Generation of ROS through the Ang II type 1 (AT1) receptor can result in oxidative stress leading to cardiovascular pathologies. A wealth of experimental evidence reveals that inhibition of the Ang II-generating axis of the renin-angiotensin-aldosterone system (RAAS) either by selective AT1 receptor antagonists or inhibition of the formation of Ang II by angiotensin-converting enzyme (ACE2), which metabolizes Ang II to Ang-(1-7). The ACE2 inhibitor MLN4760 also exacerbated the Ang II–dependent formation of ROS (156±15%) and abolished the generation of Ang-(1-7) from Ang II. We conclude that an ACE2-Ang-(1-7)-AT7R pathway modulates Ang II–dependent ROS formation within the nucleus, providing a unique protective mechanism against oxidative stress and cell damage. (Hypertension. 2010;55: 166-171.)

Key Words: angiotensin • reactive oxygen species • kidney • angiotensin-(1-7) receptor • intracellular RAS

It is well-established that reactive oxygen species (ROS) play an important role as signaling molecules in a variety of cellular responses. Sustained perturbations in redox homeostasis can result in oxidative stress leading to cardiovascular damage and cellular injury. Angiotensin (Ang) II stimulates the generation of ROS through the Ang II type 1 (AT1) receptor isoform. Blockade of the renin-angiotensin-aldosterone system (RAAS) either by selective AT1 receptor antagonists or inhibition of the formation of Ang II by angiotensin-converting enzyme (ACE) inhibitors is the leading therapeutic approach to lower blood pressure and reduce tissue injury in various cardiovascular pathologies. A wealth of experimental evidence reveals that inhibition of the Ang II-generating axis of the RAAS is associated with a reduction in oxidative stress within the kidney and other tissues.

The Ang II–dependent formation of ROS occurs by stimulating the assembly of the NAD(P)H (NOX) complex associated with the cell membrane. Chronic stimulation by Ang II also promotes the synthesis of several NOX components, including p22phox and p47phox, and attenuates the expression of various scavenging proteins within the cell, resulting in higher levels of intracellular ROS. Moreover, increased levels of ROS may stimulate the expression of components of the RAAS favoring Ang II, thus leading to a potentially vicious feedback loop that would exacerbate tissue injury. However, there are additional pathways within the RAAS that may functionally antagonize an activated Ang II-AT1 receptor pathway. One alternative product of the RAAS is the peptide Ang-(1-7), which is formed from either Ang I or Ang II. In contrast to the Ang II-AT1 receptor–mediated actions, Ang-(1-7) exhibits vasodilatory properties through the stimulation of NO or prostaglandins, stimulates natriuresis and diuresis, and conveys antifibrotic and antioxidant actions.

The inflammatory, fibrotic, and pressor actions of Ang II are assumed to originate at the cell surface by activation of the AT1 protein, a prototypic 7-transmembrane, G-protein coupled receptor; however, we and others have found a significant density of intracellular AT1 receptors on isolated nuclei obtained from the renal cortex and medulla of both the young adult (1.5 years) and older adult (3.0 to 5.0 years) sheep. Binding studies in renal nuclei revealed the AT2Ra s as the predominant receptor subtype (∼80%) in young sheep, with the Ang-(1-7) (AT7R; Mas protein) and AT1Ra antagonists competing for the remaining sites. Conversely, in older sheep, the AT1R accounted for ∼85% of nuclear sites, whereas the Ang type 2 receptor and AT1R subtypes comprise ∼20% of remaining sites. Ang II increased nuclear ROS to a greater extent in older (97±22%; n=6) versus young animals (7±2%; P=0.01; n=4), and this was abolished by an AT1R antagonist. The AT-R antagonist D-Ala7-Ang-(1-7) increased ROS formation to Ang II by ∼2-fold (174±5% versus 97±22%; P<0.05) in older adults. Immunoblots of renal nuclei revealed protein bands for the AT7R and Ang-converting enzyme 2 (ACE2), which metabolizes Ang II to Ang-(1-7). The ACE2 inhibitor MLN4760 also exacerbated the Ang II–dependent formation of ROS (156±15%) and abolished the generation of Ang-(1-7) from Ang II. We conclude that an ACE2-Ang-(1-7)-AT7R pathway modulates Ang II–dependent ROS formation within the nucleus, providing a unique protective mechanism against oxidative stress and cell damage.
Materials and Methods

Animals

Tissues were obtained from 4 young adult (1.5 years of age) and 6 older adult (3.0 to 5.0 years) female mixed-breed sheep that were pasture reared and then housed in the animal facility of Wake Forest University for 1 week before the study. Animals were synchronized by estrus cycles and maintained on a 12:12-hour light-dark cycle with access to food and water ad libitum. Kidney cortices were obtained fresh from animals anesthetized with ketamine and isoflurane and processed immediately for the isolation of the cortical nuclei. All of the procedures were approved by the institutional animal care and use committee at Wake Forest University School of Medicine.

Preparation of Nuclei

Cortical nuclei were prepared as described previously. Briefly, fresh tissue was homogenized in buffer containing 25 mmol/L of KCl, 5 mmol/L of MgCl2, 20 mmol/L of Tricine-KOH, and 25 mmol/L of sucrose (pH 7.8), filtered through a 100-μm mesh and centrifuged twice at 1000g (4°C) for 10 minutes. The pellet was resuspended in 20% OptiPrep solution (Accurate Chemical and Scientific), layered on a discontinuous density gradient column of 10%, 20%, 25%, 30%, and 35% OptiPrep solution and centrifuged at 10 000g for 20 minutes (4°C). The enriched fraction of nuclei was recovered at the 30% to 35% layer interface.

Characterization of Ang Receptors in Female Sheep Kidney

Ang receptor binding was performed as described previously. Briefly, isolated nuclei were suspended in HEPES buffer and incubated with the radioligand [125I]-[Sar1Thr8]-Ang II in the presence of losartan (the AT1-receptor antagonist), D-Ala5-(Ang 1-7) (A779 or DALA, the Ang-[1-7] receptor antagonist), PD123319 (the Ang II type 2 [AT2]-receptor antagonist), or nonlabeled Sarthran, each at a final concentration of 10 μmol/L.

Western Blotting

Nuclei isolated from OptiPrep gradient separation were suspended in PBS and added to a Laemmli buffer containing mercaptoethanol. Proteins were separated on 10% SDS polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes. Immunoblots were blocked for 1 hour with 5% dry milk (Bio-Rad Laboratories) and Tris-buffered saline containing 0.05% Tween, then probed with antibodies against AT1 (1:5000; Alpha Diagnostics); the Ang-(1-7) receptor, Mas (1:200, Alomone Laboratories); the ACE2 (1:2000; prepared at the Hypertension and Vascular Research Center, No. AN212); NOX2 (goat pIκBα, 1:1000; BD Transduction Laboratories); and p47phox (1:200; Cell Signaling). To confirm specificity of the Mas receptor antibody, immunogenic Mas peptide was incubated (1 μg of peptide per 1 μg of antibody) in 1% BSA at room temperature for 1 hour and then added to immunoblots of purified nuclear extracts and incubated at 4°C overnight. Reactive proteins for all of the immunoblots were detected with Pierce Super Signal Chemiluminescent substrates and exposed to Amersham Hyperfilm enhanced chemiluminescence.

Measurement of ROS Production

Isolated cortical nuclei were preincubated with the fluorescence dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester (DCF; 20 μg/mL; Molecular Probes) in buffer containing 100 mmol/L of KH2PO4, 1 mmol/L of Na2HPO4, 1 mmol/L of EGTA, 100 μmol/L of flavin adenine dinucleotide, and 100 μmol/L of reduced nicotinamide-adenine dinucleotide (pH 7.4) for 30 minutes at 37°C.13 Nuclei were washed twice in HEPES buffer to remove any unbound dye and then incubated with 1 nmol/L of Ang II in the presence of losartan (the AT1-receptor antagonist), DALA (the Ang-[1-7]-receptor antagonist), PD123319 (the AT2-receptor antagonist), the ACE2 inhibitor MLN4760 (MLN) or buffer alone. Increases in DCF fluorescence, as an indicator of ROS production, were measured using a SpectraMax M2e microplate reader (Molecular Devices) at wavelengths of 488 nm (excitation) and 510 nm (emission).

Determination of ACE2 Activity

ACE2 enzymatic activity was determined in isolated cortical nuclei of an older adult sheep kidney. Nuclei were purified by OptiPrep density gradient separation and analyzed at 37°C in 10 mmol/L of HEPES, 125 mmol/L of NaCl, and 10 mmol/L of ZnCl2 (pH 7.4), with inhibitors, as described previously. Briefly, nuclei were coincubated with 0.5 mmol/L of iodinated [125I]-Ang I or [125I]-Ang II, washed with ice-cold 1.0% phosphoric acid and filtered before separation by reverse-phase, high-performance liquid chromatography.

Statistical Analysis

Data are represented as mean±SEM. Paired Student t test and 1-way ANOVA with Tukey multiple comparison posthoc and nonlinear regression were performed using GraphPad Prism 5.0 plotting and statistical software.

Results

We initially determined the profile of Ang receptor subtypes in nuclei freshly isolated from the renal cortex of younger and older adult female sheep by radioligand binding of the nonselective antagonist [125I]-[Sar1Thr8]-Ang II and competition with selective isotype antagonists. In 1.5-year-old sheep (equivalence of 200 to 250 years of human age), the AT2 antagonist competed for the greatest proportion of Ang binding sites in renal cortical nuclei (80±6% competition with PD123319), consistent with our previous results, whereas antagonists for the Ang-(1-7) receptor (AT7) and AT1 subtypes competed for (52±5% and 25±2%), respectively (Figure 1). Conversely, the AT1 isoform is the predominant receptor subtype (84±5% competition with losartan) in cortical nuclei isolated from older sheep (3 to 5 years or 45 to 60 years of human age). In comparison, both PD123319 (the AT2 subtype antagonist) and DALA (the AT2 receptor antagonist) competed to a similar extent (21±2% versus 21±1%) for [125I]-[Sar1Thr8]-Ang II binding.

We next assessed the generation of ROS in response to Ang II in freshly isolated cortical nuclei from both younger and older adult female sheep using the fluorescent dye DCF. As shown in the fluorescent tracing of Figure 2A, Ang II (1 nmol/L) stimulated a sustained increase in DCF fluorescence over control nuclei (buffer alone) from older sheep that was abolished by the NADPH oxidase inhibitor diphenyleneiodonium chloride (10 μmol/L). Basal level ROS production in older adult sheep was 7.3-fold higher than that of young adult sheep (P<0.01; data not shown). Moreover, in Figure 2B, Ang II stimulated the DCF signal to a greater extent in the cortical nuclei isolated from the older sheep in comparison with the young adult sheep (97±22% versus 7±1%; P<0.05;
In the nuclei of older animals, losartan essentially abolished the Ang II–dependent increase in DCF (Figure 3B). In contrast to the ROS-enhancing effects in younger animals, PD123319 did not alter the Ang II response in older sheep; however, DALA increased the Ang II stimulation of ROS (174 ± 5%; n = 6; *<P<0.05 vs young). Losartan; PD, PD123319) or unlabeled Sarthran at a final concentration of 10 μmol/L. Data are expressed as mean ± SEM (n=4; *P<0.05 vs young).

To assess whether NOX components are expressed in the sheep nuclei, we performed Western blot analysis on purified nuclei from the renal cortex of older animals. In the blot of Figure 2C, we show a single immunoreactive band at approximately 80 kDa for gp91phox (NOX2), a membrane-bound glycoprotein component of the NAD(P)H oxidase complex that functions in electron transport. Moreover, we demonstrate doublet bands of 47 and 50 kDa for p47phox, the cytosolic component of the NOX complex that is required for activation of NOX2. To identify the Ang receptor subtypes that elicit the formation of ROS, we preincubated DCF-loaded nuclei from younger and older sheep with losartan, PD123319, or DALA (1 μmol/L each). As shown in Figure 3A, the AT1 antagonist losartan tended to reduce the small increase in Ang II–stimulated DCF fluorescence. However, pretreatment with the AT2 antagonist PD123319 significantly enhanced the DCF response to Ang II (14 ± 1% versus 7 ± 1%; P<0.05), whereas the AT1 receptor antagonist DALA had no effect. In the nuclei of older animals, losartan essentially abolished the Ang II–dependent increase in DCF (Figure 3B). In contrast to the ROS-enhancing effects in younger animals, PD123319 did not alter the Ang II response in older sheep; however, DALA increased the Ang II stimulation of ROS ∼2-fold (174 ± 5% versus 97 ± 22%; P<0.05) in the latter group. The addition of the Ang II or Ang-(1-7) receptor antagonists alone to DCF-loaded nuclei had no effect on control DCF levels in older animals (data not shown).

Treatment of nuclei with the specific ACE2 inhibitor MLN4760 (MLN) increased Ang II–dependent DCF fluorescence to a similar extent as that of DALA (174 ± 5% versus 156 ± 15%; P>0.05; Figure 3B).

In purified nuclei from the sheep renal cortex, immunoblots reveal the presence of the AT1/Mas receptor protein (Figure 4A). Preincubation of the immunoblot with the Mas peptide blocked this immunoreactive band. Western blot analysis of purified nuclei also revealed a single 120-kDa band for ACE2 (Figure 4A). Lastly, we demonstrate ACE2 activity in purified nuclei by the conversion of 125I-Ang II to 125I-Ang-(1-7) (17 ± 4 fmol · min⁻¹ · mg of protein⁻¹; n = 3). Nuclear ACE2 activity was abolished by the ACE2 inhibitor MLN (Figure 4B) but not by the addition of the DALA peptide (Figure 4C).

Discussion

Evidence for the intracellular expression of the RAAS within the kidney is becoming increasingly apparent, particularly the

Figure 1. Characterization of Ang receptor subtypes in isolated renal cortical nuclei of young (1.5 years of age) and older (3.0 to 5.0 years of age) female sheep isolated by OptiPrep density gradient. Competition binding was carried out using 0.5 nmol/L 125I-[Sar¹Thr⁸]-Ang II and receptor antagonists (LOS indicates losartan; PD, PD123319) or unlabeled Sarthran at a final concentration of 10 μmol/L. Data are expressed as mean ± SEM (n=4; *P<0.05 vs young).

Figure 2. Ang II stimulation of ROS in renal nuclei. Renal cortical nuclei were freshly isolated by OptiPrep density gradient separation and preincubated with the fluorescent dye, DCF. Isolated nuclei were stimulated with Ang II (1 nmol/L), Ang II plus the NOX inhibitor, diphenyleneiodonium chloride (10 μmol/L), or buffer alone. A. Representative tracing of DCF fluorescence. B, Comparison of ROS generation in nuclei from younger (n=4) and older (n=6) animals. Nuclei were stimulated with Ang II (1 nmol/L), the PKC agonist PMA (1 μmol/L), or buffer alone. Values represented are expressed as the percentage of change in fluorescence intensity over control (baseline) measured at 45 minutes. Data are the mean ± SEM (*P<0.05 vs young). C, Immunoblots of 3 distinct preparations of purified nuclei from older animals with antibodies directed against gp91phox (NOX2) and p47phox, the cytosolic subunit of the NOX2 complex required for activation.
functional expression of the AT₁ receptor on the cell nucleus.²²–²⁴ We reported previously that the AT₁ receptor stimulates ROS formation in isolated nuclei of the rat renal cortex.¹⁴ In contrast, nuclei prepared from the renal cortex of young adult sheep expressed primarily the AT₂ receptor subtype, which is functionally linked to the generation of NO.¹⁵ The present studies demonstrate age-dependent changes in Ang receptors where an increase in AT₁ and a corresponding decrease in AT₂ and AT₇ receptor subtypes are apparent in renal nuclei of older adult sheep. Associated with increased AT₁ receptor expression, ROS levels after Ang II or PMA stimulation were significantly higher in the nuclei obtained from the kidneys of the older sheep. Moreover, blockade of the AT₁ receptor or ACE2 enhanced the Ang II–dependent stimulation of ROS. To our knowledge, these studies are the first to demonstrate a regulatory role of Ang-(1-7) that originates from the proteolytic conversion of Ang II by ACE2 within the nucleus. Demonstration of the nuclear expression for both the AT₇ receptor and ACE2 suggests that Ang-(1-7) may well function as an endogenous buffer within the cell to modulate the actions of Ang II in the production of ROS. Indeed, these data support previous findings that exogenous Ang-(1-7) attenuates either Ang II- or hyperglycemic-induced increases in ROS.²⁵,²⁶

The binding studies in isolated nuclei revealed a shift in the Ang receptor profile from the AT₂ and AT₇ subtypes to the AT₁ isoform with age. The PD compound may shunt more Ang II to the AT₁ receptor or inhibit an AT₂ receptor–dependent pathway that normally attenuates ROS, particularly given the predominance of the AT₂ subtype at the younger age. In the presence of PD123319, Ang II would preferentially bind to the AT₁ rather than the AT₇ receptor, which may account for the lack of an effect of the AT₇ antagonist. In older animals, the AT₇ antagonist DALA, but not by the AT₁ receptor antagonist PD123319, enhanced the Ang II-AT₁ effect on ROS despite the similar extent of competition for Sarthran binding with both antagonists. In this case, the AT₂ receptor may no longer be functionally coupled to attenuate AT₁-dependent stimulation of ROS in older animals. Ang-(1-7), formed by ACE2 processing of Ang II, may preferentially bind to the

**Figure 3.** Influence of Ang receptor antagonists on Ang II stimulation of ROS in renal nuclei. Measurement of Ang II–stimulated ROS (increase in DCF fluorescence) in the presence of losartan (LOS), PD123319 (PD), DALA, or the ACE2 inhibitor MLN in nuclei from younger (n=4; A) or older (n=6; B) animals. All of the antagonists or inhibitors are at 1 μmol/L final concentration. Data are expressed as mean±SEM; *P<0.05 vs Ang II.

**Figure 4.** Immunoblots reveal the AT7 receptor Mas and blockade of Mas immunoreactivity by preincubation with the Mas peptide, as well as the enzyme ACE2 in 3 distinct preparations of purified nuclei from older animals (A). Demonstration of ACE2 activity in purified cortical nuclei by the conversion of ¹²⁵I-Ang II to ¹²⁵I-Ang-(1-7), as detected by high-performance liquid chromatography (B and C). ACE2 activity was abolished by the ACE2 inhibitor MLN (B) but not by the AT₁ receptor antagonist DALA (C).
AT$_7$ rather than the AT$_2$ subtype to antagonize the Ang II–AT$_1$ receptor pathway within the nucleus. Pretreatment of nuclei with the ACE2 inhibitor MLN exacerbated Ang II–induced ROS formation. This could be attributed to increasing the availability of Ang II to the AT$_1$ receptor by preventing metabolism to Ang-(1-7), but blockade of the AT$_7$ receptor with DALA yielded identical results. Pinheiro et al$^{32}$ reported recently that Mas knockout mice exhibit increased AT$_1$ receptor expression; however, to our knowledge, there are no reports that acute treatment with DALA increases AT$_7$ receptor expression. Thus, this counterregulation of Ang II on ROS production is likely mediated by the direct actions of Ang-(1-7) on nuclear AT$_7$ receptors.

The pathways that mediate the AT$_1$-dependent stimulation of ROS or the attenuation of this effect by Ang-(1-7) within the nucleus are not known. We initially reported the functional involvement of both PKC and phosphoinositol 3-kinase in ROS generation by Ang II in rat renal nuclei consistent with studies in intact cells on ROS formation, as well as evidence for a phospholipid signaling pathway within the nucleus.$^{28–30}$ The increase in ROS with the PKC agonist PMA in sheep nuclei suggests that PKC may contribute to the nuclear actions of Ang II, although we did not attempt to block the Ang II response with PKC inhibitors or to determine whether the effects of PMA and Ang II are additive. PMA responsiveness was also greater in the older animals, which may reflect an increase in PKC in the cortical nuclei. Asghar et al$^{31}$ reported that older Fisher 344 rats express higher levels of PKC-β and PKC-δ in the kidney cortex. Moreover, these investigators found that chronic antioxidant treatment reduces the elevated PKC activity in the proximal tubules of the older rat kidney.$^{32}$ Although it is not clear from the current studies what mechanism contributes to the alteration in Ang receptor subtypes in renal nuclei of older animals, the enhanced ROS response to Ang II likely reflects the participation of additional pathways in conjunction with the increased expression of the AT$_1$ receptor subtype.

With regard to Ang-(1-7), exogenous treatment with this peptide reduced the increase in ROS and phosphorylation of c-Src kinase by Ang II in intact endothelial cells.$^{22}$ The Ang-(1-7)–dependent reduction in ROS was associated with an increase in the interaction of Src homology protein, SHP-2 phosphatase with c-Src. Moreover, knockdown of SHP-2 abolished the inhibitory influence of Ang-(1-7). In LLC-PK proximal tubule cells, Ang-(1-7) reduced both mitogen-activated protein kinase activation and the increase in transforming growth factor β in response to high glucose conditions.$^{33}$ The Ang-(1-7) response in the proximal tubule cells was associated with an increase in the tyrosine phosphatase SHP-1, and the phosphatase inhibitor phenylarsine oxide reversed the inhibitory actions of Ang-(1-7).$^{33}$ Finally, Gallagher et al$^{34}$ demonstrated that Ang-(1-7) stimulated a mitogen-activated protein kinase phosphatase that prevented the downregulation of ACE2 by Ang II in cardiomyocytes. In lieu of these data in intact cells, we speculate that the acute response to the Ang-(1-7) antagonist or the ACE2 inhibitor may involve a reduction in “phosphatase tone” that would normally attenuate the kinase-dependent Ang II activation of ROS, because various phosphatases have been shown to localize to the cell nucleus.$^{35,36}$ Alternatively, our preliminary studies demonstrate that Ang-(1-7) stimulates nuclear production of NO, which may contribute to its capacity to buffer Ang II–induced ROS.$^{37}$ However, the identity of the nuclear pathway responsible for the inhibitory actions of Ang-(1-7) is not currently known and is the focus of ongoing investigation.

The present studies provide evidence for the expression of an ACE2-Ang-(1-7)-AT$_7$ receptor pathway on nuclei of the sheep kidney. One potential function of this pathway may be to attenuate the activity of the Ang II–AT$_1$ receptor axis and the accumulation of ROS within the nuclear environment. In the perfused kidney and LLC-PK cell, Ang II–induced DNA damage was attenuated by AT$_1$ receptor blockade, as well as the antioxidants N-acetyl cysteine and α-tocopherol.$^{38,39}$ Associated with the increase in DNA damage, Ang II increased intracellular DCF fluorescence 2-fold, which is similar to the increase in fluorescence observed in the present study, although the Ang II concentration in the intact cells was 170-fold greater than that used in the nuclei.$^{38}$ Moreover, treatment with exogenous H$_2$O$_2$, the primary ROS detected by DCF, increased DNA damage to a similar extent as that for Ang II in the proximal tubule cells.$^{38}$ Although mitochondria are considered the predominant source of cellular ROS,$^{40}$ the localized increase in ROS within the cell nucleus could potentially contribute to Ang II–dependent DNA damage and cell senescence.$^{41}$ The exact role(s) of the nuclear RAAS in cellular damage and senescence is not currently known. Indeed, further studies are required to determine whether certain transcriptional factors are upregulated in response to activation of the nuclear Ang receptors. However, evidence for reduced tissue levels of Ang-(1-7) with increasing age may mitigate the counterregulatory actions of the heptapeptide on Ang II–mediated ROS within the nuclear compartment of the cell.$^{42}$

Perspectives

Long-term blockade of the RAAS by ACE inhibitors or AT$_1$ receptor antagonists reduces oxidative stress and deters age-associated cellular or tissue damage.$^{43,44}$ Indeed, AT$_1$ receptor–deficient mice exhibit a 20% increase in life span associated with reduced tissue levels of nitrotyrosine.$^{45}$ In addition to inhibiting the Ang II–AT$_1$ receptor axis, RAAS blockade with ACE inhibitors or receptor antagonists increases the levels of Ang-(1-7). The present studies suggest that the activation of the intracellular ACE2-Ang-(1-7)-AT$_7$ receptor may constitute a pathway to convey additional therapeutic benefit in the treatment of cardiovascular disease.

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Disclosures

None.

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Angiotensin-(1-7)-Angiotensin-Converting Enzyme 2 Attenuates Reactive Oxygen Species Formation to Angiotensin II Within the Cell Nucleus
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