Regulation of ecto-5′-nucleotidase by NaCl and nitric oxide: potential roles in tubuloglomerular feedback and adaptation

Joseph Satriano, Lucinda Wead, Anna Cardus, Aihua Deng, Gerry R. Boss, Scott C. Thomson, and Roland C. Blantz. Regulation of ecto-5′-nucleotidase by NaCl and nitric oxide: potential roles in tubuloglomerular feedback and adaptation. Am J Physiol Renal Physiol 291: F1078–F1082, 2006. First published May 16, 2006; doi:10.1152/ajprenal.00043.2006.—The tubuloglomerular feedback (TGF) system serves to establish an appropriate balance between tubular reabsorption and glomerular filtration rate (GFR). High salt at the macula densa activates TGF to decrease GFR. Effector molecules for the tubular reabsorption and glomerular filtration rate include ATP and adenosine. We found that the concentration of ATP can be regulated by dietary salt, with high salt increasing activity. Conversely, NO decreases ecto-5′-NT activity in isolated glomeruli and that this activity can be regulated by dietary salt, with high salt increasing activity. Conversely, NO decreases ecto-5′-NT activity in isolated glomeruli. Moreover, NO inhibition of ecto-5′-NT activity is suppressed in the presence of dithiothreitol, suggesting nitrosylation as a reversible, oxidative stress-sensitive mechanism. The salt-induced activation of ecto-5′-NT correlates with high salt resetting of TGF. NO inhibition of enzymatic activity could be part of the adaptive phase.

The role and importance of tubuloglomerular feedback (TGF) systems in the kidney are increasingly appreciated among kidney physiologists and nephrologists. The identification and regulation of the endogenous substance(s) mediating TGF remain an active area of investigation (1, 4, 10, 12, 20, 31, 33, 37). Clearly, the major candidates mediating the effector response, glomerular filtration rate (GFR), and afferent arteriolar resistances and renal blood flow, do not remain static, but rather adapt (32). Studies examining this TGF adaptation reveal that by 60 min the system readjusts, permitting either a higher macula densa NaCl concentration at the same GFR or increased GFR while NaCl remains essentially the same (34, 36). Several examples of normal TGF adaptation exist in physiology including growth (3), contralateral nephrectomy (2), and changes in NaCl intake and volume status (4, 34), to name only a few. Results from our laboratory have suggested a critical role for modulators of TGF in the process of TGF adaptation, and these substances include nitric oxide (NO) derived from NO synthase (NOS)-1 (6, 32), cyclooxygenase (COX)-2 products (7), and ANG II (6). Understanding the mechanisms of TGF adaptation is important to a variety of pathophysiological processes including hypertension, acute renal failure, the kidney in diabetes, and progressive kidney disease. It is likely that these modulators of TGF adaptation exert this influence on the activity or generation of the purported primary mediators, ATP and adenosine.

We have developed an ecto-5′-NT assay for application to freshly harvested glomeruli. We demonstrate glomerular enzyme activity and evaluate the chronic effects on the enzyme under conditions of TGF adaptation or resetting by altering approaches or blockade of adenosine generation in studies employing adenosine AR-1 receptor knockout mice prevent normal TGF responses, suggesting a primary role for adenosine in producing afferent arteriolar vasoconstriction (30, 31). The two seemingly disparate views of mediator mechanisms of TGF are not as far apart as it may first appear. Clearly, the released ATP can and will be rapidly metabolized to AMP, which can be further dephosphorylated to adenosine by ecto-5′-nucleotidase (ecto-5′-NT) in the extracellular environment. The glomerular/macula densa compartment requires ecto-5′-NT activity for this scenario to operate. Prior studies have demonstrated ecto-5′-NT within the proximal tubular brush border, as well as in proximal tubule, fibroblast, and mesangial cell lines (16). Recent evidence utilizing ecto-5′-NT-deficient mice demonstrates that both ATP release and the subsequent hydrolysis to adenosine are critical to TGF function.

Investigations of TGF have primarily focused on the acute vasoconstrictor responses to elevations in macula densa flow rate and/or NaCl concentration. However, it is clear that the relationship between macula densa NaCl and flow and the effector response, glomerular filtration rate (GFR), and afferent arteriolar resistances and renal blood flow, do not remain static, but rather adapt (32). Studies examining this TGF adaptation reveal that by 60 min the system readjusts, permitting either a higher macula densa NaCl concentration at the same GFR or increased GFR while NaCl remains essentially the same (34, 36). Several examples of normal TGF adaptation exist in physiology including growth (3), contralateral nephrectomy (2), and changes in NaCl intake and volume status (4, 34), to name only a few. Results from our laboratory have suggested a critical role for modulators of TGF in the process of TGF adaptation, and these substances include nitric oxide (NO) derived from NO synthase (NOS)-1 (6, 32), cyclooxygenase (COX)-2 products (7), and ANG II (6). Understanding the mechanisms of TGF adaptation is important to a variety of pathophysiological processes including hypertension, acute renal failure, the kidney in diabetes, and progressive kidney disease. It is likely that these modulators of TGF adaptation exert this influence on the activity or generation of the purported primary mediators, ATP and adenosine.

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dietary NaCl intake, and the modification of enzyme activity by NO. We postulate that whereas high salt increases ecto-5'-NT activity, which would activate TGF via increased production of adenosine, locally generated NOS-1-derived NO functionally modifies TGF during the temporal adaptation phase.

MATERIALS AND METHODS

Sample preparation. Animal studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. These studies were conducted in conformance with the local VA/UCSD IACUC protocol (A3659-01) dated 11-5-2005. Adult male Wistar rats (Harlan, Indianapolis, IN), weighing 250–350 g, were maintained on 1) standard rat chow of normal NaCl intake, 2) low-NaCl diet chow (ISN), and 3) normal chow plus 1% NaCl in the drinking water for the normal, low, and high NaCl dietary intake studies. Basal studies were performed in rats on standard rat chow with normal NaCl content. Studies on the effect of dietary NaCl intake utilized a low and high NaCl intake for a period of 7 days before enzyme analysis.

Male Wistar rats from the normal and varied NaCl intake groups were anesthetized with Inactin (100 mg/kg ip, Research Biochemical, Natick, MA) and placed on a thermostatically controlled surgical table to maintain body temperature at 37°C. Freshly harvested kidneys were then placed in chilled PBS, pH 7.4. The kidneys were decapsulated, the cortex was removed, and the tissue was minced with a razor blade. Glomeruli were isolated using the sequential sieving technique, as previously described in detail (24, 29). Briefly, minced cortex was gently passed through an ice-cold 106-mesh (no. 140) sieve washed with ice-cold Krebs buffer, pH 7.4, and collected through a syringe fitted with a 20-gauge needle to decapsulate the glomeruli, and then collected on an ice-cold 75-mesh sieve and washed with Krebs buffer (2×). The glomerular preparation was then resuspended in 1 ml ice-cold HEPES/HBSS buffer (1:1), with an aliquot taken for protein determination (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA).

Ecto-5'NT activity determination. Samples (20 μl) were added to a tube containing 20 μM dipyridamole and either buffer or 50 μM methyleneadenosine-5'-diphosphate (MADP), a specific blocker of ecto-5'-NT, in HEPES/HBSS buffer. Dipyridamole prevents cellular uptake of adenosine or inosine. This mixture was preincubated for 10 min at 37°C in a shaking heat block. The reaction period was initiated with the addition of 10 μM [3H]IMP for 20 min at 37°C. The addition of 5 μl of 17 N formic acid stopped the reaction. Both IMP and AMP are substrates for ecto-5'-NT. As IMP and inosine standards demonstrated better separation in our hands than AMP and adenosine by thin-layer chromatography (TLC), we utilized labeled IMP as a substrate for assay purposes. In specific studies, 150 μM spermine NONOate (Alexis Biochemicals, San Diego, CA) and/or 1 mM DTT was added at the start of the reaction to test the impact of NO on enzyme activity.

The reaction tubes were spun down, and a portion of each reaction supernatant was separated by TLC on cellulose sheets with a fluorescent indicator (Cellulose 300 plates w/UV254; Sorbent Technologies, Atlanta, GA) in Tris base (pH 10)-methanol (4:1). The TLC lanes were cut into 0.5-cm fractions and quantified by liquid scintillation counting. Nonradioactive markers of the reaction product (inosine) and substrate (IMP) were run by TLC, visualized by UV detection, and used as a reference for the labeled compounds. Ecto-5'NT activity represents MADP-inhibitable phosphate activity. Noninhibitable activity was typically 10% of total enzymatic activity.

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Statistical analysis of data. Statistical analysis was performed by Student’s t-test using StatView (ver. 5.0.1) and KaleidaGraph (ver. 4.02) software for Macintosh.

RESULTS

Demonstration of ecto-5'-NT activity in glomeruli. Studies readily defined significant phosphatase activity in our isolated glomerular preparations. TLC demonstrated a clean separation of substrate IMP from a single, converted product, inosine. In parallel assays, application of MADP, a specific inhibitor of ecto-5'-NT, was utilized to distinguish enzymatic from nonenzymatic conversion. We observed ecto-5'-NT enzyme activity of 383 ± 25 fmol·min⁻¹·μg protein⁻¹. This is the first demonstration of ecto-5'-NT activity in isolated glomeruli. A prior study demonstrated enzyme activity in whole kidney homogenates (26). Because our glomeruli were decapsulated and tubular contamination was extremely low, our results likely represent glomerular activity only. Enzyme activity was previously described in mouse mesangial cells (16), which is a likely source of enzyme activity in the isolated glomeruli. Isolated glomeruli possess short portions of the afferent arteriole and undoubtedly exhibit both intraglomerular and some extraglomerular mesangial cells. The specific cell types generating this glomerular activity were not defined in this study. However, immunohistochemical localization of glomerular ecto-5'-NT appears predominately in the glomerular tuft/mesangium (5).

Chronic effects of NaCl intake on ecto-5'-NT activity. Previous studies demonstrated increased kidney adenosine content in response to a high-salt diet (28, 42). As increased adenosine would be in accord with increased TGF-mediated vasoconstriction, although not with TGF adaptation, we evaluated the effects of a high-salt diet on ecto-5'-NT activity in isolated glomeruli. Rats were maintained on either low or high NaCl intake for 7 days. Enzyme activity was 27% higher in rats on a high-salt diet compared with values on a low-salt diet (Fig. 1). Values for a low and high NaCl intake were 338 ± 33 and 467 ± 20 fmol·min⁻¹·μg protein⁻¹, respectively. As one can observe, values reported in rats on a normal NaCl intake fall at values intermediate between those for high and low NaCl intake, demonstrating the highly reproducible nature of the results.

Our results show that a high-salt diet increases ecto-5'-NT activity, which agrees with previous findings of increased kidney adenosine concentrations. Increased ecto-5'-NT and adenosine mediating of TGF vasoconstriction would seem...
counterproductive to long-term TGF adaptation; however, we observed preservation of normal TGF activity in rats maintained on a high NaCl intake (35). NO is a vasodilatory molecule elevated in response to a high NaCl intake (25) and is considered an important modulator of TGF. Increases in NO activity might act in vivo to partially inhibit ecto-5'-NT activity. However, if the effect of NO is via nitrosylation rather than nitration, the effect could be readily lost in the sample preparation procedure. We therefore examined this issue of NO inhibition of enzyme activity in isolated glomeruli by direct administration of an NO donor.

Effects of NO donors on ecto-5'-NT activity. We administered spermine NONOate as an NO donor because it is a source of NO only, and the absence of contamination by reactive oxygen and other adjunct chemicals with metabolic activity, such as has been observed with sodium nitroprusside (SNP). Addition of 150 μM spermine NONOate at the onset of incubation reduced baseline enzyme activity by 28 ± 3% from 383 ± 25 to 269 ± 14 fmol·min⁻¹·μg protein⁻¹ (Fig. 2). However, inhibition of ecto-5'-NT activity was not observed if application of DTT occurred concurrently with the NO donor (Fig. 3). Suppressing effects of NO with DTT implies that NO mediates this inhibitory effect via nitrosylation of critical sulphydryl groups rather than by nitration.

Discussion

The TGF system within the kidney is an important regulatory mechanism that coordinates the relationship between filtered load and tubular reabsorption. Recent studies have demonstrated that this system is not dictated by a static relationship between a macula densa NaCl signal and the nephron filtration rate but is a kinetic system that adapts to physiological circumstances (2, 3, 6, 7, 32, 34, 36). Changes in the relationship of the macula densa NaCl signal and filtered load are termed TGF adaptation. This adaptation must involve either modification of the actions of proposed endogenous mediators, ATP and adenosine, and/or activities of the modulators of TGF, including NOS-1-derived NO, COX products, and ANG II. In vivo and in vitro studies provide strong support that ATP release and its metabolism to adenosine are critical to the acute TGF response (1, 5, 11, 12, 20, 21, 30, 31). The mechanisms leading to TGF adaptation have primarily involved the activities of modulator substances such as NOS-1-dependent NO generation and products of the COX-2 enzyme. Changes in ANG II activity have been logically considered as candidates because elevations in macula densa NaCl normally suppress renin and could reduce local ANG II activity and contribute to relaxation of the afferent arteriole. However, specific evidence in support of the latter mechanism has not been provided. Modulators may in fact contribute to TGF adaptation by modifying the local concentration or activity of the proposed mediators, either ATP release or the metabolism of those substances to adenosine.

An enzyme critical to the metabolism of AMP to adenosine is ecto-5'-NT. Recent studies utilizing ecto-5'-NT-deficient mice demonstrate that this enzyme is essential to the acute TGF response (5, 11). Modification of enzyme activity may then be involved in the process of TGF activation and temporal adaptation. The latter adaptive phase may be regulated by the action(s) of the designated modulators of TGF activity. We demonstrate here that a high-salt diet and NO have opposing effects on ecto-5'-NT activity in isolated glomeruli.

The current study is the first to demonstrate enzyme activity for ecto-5'-NT within freshly harvested glomeruli and that this activity is responsive to dietary salt intake. The data are quite reproducible, and a high NaCl intake generated values for enzyme activity that are ~28% higher than in rats on a low NaCl intake (Fig. 1). These results of enzyme activity correlate well with the two studies in the literature that observe higher values for kidney cortical and interstitial adenosine content with a high NaCl intake (28, 42). Although these changes in adenosine generation would appear to promote TGF activity, they are contradictory to our concepts of temporal adaptation of TGF. Several investigators have suggested that during acute volume expansion, and by inference, high NaCl intake, TGF should be suppressed, reset, or adapted (4, 33). Thus TGF adaptation should logically not be associated with increased generation of a proposed activator of TGF.

A high NaCl intake represents a physiological example of TGF resetting or temporal adaptation. Recent studies from our laboratory have shown that normal TGF activity persists in rats maintained on a high NaCl intake. In these studies, administration of 1% salt in the drinking water does not reduce the range of TGF response, as determined by changes in single-nephron GFR that occur during perfusion of Henle’s loop with artificial tubular fluid. Similarly, rats fed a high-salt diet maintained nearly identical TGF responses as those on a normal diet (35, 39). Furthermore, ANG II receptor blockade attenuated the TGF responses in both high- and normal-NaCl diet animals. These results imply that maintained adenosine generation...
reflects a capacity to mount a normal TGF response after TGF resetting. Extracellular ATP levels could also be influenced by alterations in ecto-5'-NT activity.

A well-studied modulator substance of TGF at the macula densa is NO derived from NOS-1 (38, 40). In the pathophysiological condition of kidney ischemia-reperfusion, the generation of NO appears to inhibit function of ecto-5'-NT, because downregulation of the enzyme after ischemia-reperfusion can be prevented by application of a NOS inhibitor (26). This previous study demonstrates that a nitrodonor, SNP, decreases activity of ecto-5'-NT in renal homogenates by 20–25% (26). However, in rat hippocampal synaptosomes where SNP inhibits ecto-5'-NT activity, other NO donors do not have this effect (13). We therefore chose the nitrodonor spermine NONOate, which donates only NO and no other reactive oxygen species or potentially nonspecific toxic materials. We find that NONOate inhibits ecto-5'-NT enzyme activity in isolated glomeruli by ∼28% with 150 μM NONOate (Fig. 2).

Both high salt and ANG II activate PKC (17, 19, 41). Consequently, PKC increases ecto-5'-nucleotidase activity and adenosine release in rat cardiomyocytes (14), and in renal epithelial cells in culture (27). NO inhibits PKC via nitrosylation of a critical cysteine residue (8), whereas inhibition of NOS activates PKC with resultant increases in adenosine production and ecto-5'-NT activity (18). In our studies, 1 mM DTT decreases ecto-5'-NT activity. This response is typical of cysteine-containing enzymes that require reducing agents for proper function, where excess DTT causes a concentration-dependent progressive decrease in enzyme activity by interaction with critical sulfhydryl groups. At 10 mM DTT, ecto-5'-NT activity is reduced to 51.3 ± 6.0 fmol·min⁻¹·μg protein⁻¹. Ornithine decarboxylase (ODC), the first and rate-limiting enzyme of polyamine biosynthesis, which has a critical cysteine in its active center, displayed a similar response to DTT (23). That NONOate inhibition of ecto-5'-NT activity is abrogated in the presence of DTT (Fig. 3) supports the concept that NO is mediating this inhibitory effect via nitrosylation of sulfhydryl groups. Nitrosylation, unlike nitration, is readily reversible and dependent on the oxidative state of the cell. Because NO activity is increased in animals on a high NaCl intake (25), there may be significant functional suppression of ecto-5'-NT activity in vivo. This suppression may not persist in the in vitro assay if NO is removed during tissue preparation. Importantly, NO inhibition of ecto-5'-NT activity in vivo can be reversibly influenced by local redox factors (22). Whether the balance between PKC activation and subsequent suppression by nitrosylation affects ecto-5'-NT activity in glomeruli, or whether there are other targets for NO’s effects, such as the ecto-5'-NT enzyme, has yet to be determined.

These studies demonstrate that ecto-5'-NT is present in glomeruli and that glomerular ecto-5'-NT can be physiologically regulated. The high-salt results do not seem intuitively logical because a high NaCl intake confers a TGF-adapted condition, or a condition in which a higher GFR is associated with modestly increased macula densa NaCl luminal concentration. The finding of inhibition of enzyme activity in isolated glomeruli in the presence of nitrodonors is important to our understanding of the effects of NO on the TGF system, and particularly the impact of NO from NOS-1 on producing TGF temporal adaptation, as previously demonstrated by our laboratory (6, 32). Generation of NO during TGF activation of NOS-1 could lead to local inhibition of ecto-5'-NT, thereby influencing adenosine generation at the juxtaglomerular apparatus and leading to TGF temporal adaptation. However, it is unlikely that the readily reversible nitrosylation of the enzyme would be maintained throughout tissue preparation of glomeruli and the enzyme assay. Support for a localized effect is the observation that Ca²⁺/calmodulin-dependent NOS and ecto-5'-NT colocalize on the plasma membrane (9). However, questions remain on the status of ecto-5'-NT activity in vivo with a high NaCl intake. Although enzyme activity is increased, NO activity is also increased with a high NaCl intake, generating potential functional suppression of ecto-5'-NT in this condition.

The current results demonstrate that ecto-5'-NT is present in significant activity in isolated glomeruli from the rat kidney. In addition, wide variations in NaCl intake exert significant effects on ecto-5'-NT activity with a high NaCl intake increasing enzyme activity. In addition, NO donors administered in vitro in isolated glomeruli significantly decrease enzyme activity. NO inhibition of ecto-5'-NT activity should reduce the local production of adenosine and thereby attenuate the TGF-mediated vasoconstrictor responses. Ecto-5'-NT activity and its regulation may be critical to the metabolic fate of ATP, reported to be released by macula densa cells during TGF activation (1, 6), because the generation of adenosine, a purported mediator of TGF, may be the mechanism whereby TGF adapts to physiological conditions.

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