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Oxidative stress enhances the production and actions of adenosine in the kidney

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Chen, Ya-Fei, Pin-Lan Li, and Ai-Ping Zou. Oxidative stress enhances the production and actions of adenosine in the kidney. *Am J Physiol Regulatory Integrative Comp Physiol* 281: R1808–R1816, 2001.—The purpose of this study was to determine whether superoxide anions ($O_2^-\cdot$) activate 5′-nucleotidase (5′-ND), thereby increasing the production of renal adenosine and regulating renal function. Using HPLC analysis, we found that incubation of renal tissue homogenate with the $O_2^-\cdot$ donor KO_2 doubled adenosine production and increased the maximal reaction velocity of 5′-ND from 141 to 192 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$. The $O_2^-\cdot$-generating system, xanthine/xanthine oxidase increased the maximal reaction velocity of 5′-ND from 122 to 204 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$. Superoxide dismutase (SOD) with catalase produced a concentration-dependent reduction of 5′-ND activity in renal tissue homogenate, while the SOD inhibitor diethyldithiocarbamic acid significantly increased 5′-ND activity. Inhibition of disulfide bond formation by thioredoxin or thioredoxin reductase significantly decreased xanthine/xanthine oxidase-induced activation of renal 5′-ND. In in vivo experiments, inhibition of SOD by diethyldithiocarbamic acid (0.5 $mg \cdot kg^{-1} \cdot min^{-1}$ iv) enhanced renal vasoconstriction induced by endogenously produced adenosine and increased renal tissue adenosine concentrations under control condition and in ischemia and reperfusion. We conclude that oxidative stress activates 5′-ND and increases adenosine production in the kidney and that this redox regulatory mechanism of adenosine production is important in the control of renal vascular tone and glomerular perfusion.

redox signaling; renal hemodynamics; reactive oxygen species; nucleotide

ADENOSINE SERVES IN A PARACRINE role to constrict renal preglomerular arterioles (6, 17) and dilate postglomerular vessels (2, 20, 31), thereby reducing glomerular filtration rate and increasing medullary blood flow (6, 9, 13, 17). These hemodynamic effects of adenosine in the kidney work together with its inhibitory effect on tubular ion transport to adjust the metabolic supply and demand toward a level of transport activity appropriate for the oxygen and substrate availability of the tissue (28, 41). Therefore, adenosine plays a critical role in the control of renal vascular tone and tubular function. It has been demonstrated that adenosine is produced in the kidney in response to tissue metabolic activity under physiological conditions (14, 41). Tissue ischemia and reperfusion or cell hypoxia and reoxygenation markedly increased adenosine production in the kidney (33, 37). Despite intensive exploration of the physiological significance or pathological relevance of adenosine, the mechanism regulating adenosine production and metabolism in the kidney remains poorly understood.

It is well known that 5′-nucleotidase (5′-ND)-mediated AMP hydrolysis is a primary pathway responsible for adenosine production in the kidney (41). There is substantial evidence that ADP or/and AMP is accumulated in tissue subjected to ischemia and reperfusion or to increased metabolic activity (18, 40, 41, 49). Accumulation of ADP or AMP would provide more substrates for the production of tissue adenosine, which has been well accepted as an important mechanism increasing adenosine production during tissue ischemia or hypoxia (1, 7, 8, 15, 18, 28). However, it was reported that the concentrations of ADP or AMP under physiological conditions were close to or even higher than the Michealis-Menten constant (K_m) of 5′-ND (22, 27, 45, 49). This raised the question whether only an increase in substrate or accumulation of AMP and ADP can result in the production of a large amount of adenosine in tissues subjected to ischemia-reperfusion. It seems that, in addition to the accumulation of its substrates, enhanced 5′-ND activity is required to produce a large amount of adenosine under these circumstances. The present study was designed to test this hypothesis and to explore the mechanism related to this hypothesis.

Recently, redox-mediated signaling is emerging as a fundamental regulatory mechanism in cell biology (38). Many cellular proteins, such as transcription factors, receptors, and enzymes, are sensitive to reactive oxygen species (ROS) (38). In regard to the enzyme activity, ROS can react with thiol groups within the enzyme protein to form disulfide bonds, forming dimers and thereby changing the activity of enzymes (11, 16). Because ROS are increasingly produced during tissue ischemia and reperfusion or in response to enhanced tissue metabolic rate (1, 12), we hypothesize that in-

creased ROS may activate 5'-ND and thereby enhance production of adenosine under these conditions. In the present study, HPLC analysis was used to determine the effects of an $O_2^-\cdot$ donor or the $O_2^-\cdot$-generating reaction, superoxide dismutase (SOD), and the SOD inhibitor diethyldithiocarbamate (DETC) on the activity of 5'-ND by measuring the conversion rate of 5'-AMP to adenosine in renal tissue homogenate or purified 5'-ND. To explore the possibility of $O_2^-\cdot$-induced dimerization of 5'-ND through the formation of disulfide bonds, the effects of thioredoxin (Trx) and thioredoxin reductase (TR) on $O_2^-\cdot$-induced activation of 5'-ND were examined. We also performed in vivo experiments in anesthetized rats to determine whether increased oxidative stress by inhibition of SOD alters the production and actions of renal adenosine.

MATERIALS AND METHODS

Preparation of Renal Tissue Homogenate

Male Sprague-Dawley rats (Harlan Sprague Dawley, Madison, WI) were housed in the Animal Resource Center at the Medical College of Wisconsin. They were fed pellet diets with normal salt (1% NaCl), and water was provided ad libitum. Renal tissue homogenates were prepared as described previously (46, 49). Briefly, the renal cortex was homogenized with a glass homogenator in ice-cold HEPES buffer containing (in mM) 25 Na-HEPES, 1 EDTA, and 0.1 phenylmethylsulfonyl fluoride (PMSF). PMSF was dissolved in ethanol (10 mM) as a stock solution and stored at $-20°C$. When we prepared renal tissue homogenates, the PMSF stock solution was directly diluted 100-fold to 0.1 mM in HEPES buffer as the working solution. The homogenate was centrifuged at 6,000 g for 5 min at 4°C, and then the supernatant containing membrane and cytosolic components, termed the homogenate, was aliquoted, frozen in liquid nitrogen, and stored at $-80°C$ until used. All reagents were purchased from Sigma Chemical (St. Louis, MO). Acetonitrile was HPLC grade, and all other reagents were analytic grade.

HPLC Analysis of Adenosine and 5'-ND Activity

Assay of adenosine was performed as described previously (48, 49). This purine nucleoside and its substrate were separated and quantitated by reverse-phase HPLC. To measure 5'-ND activity, 20 μg of renal cortical tissue homogenate were incubated with 0.01–1.0 mM 5'-AMP and 3 mg/ml of the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA). EHNA was used to block the catabolism of adenosine in the reaction mixture. Then an ultrafiltration was performed to remove protein from the reaction mixture and to terminate the reaction.

The sample ultrafiltrates or dialysates obtained from in vivo experiments were injected and chromatographed on a liquid chromatograph (model 1090, series II, Hewlett-Packard, Palo Alto, CA) using an autosampler. The reverse-phase HPLC column was a C_{18} Absorbosphere cartridge (LC-18-T, 150 × 4.6 mm, 3 μm; Supelco, Bellefonte, PA) with an MTO Supelguard (LC-18-T, 20 mm; Supelco). A mobile phase of 5% acetonitrile with 5 mM potassium dihydrogen phosphate and 2.5 mM tetrabutylammonium hydrogen sulfate was used for separation of these purine nucleotides and substrates, and the total flow rate was 0.8 ml/min. The effluent was detected using an ultraviolet detector at 254 nm, and the chromatograph was recorded. The peaks were then integrated on an integrator (model 3392, Hewlett-Packard). Retention time of adenosine was 6.8 min. At each assay, adenosine synthetic standard was serially diluted to construct a standard curve used for quantitation of adenosine from a tissue sample or the dialysate.

Protocols for In Vitro Biochemical Experiments

Protocol 1: effects of $O_2^-\cdot$ donor on 5'-ND activity. KO2, an $O_2^-\cdot$ donor, releases $O_2^-\cdot$ at 25 μM $O_2^-\cdot$ per 1 mM at pH 7.4 (10). Renal tissue homogenate (20 μg) was mixed with 3 mg/ml EHNA and incubated with different concentrations of 5'-AMP (0.01–1.0 mM) at 37°C for 30 min. In another group of experiments, 5 mM KO2 was added to the reaction mixtures, and substrate concentration-dependent production of adenosine was examined.

Protocol 2: effects of xanthine and xanthine oxidase on 5'-ND activity. Xanthine/xanthine oxidase (X/XO) is a well-known $O_2^-\cdot$-generating system and is widely used to study the biological activity of $O_2^-\cdot$ in vitro. It has been reported that XO (50 mU) with X (100 mM) may produce 100 μM $O_2^-\cdot$ (21, 43, 47). Renal tissue homogenate (20 μg) was mixed with 3 mg/ml EHNA and incubated with 5'-AMP (0.01–1.0 mM) at 37°C for 30 min. To observe the effect of X/XO on 5'-ND activity in renal homogenate, 0.1 mM X and 50 mU of XO were added to the reaction mixtures, which may produce a 100 nM steady-state $O_2^-\cdot$ concentration in the reaction mixture during 30 min of incubation (43, 47). The substrate concentration-dependent production of adenosine was examined. To determine the effect of X/XO on the activity of purified 5'-ND, a purified bovine liver 5'-ND was incubated with 5'-AMP (0.5 mM) and X (0.01–1 mM) with and without XO (50 mU).

Protocol 3: effects of SOD and catalase on basal and X/XO-increased 5'-ND activity. To determine the role of endogenously produced $O_2^-\cdot$ in regulation of 5'-ND activity, SOD (100–600 mU) and catalase (1 U) were added to the reaction mixture, and the production of adenosine was examined. In another group of experiments, SOD and catalase were added to the reaction mixture with X/XO, and adenosine production was examined. To determine the effect of tissue SOD inhibition on the activity of 5'-ND, DETC (0.1–1 mM) was added to the reaction mixture with or without the X/XO system, and adenosine production was examined. DETC has been reported to inhibit the SOD activity in endothelial cells, vessels, and different renal tissues. It inhibits SOD activity by blocking the binding of metal ions to SOD or by depleting these metal ions, such as Zn or Cu, from SOD (30, 36, 47). The doses of DETC chosen for the present study were based on our previous studies showing that DETC significantly decreased medullary blood flow and effectively increased $O_2^-\cdot$ levels produced by NADH oxidase in the kidney tissue. This dose has been reported to inhibit SOD activity by >80% (47).

Protocol 4: effects of blockade of disulfide bond formation on X/XO-induced increase in 5'-ND activity. It has been reported that protein dimerization is an important mechanism mediating the oxidant-induced increase in the enzyme activity (16, 38). Trx and TR can block or reverse the dimerization of the enzyme molecules (19, 38). In the present experiments, Trx and TR were used to study the effect of the dimerization on 5'-ND activity. Renal tissue homogenate (20 μg) was incubated with 3 mg/ml EHNA, 1.0 mM 5'-AMP, 0.1 mM X, and 50 mU of XO, with and without 15 μM Trx and 7.5 μM TR, at 37°C for 10 min. The concentrations of adenosine in the reaction mixtures were measured as described above. Trx and TR concentrations were chosen on the basis of previous studies (42).

Protocols for In Vivo Animal Experiments

Protocol 5: effects of SOD inhibition by DETC on production and action of endogenous adenosine in the kidney. Sprague-Dawley rats ($n = 6$) weighing 250–300 g were anesthetized with ketamine (Ketaject, 30 mg/kg body wt im) and thiobutabarbital (Inactin, 50 mg/kg body wt ip) and then placed on a thermostatically controlled warming table to maintain body temperature at 37°C. A catheter was placed in the left external jugular vein for continuous intravenous infusion of 0.9% NaCl solution containing 2% albumin at a rate of 3 ml/h to replace fluid losses and maintain a euvolemic state. Another catheter connected to a Statham pressure transducer was inserted in the left femoral artery for measurement of arterial blood pressure. A midline abdominal incision was made, the left renal artery was isolated, and a flow probe (2 mm ID) was placed around the renal artery for continuous measurement of renal blood flow (RBF) using an electromagnetic flowmeter (model 501, Carolina Medical Instruments, King, NC). After a 90-min equilibration period, the response of RBF to endogenously produced adenosine was assessed by a postocclusive response of RBF (POR) as described previously (34, 35). This POR was determined by release of an occlusion (30 s) of the renal artery. In this model, renal adenosine production was increased by hydrolysis of ATP throughout the kidney during renal artery occlusion, and the accumulated adenosine produced renal vasoconstriction and thereby reduced RBF after release of the renal artery occlusion. The extent of the POR was determined via the ratio of minimal RBF after the occlusion to basal RBF (34, 35). A recovery period of $\geq$10 min followed each occlusion. To examine the effect of SOD inhibition on the POR, DETC was administrated intravenously at an infusion rate of 0.5 mg·kg^{-1}·min^{-1} for 30 min, and then the POR was redetermined.

Protocol 6: effects of SOD inhibition by DETC on production of renal adenosine during ischemia and reperfusion. In vivo microdialysis was performed as described previously (4, 40, 48). Briefly, the rats ($n = 7$) were anesthetized and surgically prepared as described above. The left kidney was immobilized by placing it dorsal side up in a kidney cup. A microdialysis probe (Bioanalytical Systems, West Lafayette, IN) with 0.5-mm tip diameter, 1-mm dialysis length, and 20-kDa transmembrane diffusion cutoff was gently implanted into the renal outer medulla (5 mm deep from the dorsal surface). This probe was connected to a microinfusion pump and perfused with phosphate-buffered saline containing (in mM) 205 NaCl, 40.5 Na$_2$HPO$_4$, and 9.5 NaH$_2$PO$_4$ (pH 7.4) at a rate of 2 µl/min throughout the experiment. To produce ischemia and reperfusion in the kidney, an occluder was placed around the aorta above the renal arteries. After a 90-min equilibration period, two 30-min control dialysate samples were collected for analysis of renal interstitial adenosine concentrations. Then the aorta was occluded until femoral arterial pressure decreased to 50 mmHg, and 10 min later a 30-min dialysate sample was collected. Thereafter, the aortic clamp was released to reperfuse the kidney for 70 min, and two 30-min dialysate samples were collected. Blood samples were also collected from the venous catheter for measurement of plasma adenosine concentrations under control, ischemia, and reperfusion conditions. Because our preliminary experiments demonstrated that adenosine concentrations after ischemia and reperfusion were maintained at high levels for a long period, the effects of DETC or a combination of DETC and 4-hydroxytetramethylpiperidine-1-oxyl (TEMPOL) on ischemia- and reperfusion-induced adenosine production were determined in additional experiments. In

one group of rats ($n = 7$), DETC (0.5 mg·kg^{-1}·min^{-1}) was infused intravenously for 30 min, and then the dialysate samples under control condition and during ischemia and reperfusion were collected as described above. In another group of rats ($n = 5$), TEMPOL (30 µmol·kg^{-1}·min^{-1}) and then DETC (0.5 mg·kg^{-1}·min^{-1}) were intravenously infused for 30 min. Then the collection of dialysates was repeated as described above. To determine the role of 5′-ND in DETC-enhanced adenosine production, the effect of the 5′-ND inhibitor α,β-methylene-adenosine diphosphate (AOPCP) on renal interstitial adenosine production was examined. After AOPCP (0.75 mg·min^{-1}·kg^{-1}) was infused intravenously for 30 min, the increase in adenosine in renal interstitial dialysate during ischemia and reperfusion was measured in the presence or absence of DETC as described above. All samples were stored at -80°C until HPLC analysis. At the end of the experiments, an excess dose of pentobarbital sodium (150 mg/kg) was given intravenously to euthanize the animals. The left kidney was weighed and excised to confirm the position of the dialysis probe. If the probe was positioned incorrectly in the outer medulla, we omitted the data of these dialysates.

Statistics

Values are means $\pm$ SE. The significance of differences within and between groups in multiple groups of experiments was evaluated using an analysis of variance for repeated measures followed by Duncan's multiple-range tests. The significance of differences between two groups was evaluated by Students' t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of the O$_2^-\cdot$ Donor KO$_2$ on 5′-ND Activity

The results of these experiments are presented in Fig. 1. When renal tissue homogenates were incubated with the adenosine deaminase inhibitor EHNA and different concentrations of 5′-AMP, a concentration-dependent production of adenosine was observed. The maximal conversion rate of adenosine was 131.1 ± 12.1 nmol·min^{-1}·mg protein^{-1}. When 5 mM KO$_2$ was added to the reaction mixtures, the conversion rate of adenosine was significantly increased. The maximal conversion rate of adenosine was increased to 216.8 ± 7.3 nmol·min^{-1}·mg protein^{-1} (Fig. 1A). The kinetic analyses demonstrated that the maximum velocity (V_{max}) and K_m of 5′-ND in renal tissues were 141 nmol·min^{-1}·mg protein^{-1} and 0.268 mM, respectively. KO$_2$ significantly increased the V_{max} of this enzyme to 192.3 nmol·min^{-1}·mg protein^{-1}, but it had no significant effect on the K_m of 5′-ND (Fig. 1B).

Effects of X/XO on 5′-ND Activity

When X/XO (an O$_2^-\cdot$-generating system) was added to the reaction mixtures of renal homogenate, the maximal conversion rate of adenosine was increased from 124.4 ± 9.4 to 208.1 ± 6.4 nmol·min^{-1}·mg protein^{-1} (Fig. 2A). The kinetic analyses demonstrated that X/XO increased the V_{max} of 5′-ND from 121.95 to 204.1 nmol·min^{-1}·mg protein^{-1}, but it was without effect on the K_m of 5′-ND (Fig. 2B).
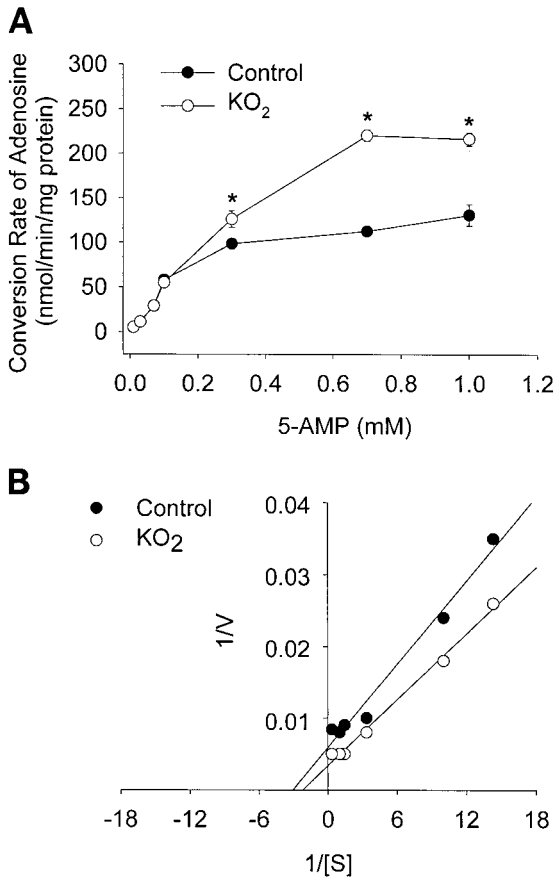
A

B

Fig. 1. Effects of the $O_2^-\cdot$ donor KO_2 on 5′-nucleotidase (5′-ND) activity. *A*: 5′-AMP concentration-dependent production of adenosine in rat renal homogenates in the presence or absence of KO_2 ($n = 6$ rats). HPLC analysis was performed to measure adenosine concentrations. *B*: Lineweaver-Burk plots showing kinetic analyses of 5′-ND activity in renal tissue homogenate. V, velocity; [S], concentration of substrate. *$P < 0.05$ vs. control.

Effects of SOD and Catalase on Basal and X/XO-Increased 5′-ND Activity

To determine the role of endogenous $O_2^-\cdot$ in the regulation of 5′-ND activity in the kidney, SOD and catalase were added to the reaction mixtures to scavenge endogenously produced $O_2^-\cdot$. SOD at 600 mU with catalase (1 U) significantly decreased 5′-ND activity in renal homogenate. In the presence of SOD and catalase, the X/XO-induced increase in 5′-ND activity was attenuated. SOD (600 mU) with catalase (1 U) completely blocked the effect of X/XO on 5′-ND activity (Fig. 3*A*). In contrast, the SOD inhibitor DETC increased 5′-ND activity under control condition and during incubation with X/XO (Fig. 3*B*).

Effects of X/XO on Activity of Purified 5′-ND

The results of these experiments are presented in Fig. 4. Purified bovine liver 5′-ND (1 mU) was incubated with 5′-AMP, and the production of adenosine was determined. This purified 5′-ND at 1 mU produced an amount of adenosine comparable to 100 μg of renal homogenate. In the presence of X alone in the reaction

mixtures, the production of adenosine was not altered. When 50 mU of XO and X in combination were added to the reaction mixture, the production of adenosine by this purified 5′-ND was significantly increased. A five-fold increase in 5′-ND activity was observed in the presence of 50 mU of XO and 1 mM X in the reaction mixtures.

Effects of Blockade of Disulfide Bond Formation on X/XO-Induced Increase in 5′-ND Activity

Incubation of renal tissue homogenates with the inhibitors of disulfide bond formation Trx and/or TR significantly inhibited the X/XO-induced increase in 5′-ND activity. Trx or TR decreased the X/XO-induced increase in the conversion rate of adenosine in renal tissue by 38.1% or 43.9%, respectively. A combination of Trx and TR had no marked additive effect on the X/XO-induced increase in 5′-ND activity (Fig. 5).

Effects of SOD Inhibition by DETC on Production and Action of Renal Adenosine in Anesthetized Rats

RBF exhibited a POR that represents the production and action of adenosine in the kidney (Fig. 6*A*). Intra-

A

B

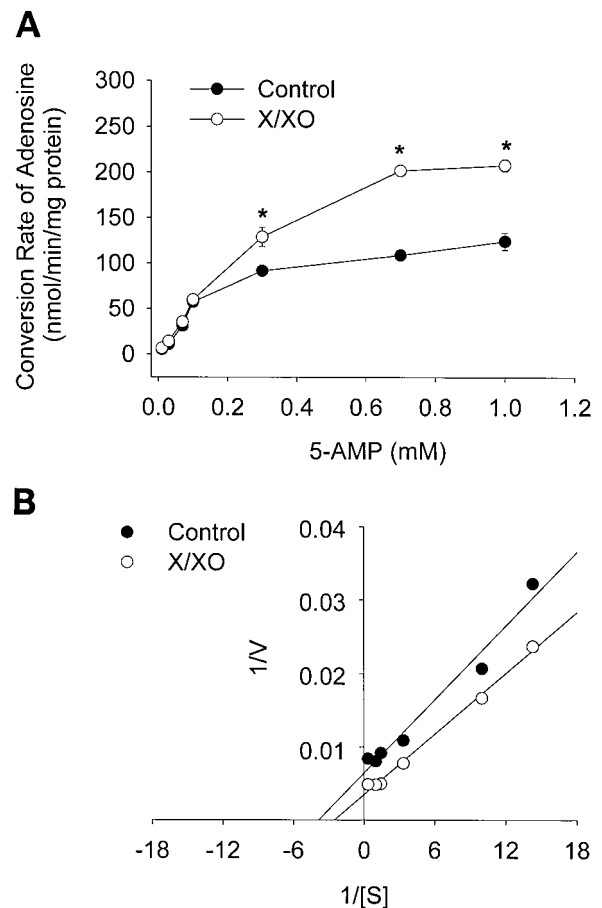

Fig. 2. Effects of the $O_2^-\cdot$-generating system xanthine/xanthine oxidase (X/XO) on 5′-ND activity. *A*: 5′-AMP concentration-dependent production of adenosine in rat renal homogenates in the presence or absence of X/XO ($n = 6$ rats). *B*: Lineweaver-Burk plots showing kinetic analyses of 5′-ND activity in renal tissue homogenates. *$P < 0.05$ vs. control.
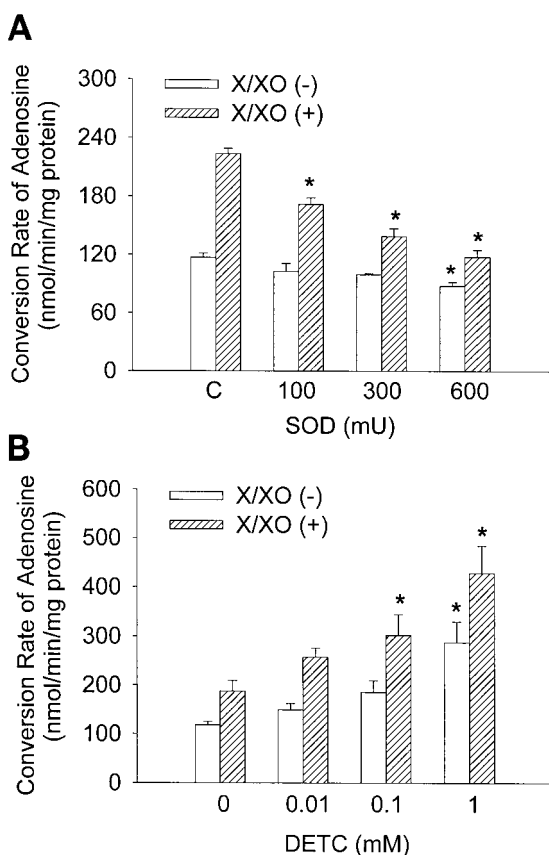
A

B

Fig. 3. Effects of superoxide dismutase (SOD) and diethyldithiocarbamate (DETC) on 5′-ND activity. *A*: effect of SOD on 5′-ND activity with (+) or without (−) X/XO (*n* = 6). *B*: effect of SOD inhibition by DETC on 5′-ND activity. *P < 0.05 vs. control.

venous infusion of DETC markedly enhanced this POR. The results of these experiments are summarized in Fig. 6*B*. Under control condition, RBF after release of occlusion decreased by 21.9 ± 1.9%, while this POR was significantly enhanced (49.1 ± 4.56%) in the presence of DETC (Fig. 6*B*).

Effects of SOD Inhibition by DETC on Ischemia- and Reperfusion-Induced Production of Renal Adenosine

To further confirm that oxidative stress enhances the production of renal adenosine, in vivo microdialysis experiments were performed to directly measure adenosine concentrations in renal interstitial fluid. The results of these experiments are presented in Fig. 7. Before intravenous infusion of DETC, interstitial adenosine concentration in the dialysate from the renal outer medulla measured by HPLC was 161.7 ± 13.6 nM. Kidney ischemia, by decreasing renal perfusion pressure to 50 mmHg, resulted in elevation of adenosine concentration in renal microdialysate to 366.5 ± 37.0 nM. Reperfusion for a total of 70 min after ischemia increased the concentrations of adenosine in the dialysate to 549.8 ± 68.8 nM. However, the plasma adenosine levels were not altered under control (127.8 ± 31.3 nM) and during ischemia (129.6 ± 13.1 nM) and reperfusion (102.5 ± 9.7 nM). When the rats
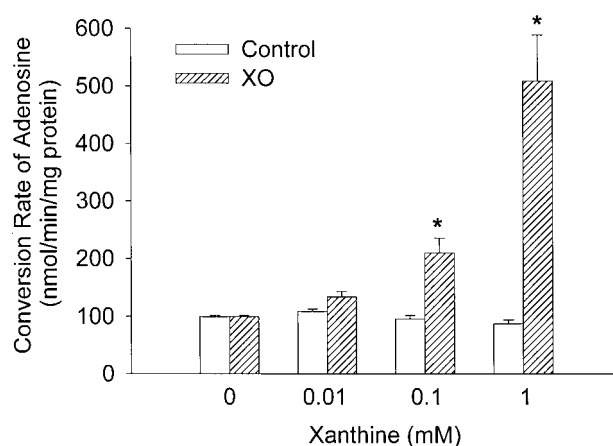

Fig. 4. Effects of X/XO on activity of purified 5′-ND. *P < 0.05 vs. control (*n* = 6).

were pretreated with intravenous DETC, adenosine concentrations in the microdialysate were markedly increased even before tissue ischemia. This adenosine increase continued during ischemia and reperfusion. The DETC-induced increase in renal interstitial adenosine concentrations under control, ischemia, and reperfusion conditions was completely blocked by TEMPOL and AOPCP.

DISCUSSION

In the present study, we demonstrated that the $O_2^-\cdot$ donor KO_2 or the $O_2^-\cdot$-producing system X/XO significantly increased the activity of 5′-ND in renal tissue homogenates. The kinetic analyses showed that the enhancement of 5′-ND activity was associated with increased V_{max} of this enzyme, suggesting that the turnover of this enzyme is increased during oxidative stress. Given that adenosine can reduce glomerular filtration rate, decrease tubular loading, inhibit tubular ion transport activity, and increase medullary blood perfusion (41, 48), this oxidant-induced increase in 5′-ND activity or adenosine production may represent an important adaptive mechanism during oxidative stress under pathological conditions such as ischemia and/or reperfusion (1, 2, 6). Previous studies showed
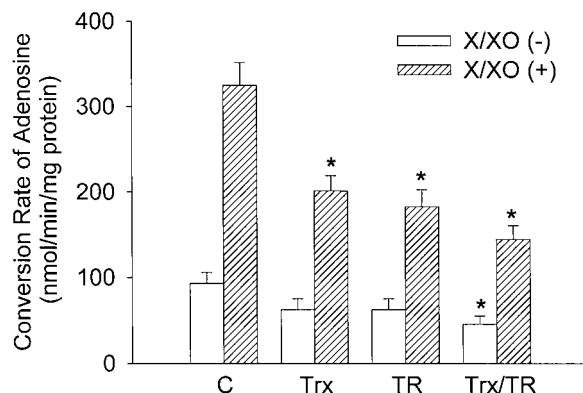

Fig. 5. Effects of thioredoxin (Trx) and thioredoxin reductase (TR) on 5′-ND activity. *P < 0.05 vs. control (*n* = 6).
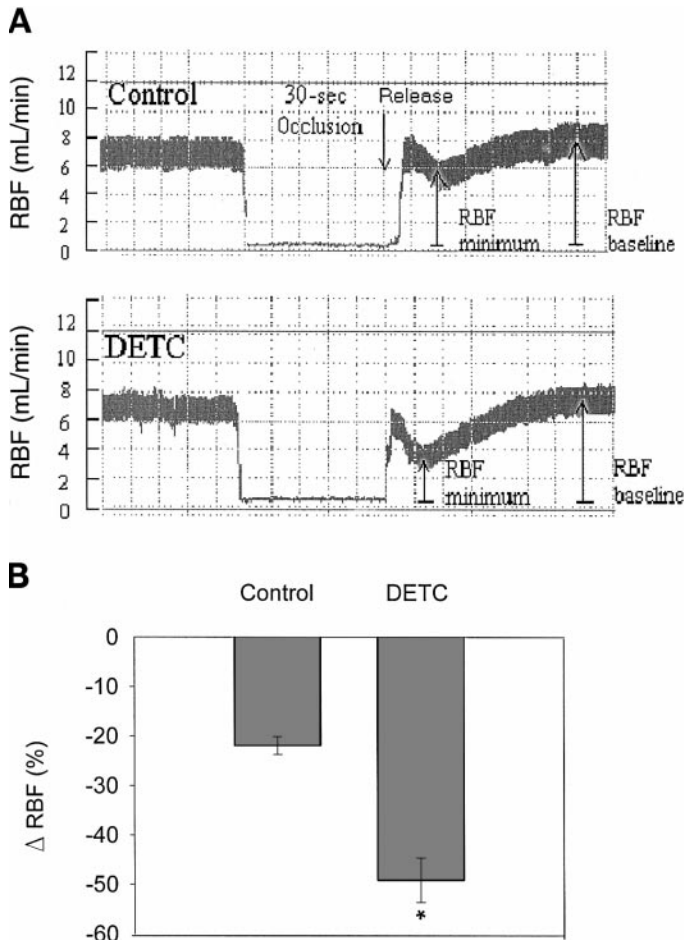
Fig. 6. Effects of SOD inhibition by DETC on production and action of endogenous adenosine in the kidney in anesthetized rats. A: representative original trace of postocclusive reduction of renal blood flow (RBF) after release of a 30-s renal artery occlusion clamp before and after intravenous infusion of DETC. B: summarized data showing postocclusive reduction of RBF in the absence or presence of DETC. *$P < 0.05$ vs. control ($n = 6$).

Considering the fact that nitric oxide (NO) inhibits 5'-ND activity in the kidney and other tissues (29, 39), it is possible that NO interacts with $O_2^-\cdot$ and, consequently, blocks $O_2^-\cdot$-induced activation of 5'-ND, thereby decreasing the production of adenosine. This interaction of NO and $O_2^-\cdot$ may importantly contribute to the regulation of 5'-ND-mediated adenosine production in the kidney.

Conventionally, it is well accepted that ROS are of only pathological consequence. However, recent studies have indicated that, under physiological conditions, low concentrations of ROS such as $O_2^-\cdot$ play an important role in the normal regulation of cell and organ function. Redox-mediated signaling is emerging as a fundamental regulatory mechanism in cell biology and physiology (38, 47). Despite the presence and ubiquity of the $O_2^-\cdot$-scavenging enzymes, intracellular steady-state levels of $O_2^-\cdot$ are ~0.1–1 nM. This small amount of $O_2^-\cdot$ in the cells can regulate the activity of a number of cellular enzymes and, hence, influence cell functions. In the present study, we determined the effects of SOD and SOD inhibitor on renal 5'-ND activity. It was found that a combination of SOD and catalase not only markedly reduced the X/XO-induced increase in adenosine production but also decreased basal 5'-ND activity in renal tissue homogenate. In contrast, SOD inhibition by DETC enhanced the activity of 5'-ND regardless of the absence or presence of X/XO. These results suggest that endogenous $O_2^-\cdot$ and its scavenging system normally present in renal tissues or cells may regulate 5'-ND activity. Therefore, adenosine levels in the kidney may be associated with redox status.

The present study also explored the mechanism by which ROS activate 5'-ND. We found that X/XO markedly increased the activity of purified 5'-ND, suggesting that $O_2^-\cdot$-induced activation of 5'-ND is related to its direct effect on this enzyme. Previous studies have indicated that membrane lipids are the major target of ROS and that lipid peroxidation is an important mech-

that 5'-ND was activated during myocardial ischemia and/or reperfusion and that increased production of adenosine in the myocardial tissue counteracted the cell injury under these circumstances (1, 24, 25). Inhibition of 5'-ND largely increased myocardial infarction induced by a 30-min coronary occlusion and 3-h reperfusion (24). These results support the view that enhanced 5'-ND activity protects the tissues or cells from the ischemic injury in the myocardium. This protective effect of 5'-ND activation has been documented in other tissues or organs (8, 26). However, the mechanism activating 5'-ND during ischemia and/or reperfusion is poorly understood. Although it is well known that tissue ischemia and/or reperfusion produces a large amount of ROS and that ROS are involved in ischemic injury (1), it remains unclear whether increased ROS could activate 5'-ND and increase adenosine production, thereby counteracting the detrimental action of those ROS. Our findings that increased production of $O_2^-\cdot$ activated 5'-ND in the kidney tissue provide direct evidence supporting this hypothesis.
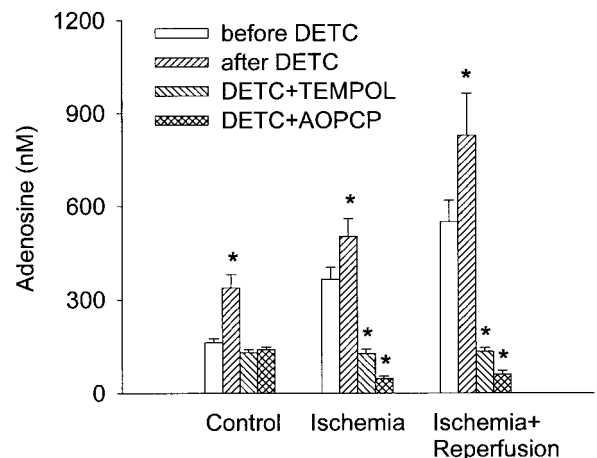

Fig. 7. Effects of SOD inhibition by DETC on production of renal adenosine during kidney ischemia and reperfusion in the presence or absence of the SOD mimetic 4-hydroxytetramethylpiperidine-1-oxyl (TEMPOL) or the 5'-ND inhibitor α,β-methylene adenosine diphosphate (AOPCP). *$P < 0.05$ vs. the values before DETC ($n = 7$).

anism resulting in cellular damage (12). However, the lipid peroxidation does not mediate ROS-induced alteration of the enzyme activity within cells. Many studies demonstrated that the change in protein structure or activity is a much more sensitive indicator of cellular exposure to ROS than lipid peroxidation (23, 38). In regard to the actions of ROS on the enzyme activity, ROS have been shown to activate many enzymes such as heme oxygenase, aconitase, tyrosine phosphatase, alkaline phosphatase, and ADP-ribosylcyclase (5, 12, 38). The dimerization of enzyme molecules induced by ROS is one of the important mechanisms mediating the effect of ROS on cellular enzyme activity (5, 16). It has been demonstrated that the dimer formation of many enzymes is due to oxidation of the cysteine residue in the enzyme molecules (5, 16, 38). Because there is an accessible cysteinyl residue in 5′-ND (3, 44), oxidation of this cysteine molecule may lead to the formation of one or several disulfide bonds of two 5′-ND monomers, which produces the dimerization of 5′-ND, resulting in the enhancement of adenosine production. To test this hypothesis, we examined the effects of a disulfide reducing system, Trx and TR, on the X/XO-induced increase in 5′-ND activity. In the presence of Trx, TR, and NADPH, disulfide bond or dimer formation of enzyme molecules would be blocked or reversed (19, 38). It was found that Trx and TR alone or in combination significantly attenuated the X/XO-induced activation of 5′-ND. This disulfide reducing system even decreased the basal activity of 5′-ND. These results suggest that formation of the disulfide bond or dimer of 5′-ND is an important mechanism mediating ROS-induced activation of 5′-ND in the kidney. ROS and the Trx/TR system may represent a redox signaling pathway in regulating the enzyme activity.

To further determine the role of redox signaling in regulating the production and actions of adenosine in the kidney, in vivo animal experiments were performed to examine the effects of SOD inhibition by DETC on the production and action of endogenous adenosine. A well-established rat model, namely, measurement of POR, was used (34, 35). In this model, renal adenosine production was increased by hydrolysis of ATP during renal artery occlusion, and the accumulated renal adenosine produced vasoconstriction and reduced RBF after release of the renal artery occlusion. Therefore, it can be used to test the effect of increased ROS on the production or action of adenosine in the kidney. Using this model, we found that the POR was significantly increased by intravenous infusion of a specific inhibitor of SOD, DETC. It seems that endogenously produced $O_2^-\cdot$ in the kidney plays an important role in regulating the production and action of adenosine in the kidney.

Although these in vivo experiments demonstrated the involvement of $O_2^-\cdot$ in the POR associated with adenosine production, it was not certain that this enhanced POR was due to increased production of adenosine. As described in previous studies, an increase in the POR can be associated with enhanced vascular reactivity to adenosine in the renal preglomerular arteries (34, 35). To further confirm the effect of oxidative stress on the production of renal adenosine in vivo, we performed microdialysis experiments to directly measure the levels of adenosine in the renal interstitium before and after inhibition of SOD by DETC. In normal or ischemic and/or reperfused kidneys, interstitial adenosine concentrations were significantly increased by intravenous infusion of DETC, which could be blocked by 5′-ND inhibition. This suggests that endogenous ROS increases adenosine production through 5′-ND and thereby exerts a tonic regulatory action on tissue adenosine levels in the kidney. By using a cell-permeable SOD mimetic, TEMPOL, DETC-induced enhancement of adenosine production in the kidney was completely blocked, further demonstrating that ROS is a specific activator of 5′-ND under different physiological or pathological conditions to produce adenosine in the kidney. These results obtained from in vivo microdialysis and HPLC analysis are consistent with those obtained from in vitro biochemical experiments supporting the view that $O_2^-\cdot$-mediated redox signaling importantly contributes to the regulation of adenosine production in the kidney.

It should be noted that renal interstitial adenosine concentrations during reperfusion after 30 min of ischemia measured in the present study were different from those obtained in a previous study, in which increased adenosine levels returned to normal after 60 min of reperfusion (32). The reason for this discrepancy remains unknown. It is possible that there is a species difference in renal adenosine response to reperfusion between rats used in the present study and dogs used in the previous study. This species difference in adenosine response may be associated with their different regulatory mechanisms. Moreover, in the previous study, renal interstitial adenosine was measured in the superficial renal cortex in dogs, but we dialyzed adenosine in the deeper cortex or outer medulla in rats. Different renal regions may have different adenosine response to reperfusion, since the deep cortex or outer medulla may restore local oxygenation more slowly than the superficial cortex. Therefore, a longer time period may be needed to restore adenosine concentrations to normal after reperfusion in our experiments.

Perspectives

The present study demonstrated that *1*) $O_2^-\cdot$ significantly increased the V_{max} of 5′-ND in the kidney tissue homogenate, *2*) scavenging of $O_2^-\cdot$ by SOD and catalase markedly attenuated the stimulative effect of endogenous or exogenous $O_2^-\cdot$ on renal 5′-ND activity, *3*) Trx and TR inhibited $O_2^-\cdot$-induced activation of 5′-ND, and *4*) inhibition of SOD by DETC produced a remarkable increase in adenosine production and adenosine-mediated reduction of RBF in response to a 30-s occlusion of the renal artery followed by release. These results indicate that redox status plays an important role in the control of 5′-ND-mediated production of adenosine in the kidney. The physiological relevance of this redox signaling in renal physiology remains to be clarified. It is plausible that this ROS-induced increase in adeno-

sine production may be an important mechanism mediating enhanced tubuloglomerular feedback response under different pathological conditions such as hypertension. In addition, the counteracting effect of adenosine increase on ROS-induced renal dysfunction and damage may participate in the adaptation response of renal tissue or cells to ischemia, hypoxia, or oxidative stress. Although adenosine has been reported to have an antioxidant effect, further studies are needed to clarify the physiological significance of adenosine-mediated antioxidant actions.

REFERENCES

1. **Abd-Elfattah AS, Ding M, and Wechsler AS.** Myocardial stunning and preconditioning: age, species, and model related differences: role of AMP-5′-nucleotidase in myocardial injury and protection. *J Card Surg* 8: 257–261, 1993.
2. **Aki Y, Shoji T, Hasui K, Fukui K, Tamaki T, Iwao H, and Abe Y.** Intrarenal vascular sites of action of adenosine and glucagon. *Jpn J Pharmacol* 54: 433–440, 1990.
3. **Bailyes EM, Soos M, Jackson P, Newby AC, Siddle K, and Luzio JP.** The existence and properties of two dimers of rat liver ecto-5′-nucleotidase. *Biochem J* 221: 369–377, 1984.
4. **Baranowski RL and Westenfelder C.** Estimation of renal interstitial adenosine and purine metabolites by microdialysis. *Am J Physiol Renal Fluid Electrolyte Physiol* 267: F174–F182, 1994.
5. **Berruet L, Muller-Steffner H, and Schuber F.** Occurrence of bovine spleen CD38/NAD$^+$ glycohydrolase disulfide-linked dimers. *Biochem Mol Biol Int* 46: 847–855, 1998.
6. **Biaggioni I.** Contrasting excitatory and inhibitory effects of adenosine in blood pressure regulation. *Hypertension* 20: 457–465, 1992.
7. **Biaggioni I and Mosqueda-Garcia R.** Adenosine in cardiovascular homeostasis and the pharmacologic control of its activity. In: *Hypertension: Pathophysiology, Diagnosis, and Management* (2nd ed.). New York: Raven, 1995, p. 1125–1140.
8. **Braun N, Lenz C, Gillardon F, Zimmermann M, and Zimmermann H.** Focal cerebral ischemia enhances glial expression of ecto-5′-nucleotidase. *Brain Res* 766: 213–226, 1997.
9. **Brezis M, Rosen SN, and Epstein FH.** The pathophysiological implications of medullary hypoxia. *Am J Kidney Dis* 13: 253–258, 1989.
10. **Carter WO, Narayanan PK, and Robinson JP.** Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J Leukoc Biol* 55: 253–258, 1994.
11. **Castilho RF, Kowaltowski AJ, and Vercesi AE.** 3,5,3′-Triiodothyronine induces mitochondrial permeability transition mediated by reactive oxygen species and membrane protein thiol oxidation. *Arch Biochem Biophys* 354: 151–157, 1998.
12. **Chakraborti T, Ghosh SK, Michael JR, Batabyal SK, and Chakraborti S.** Targets of oxidative stress in cardiovascular system. *Mol Cell Biochem* 187: 1–10, 1998.
13. **Dinour D and Brezis M.** Effects of adenosine on intrarenal oxygenation. *Am J Physiol Renal Fluid Electrolyte Physiol* 261: F787–F791, 1991.
14. **Epstein FH.** Oxygen and renal metabolism. *Kidney Int* 51: 381–385, 1997.
15. **Epstein FH, Rosen S, Galicka-Piskorska G, Spokes K, Brezis M, and Silva P.** Relation of adenosine to medullary injury in the perfused rat kidney. *Miner Electrolyte Metab* 16: 185–190, 1990.
16. **Finkel T.** Signal transduction by reactive oxygen species in non-phagocytic cells. *J Leukoc Biol* 65: 337–340, 1999.
17. **Hall JE and Granger JP.** Renal hemodynamics and arterial pressure during chronic intrarenal adenosine infusion in con-

scious dogs. *Am J Physiol Renal Fluid Electrolyte Physiol* 250: F32–F39, 1986.
18. **Headrick J, Clarke K, and Willis RJ.** Adenosine production and energy metabolism in ischaemic and metabolically stimulated rat heart. *J Mol Cell Cardiol* 21: 1089–1100, 1989.
19. **Holmgren A and Bjornstedt M.** Thioredoxin and thioredoxin reductase. *Methods Enzymol* 252: 199–208, 1995.
20. **Holz FG and Steinhausen M.** Renovascular effects of adenosine receptor agonists. *Renal Physiol* 10: 272–282, 1987.
21. **Ito A, Krinsky NI, Cunningham ML, and Peak MJ.** Comparison of the inactivation of *Bacillus subtilis* transforming DNA by the potassium superoxide and xanthine-xanthine oxidase systems for generating superoxide. *Free Radic Biol Med* 3: 111–118, 1987.
22. **Kalsi KK, Smolenski RT, and Yacoub MH.** Effects of nucleoside transport inhibitors and adenine/ribose supply on ATP concentration and adenosine production in cardiac myocytes. *Mol Cell Biochem* 180: 193–199, 1998.
23. **Kocic G, Vlahovic P, Pavlovic D, Kocic R, Cvetkovic T, and Stojanovic I.** The possible importance of the cation-binding site for the oxidative modification of liver 5′-nucleotidase. *Arch Physiol Biochem* 106: 91–99, 1998.
24. **Komamura K, Kitakaze M, Funaya H, Ueda Y, Node K, Minamino T, Kurihara T, and Hori M.** Ecto-5′-nucleotidase mediates infarct size-limiting effect by ischemic preconditioning in the rabbit heart. *J Cardiovasc Pharmacol* 30: 775–783, 1997.
25. **Lasley RD, Hegge JO, Noble MA, and Mentzer RM.** Evidence that cytosolic and ecto 5′-nucleotidases contribute equally to increased interstitial adenosine concentration during porcine myocardial ischemia. *Basic Res Cardiol* 94: 199–207, 1999.
26. **Lee HT and Emala CW.** Protective effects of renal ischemic preconditioning and adenosine pretreatment: role of A$_1$ and A$_3$ receptors. *Am J Physiol Renal Physiol* 278: F380–F387, 2000.
27. **Maessen JG, van der Vusse GJ, Vork M, and Kootstra G.** Nucleotides, nucleosides, and oxypurines in human kidneys measured by use of reversed-phase high-performance liquid chromatography. *Clin Chem* 34: 1087–1090, 1988.
28. **McCoy DE, Bhattacharya S, Olson BA, Levier DG, Arend LJ, and Spielman WS.** The renal adenosine system: structure, function, and regulation. *Semin Nephrol* 13: 31–40, 1993.
29. **Minamino T, Kitakaze M, Node K, Funaya H, and Hori M.** Inhibition of nitric oxide synthesis increases adenosine production via an extracellular pathway through activation of protein kinase C. *Circulation* 96: 1586–1592, 1997.
30. **Munzel T, Hink U, Yigit H, Macharzina R, Harrison DG, and Mulsch A.** Role of superoxide dismutase in in vivo and in vitro nitrate tolerance. *Br J Pharmacol* 127: 1224–1230, 1999.
31. **Murray RD and Churchill PC.** Effects of adenosine receptor agonists in the isolated, perfused rat kidney. *Am J Physiol Heart Circ Physiol* 247: H343–H348, 1984.
32. **Nishiyama A, Kimura S, He H, Miura K, Rahman M, Fujisawa Y, Fukui T, and Abe Y.** Renal interstitial adenosine metabolism during ischemia in dogs. *Am J Physiol Renal Physiol* 280: F231–F238, 2001.
33. **Nishiyama A, Miyatake A, Aki Y, Fukui T, Rahman M, Kimura S, and Abe Y.** Adenosine A$_1$ receptor antagonist KW-3902 prevents hypoxia-induced renal vasoconstriction. *J Pharmacol Exp Ther* 291: 988–993, 1999.
34. **Pflueger AC, Gross JM, and Knox FG.** Adenosine-induced renal vasoconstriction in diabetes mellitus rats: role of prostaglandins. *Am J Physiol Regulatory Integrative Comp Physiol* 277: R1410–R1417, 1999.
35. **Pflueger AC, Osswald H, and Knox FG.** Adenosine-induced renal vasoconstriction in diabetes mellitus rats: role of nitric oxide. *Am J Physiol Renal Physiol* 276: F340–F346, 1999.
36. **Pritsos CA, Keyes SR, and Sartorelli AC.** Effect of the superoxide dismutase inhibitor, diethyldithiocarbamate, on the cytotoxicity of mitomycin antibiotics. *Cancer Biochem Biophys* 10: 289–298, 1989.
37. **Reyes JL, Melendez E, Suarez J, Rangel M, Franco M, and Martinez F.** Modulation of the release of adenosine by glucose and chemical hypoxia in cultured renal cells. *Biochem Biophys Res Commun* 208: 970–977, 1995.

38. **Sen CK.** Redox signaling and the emerging therapeutic potential of thiol antioxidants. *Biochem Pharmacol* 55: 1747–1758, 1998.

39. **Siegfried G, Amiel C, and Friedlander G.** Inhibition of ecto-5′-nucleotidase by nitric oxide donors. Implications in renal epithelial cells. *J Biol Chem* 271: 4659–4664, 1996.

40. **Siragy HM and Linden J.** Sodium intake markedly alters renal interstitial fluid adenosine. *Hypertension* 27: 404–407, 1996.

41. **Spielman WS and Arend LJ.** Adenosine receptors and signaling in the kidney. *Hypertension* 17: 117–130, 1991.

42. **Sun QA, Wu Y, Zappacosta F, Jeang KT, Lee BJ, Hatfield DL, and Gladyshev VN.** Redox regulation of cell signaling by selenocysteine in mammalian thioredoxin reductases. *J Biol Chem* 274: 24522–24530, 1999.

43. **Vanden Hoek TL, Li C, Shao Z, Schumacker PT, and Becker LB.** Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. *J Mol Cell Cardiol* 29: 2571–2583, 1997.

44. **Worku Y, Luzio JP, and Newby AC.** Identification of histidyl and cysteinyl residues essential for catalysis by 5′-nucleotidase. *FEBS Lett* 167: 235–240, 1984.

45. **Wu F, Li PL, and Zou AP.** Microassay of 5′-nucleotidase and adenosine deaminase activity in microdissected nephron segments. *Anal Biochem* 266: 133–139, 1999.

46. **Zou AP, Billington H, Su N, and Cowley AW Jr.** Expression and actions of heme oxygenase in the renal medulla of rats. *Hypertension* 35: 342–347, 2000.

47. **Zou AP, Li N, and Cowley AW Jr.** Production and actions of superoxide in the renal medulla. *Hypertension* 37: 547–553, 2001.

48. **Zou AP, Nithipatikom K, Li PL, and Cowley AW Jr.** Role of renal medullary adenosine in the control of blood flow and sodium excretion. *Am J Physiol Regulatory Integrative Comp Physiol* 276: R790–R798, 1999.

49. **Zou AP, Wu F, Li PL, and Cowley AW Jr.** Effect of chronic salt loading on adenosine metabolism and receptor expression in renal cortex and medulla in rats. *Hypertension* 33: 511–516, 1999.