAD(P)H oxidase is in the immediate pericellular environment of the MCT cells. It has an extremely short half-life and is promptly converted to H$_2$O$_2$ either spontaneously or by superoxide dismutase. H$_2$O$_2$ then enters the cell and stimulates the signaling pathways, i.e., PI 3-kinase, Akt, mTOR, that regulate VEGF translation. Under the conditions employed by us where we limited the duration of incubation with catalase to 45 min, the enzyme is not likely to enter the cells, probably acting in the pericellular space. This further supports the notion that H$_2$O$_2$ is generated as a consequence of NAD(P)H oxidase activity in the pericellular space. Thus, there could be an autocrine loop that enables H$_2$O$_2$ to mediate ANG II regulation of VEGF synthesis.

Previous investigators have suggested involvement of ROS in ANG II stimulation of events that occur in the initiation phase of general mRNA translation. In vascular smooth muscle cells, initiation phase of protein synthesis was shown to be redox dependent (46). ROS have also been linked to ANG II-induced cell hypertrophy in renal mesangial and vascular smooth muscle cells (17, 22, 51). Similar observations have also been made with endothelin-1 (7). However, to our knowledge, ROS have not been linked to mRNA translation of a specific protein as we showed in the case of VEGF.

In summary, our data bring an improved understanding of the source of ROS in ANG II-induced injury in MCT cells. We identify ROS derived from NAD(P)H oxidase as an important mediator of ANG II stimulation of VEGF mRNA translation. VEGF and ANG II contribute to renal injury in diabetes (8, 11, 48) and ANG II regulates the injurious processes in the diabetic kidney (44), partly via VEGF (9). Our findings identify NAD(P)H oxidase-derived ROS as a new potential target for intervention in renal disease.

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REFERENCES


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