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Improved Planar Amperometric Nitric Oxide Sensor Based on Platinized Platinum Anode. 2. Direct Real-Time Measurement of NO Generated from Porcine Kidney Slices in the Presence of L-Arginine, L-Arginine Polymers, and Protamine

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Nitric oxide generation from porcine kidney slices is assessed using a new planar NO-selective amperometric sensor. The planar shape of the sensor allows for direct NO measurements near the surface (10 μm) of renal tissue slices in real time. Renal NO production may be modulated by the addition of L-arginine, arginine homopolymers (R2, R6, R10), and protamine, all of which can potentially transport across cellular membranes and provide a substrate for nitric oxide synthase within kidney parenchyma. Real-time amperometric measurements demonstrate that most L-arginine species can translocate across the cell membrane and rapidly increase NO production. However, no increase in NO generation is observed when the dimer of L-arginine (R2) is added to the solution bathing the tissue, suggesting that this species cannot permeate cell membranes. The degree of enhancement in NO generation observed for L-arginine and the larger peptides depends on the structure and follows the following sequence: R10 (decamer) > protamine > R6 (hexamer) > L-arginine. Protamine and the R10 decamer, especially, induce the largest increases in NO generation owing to their apparent rapid translocation into cells and subsequent cleavage by proteases to create high intracellular levels of L-arginine. The effect of sensor size (for sensor dimensions of 0.15- and 1-mm outer diameters) on the measured surface NO levels is also examined. The larger sensor traps more NO but hinders access of the L-arginine species to the tissue area between the flat distal plane of the sensor and the surface of the kidney slice. The use of such NO-generating peptides may be important in numerous biological systems that depend on NO production, such as ischemia−reperfusion injury and thrombogenesis.

Nitric oxide is enzymatically produced within mammalian cells via the oxidation of L-arginine by a family of enzymes known as nitric oxide synthases (NOS). The resulting NO serves as a potent vasodilator[1] and also functions as an inhibitor of platelet adhesion and activation,[2] as a mediator in a wide range of antimicrobial and antitumor activities,[3,4] and as an important neurotransmitter in the brain and peripheral nervous systems.[5] Nitric oxide is generated in a wide variety of cells and certain tissues.[6−8] Indeed, in mammalian kidneys, NO is known to play an important role in renal function by affecting both renal hemodynamics and filtration. Nitric oxide increases renal blood flow, oxygenation, and excretion of sodium and water through a decrease in pre- and postglomerular vascular resistance.[9]

Nitric oxide is also known as an important effector molecule in ischemia−reperfusion injury and long-term graft survival, which are important concerns in the transplantation of organs (e.g., kidneys,[9] heart,[10] and liver[11]). Ischemia−reperfusion injury is a major cause of organ dysfunction after transplantation. It has been suggested that stimulation of NOS activity in tissues could improve recovery from ischemic damage during transplantation.[12] These

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effects may be mediated through mechanical means, such as through vasodilation of efferent blood vessels or through alterations in the inflammatory landscape of the newly implanted organ. On the other hand, the enhanced NO generated by inducible NOS, one of the three NOS isomers, may actually have harmful effects as shown in some renal transplant models.[13] Although the exact mechanism and role of NO production in ischemia−reperfusion injury is still unclear, it is known that NOS activity and the amount of liberated NO following ischemia−reperfusion injury can affect the extent of ischemic damage, as well as the rate of repair during the injury response. Thus, the administration of a NOS substrate (L-arginine) or NOS inhibitors (e.g., N^G-monomethyl-L-arginine, N^G-nitro-L-arginine methyl ester (L-NAME), etc.), through dietary supplementation or ex vivo incubation of organs, has been suggested and its effects on functional and inflammatory characteristics in the transplantation of organs has been investigated.[14] These treatments to enhance or block the synthesis of NO from the transplanted allografts have been conducted with the ultimate goal of enhancing clinical outcomes.

In addition to L-arginine, polymers of this amino acid can be used as a substrate for NOS to play a major role in NO production in mammalian (including human) cell lines and primary tissues. Uemura et al. found by in vitro study that the cellular uptake of polyarginine (with greater than six residues) takes place not through the basic amino acid transporter but through a different, as yet unknown, translocation mechanism.[15] Uptake of L-arginine polymers has been shown to be enhanced with increasing peptide length up to 20 residues.[16] Once the polymers have translocated the cell membrane, they can provide the L-arginine substrate for NOS (after being cleaved by intracellular proteases) and thus enhance the generation of NO. Polycationic protamine may behave in a manner analogous to the L-arginine homopolymers because it consists of a group of heterogeneous peptides (average number of amino acids, 31) with ~67% arginine residues.[17] In fact, it has been reported previously that the addition of protamine to cells with NO-generating capabilities has resulted in increased production of NO.[18,19]

Quantitative studies of the NO generated from whole tissues is challenging because of the low concentrations of NO present and its short half-life due to autoxidation by reactions with endogenous oxygen or hemoglobin to form nitrite (NO_2^-) or nitrate (NO_3^-). Therefore, NO modulation induced by the presence of given species (e.g., L-arginine, polyarginine, and NOS inhibitors) has thus far been investigated indirectly through measurement of nitrite levels in the bathing medium via the Griess reaction,[15,20,21] a byproduct (L-citrulline) assay of L-arginine metabolism,[22] or by monitoring NO physiological effects (e.g., vascular tone[18]). More recently, detection of NO from the renal cortex by direct sensor insertion into the rat kidney has been attempted using a NO-selective amperometric electrode in an effort to examine the effect of a NOS inhibitor (L-NAME).[23] However, to date, there has been no comprehensive research aimed at the direct, real-time measurements of NO from tissue slices in the presence of potential substrate sources for NOS.

In the preceding paper in this series,[24] we reported an improved miniature amperometric NO sensor with a planar sensing tip based on a platinized platinum working electrode behind an outer microporous PTFE gas-permeable membrane. Direct concentration measurements (down to 2−5 nM) can be made in real time near the surface of a NO releasing/generating source (novel polymeric films that continuously release or generate (catalytically) NO at fluxes comparable to normal endothelial cells).[24,25] This sensor is an ideal tool to monitor local concentrations of NO near the surfaces of biological tissues, by positioning the small sensor (with a response time fast enough to follow NO release in real time) in close proximity to the surface of the NO-emitting tissues. Herein, we describe the application of this amperometric NO measuring method for direct, quantitative, and real-time detection of NO generation from porcine kidney tissue slices in the absence and presence of L-arginine, homopolymers of L-arginine, and protamine. The data presented may ultimately be useful in optimizing the composition of solutions that are used to store procured organs for transplantation if further studies prove the benefits of given NO levels in preventing cold storage, ischemia, and reperfusion injury.

EXPERIMENTAL SECTION

Materials. University of Wisconsin (UW) solution was purchased from Dupont Pharmaceuticals (Wilmington, DE). L-Arginine and phosphate-buffered saline (PBS, pH 7.4) were products of Sigma (St. Louis, MO). H-Arg-Arg-OH (dimer of arginine, R2) and H-Arg-Arg-Arg-Arg-Arg-Arg-OH (hexamer of arginine, R6) were purchased from Bachem Bioscience, Inc. (King of Prussia, PA). H-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-OH (decamer of arginine, R10) was obtained from New England Peptide, Inc. (Fitchburg, MA). Protamine sulfate (Clupeine) was from Sigma. Nitric oxide and argon gases were products of Cryogenic Gases (Detroit, MI). All solutions were prepared with 18 MΩ cm^{-1} deionized water with reagent grade materials as obtained without further purification.

Animal and Tissue Preparation. Fresh porcine kidneys were procured from juvenile farm swine weighing 20−35 kg. The animals were anesthetized using isoflurane and kept under mechanical ventilation on 21% oxygen. Animals were infused with lactated Ringers solution during mechanical ventilation, at a rate of 125 mL/h. Both the left and right kidneys were removed and

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immediately flushed via the renal artery with cold (under 2 °C) UW solution. The kidneys were stored in cold UW solution on ice until they were used for NO detection experiments (within 48 h). Prior to each experiment, the kidneys were placed on ice and ~2-mm-thick transverse slices were prepared using a scalpel. The kidney slices were washed extensively with PBS to remove dead cells and free proteins from the surface. Tissue slices were then placed in glass dishes containing 5 mL of PBS for the measurement of NO.

Surface NO Measurements. The amperometric gas sensors used in this work were composed of a platinized Pt working electrode (platinized Pt disk (with 76-μm o.d.) sealed in glass (with 0.15- or 1-mm o.d.)) with a Ag/AgCl wire (127-μm o.d.) reference/counter electrode, housed behind an outer PTFE gas-permeable membrane. The detailed preparation procedures for this NO sensor design were previously described (see Figure 1 in the preceding paper[24]). The sensors were polarized at +0.75 V for 3−5 h to achieve stable background currents prior to their initial use. The sensors were then calibrated before and after NO measurements using standard NO solutions (assuming a concentration of NO in the saturated stock solution of 1.9 mM) prepared by bubbling of NO gas into PBS solutions deaerated by Ar gas purging. Resulting currents were measured using a high-sensitivity two-electrode microsensor module (Diamond Electro-Tech Inc., Ann Arbor, MI) using an applied voltage of +0.75 V. Output currents were recorded using a DATAQ Instruments DATAQ-700 USB data acquisition card (Akron, OH) with accompanying WinDAQ/Lite software.

For surface NO measurements, NO sensors were positioned 10 μm above kidney tissue slices immersed in PBS using micromanipulators (World Precision Instrumentation Inc., Sarasota, FL). After the amperometric signal reached a stable steady-state baseline (~10 min), 1 mM L-arginine or its equivalent molar concentration of the various arginine-rich peptides (except for protamine, see below) was added to the buffer to achieve the same number of arginine residues in solution for all experiments. The solution was gently stirred (for a few seconds) and the response of the NO sensor observed continuously for a 30−60-min period. The measured current was converted to the concentration of NO using a prior calibration curve recorded immediately before surface measurements were started. The increases in NO levels observed over this time were compared to the initial steady-state baseline values (buffer only) and the average percent increase was calculated for all separate experiments with the same species.

Comparisons between data groups (change in NO levels for different arginine species) were carried out using a paired Student's *t*-test (StatView for Windows, SAS Institute Inc, Cary NC, version 5.0.1). A p value of <0.05 was taken as the cutoff for statistical significance between the data sets.

RESULTS AND DISCUSSION

NO Generation from Porcine Kidney Slices. The kidney slices, cut transversely, have three distinct areas, distinguished as the renal cortex, medulla, and pelvis. In initial feasibility experiments, the NO sensor was placed 10 μm over both the renal medulla and pelvis to measure the basal concentrations of NO emitted from the different sections of the kidney slices. Nitric oxide generated from both areas of the kidney slices in PBS buffer was observed to be reasonably stable for at least 1 h but began to decrease slightly after this period (Figure 1). It has been reported that kidneys can be preserved for up to 60 h in cold UW solution,[26] a solution that contains many of the nutrients required to maintain tissue cell activity. Indeed, kidney tissues preserved here were successfully used for NO measurement experiments for a period up to 2 d after they were harvested from the animals.

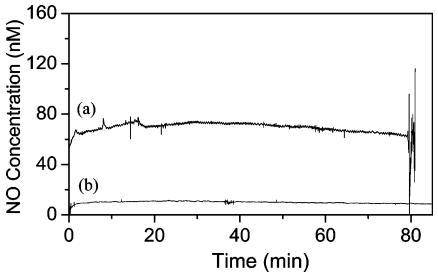

Figure 1. Nitric oxide measurements as a function of time for a porcine kidney slice immersed in PBS buffer. The NO sensor (1-mm o.d.) was positioned 10 μm above the surface of the (a) renal medulla and (b) renal pelvis to measure surface NO concentrations.

As shown in Figure 1, the detected amounts of NO over the renal medulla and pelvis were significantly different even for the same kidney slice. Much higher NO concentrations were observed for the medulla (~70 nM) compared to the pelvis (~10 nM). This observation agrees very well with previous studies on the quantitative distribution of NOS enzyme in the mammalian kidney, indicating that the renal medulla is the area most enriched in NOS compared to other sections of the kidney.[9] Because of its high NOS content, sensors were positioned over the renal medulla rather than the pelvis for all subsequent experiments. Basal NO concentrations measured using NO sensors with a 1-mm tip diameter positioned directly over the renal medulla (at 10-μm distance) were in the range of 50−150 nM depending on the exact thickness of the kidney slice as well as the condition of individual kidneys. The detected concentration of NO close to the surface of the tissue slice was also dependent on the sensor size, as described elsewhere[24] (see also below). The analytical stability of the sensors (within ±10% variations) was confirmed by calibration before and after NO measurements.

Effects of the Addition of L-Arginine Species to Bathing PBS Buffer. Supplementary administration of L-arginine has been investigated previously to enhance kidney function in renal ischemia−reperfusion injury (i.e., organ transplantation).[12] L-Arginine polymers have also been shown to have beneficial effects in other tissue types (e.g., coronary artery,[20] jugular vein,[21] and umbilical vein[15]). Here, changes in NO generation were monitored by adding L-arginine or L-arginine polymers of differing lengths (R2, R6, R10). In addition, the effect of protamine was also investigated, as it is another possible candidate as an NO enhancing material, with its high content of L-arginine (~67%).[17]

After a relatively stable basal level of NO was observed over the renal medulla, the given L-arginine species was added to the bathing PBS solution and the upper solution in the beaker containing the kidney slice was stirred gently for a few seconds to induce a homogeneous delivery of the species to the tissue. Even though stirring was as mild as possible, it often invoked a large noise spike in the sensor's output and caused baseline shifts by perturbing the pre-established steady-state NO diffusion layer around the sensing tip (in proximity to the kidney slices).

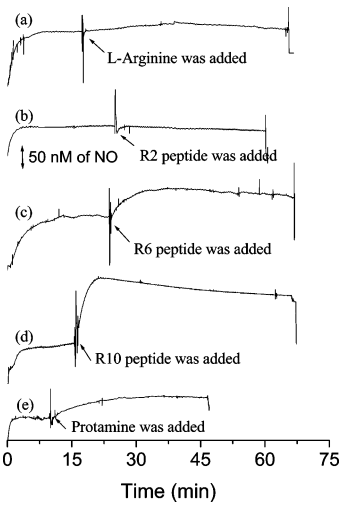

Figure 2. Representative NO release profiles from medulla portion of kidney slices as a function of time generated by the additions of 1 mM L-arginine (a) and different L-arginine homopolymers; (b) 0.5 mM R2 (dimer), (c) 0.167 mM R6 (hexamer), (d) 0.1 mM R10 (decamer), and (e) 0.1 mM protamine. The NO sensor was positioned 10 μm above the tissue slice in each experiment. (note: traces are offset for clarity, but concentration scale is the same for all).

Similarly, when experiments were terminated and the sensor was removed from near the tissue surface, a spike was also seen in the sensor output. Nonetheless, continuous NO measurements after the addition of the test species clearly provides information about the effects of the given species on relative changes in NO generation by the kidney parenchyma. The signals of NO sensors were also monitored in a PBS buffer solution (in the absence of kidney slices), and the addition of L-arginine, L-arginine polymers, and protamine exhibited no change in the sensors' signals. Thus, it was verified that the amperometric signal changes presented in the following sections were due to the variations of NO generation from the kidney cells induced by the addition of the given arginine species.

(a) L-Arginine. The addition of L-arginine (to achieve a final concentration of 1 mM in the tissue bathing solution) exhibited two different trends: either a slight increase (23 ± 15% increase compared to the initial steady-state baseline values) or no change in NO generation. A representative NO generation profile as a function of time is shown in Figure 2a. Considering that the final product of L-arginine metabolism through NOS activity is NO, these experimental results suggest that there is very little net transport of L-arginine into the cells. This may be attributed to the transport mechanism of L-arginine (basic amino acid transport), which is based upon the concentration gradient of the amino acid across the cell membrane.[27] If the kidney has a sufficient amount of basic amino acids already present inside the cell, additional L-arginine will not be transported through the cell membrane, and thus, no affect on the rate of NO generation will be observed. On the other hand, if the tissue has a somewhat lower intracellular concentration (as compared to the extracellular milieu), additional L-arginine is able to enter the cell and increase the substrate concentration for NOS. In such a manner, NO generation from the kidney slice will increase.

(b) L-Arginine Polymers and Protamine. We hypothesize that the addition of L-arginine polymers can increase NO production by kidneys (and kidney slices) if such species can be delivered into the cells and cleaved into free L-arginine monomer. We evaluated the effect of a dimer (R2), a hexamer (R6), and a decamer (R10) of L-arginine on NO generation from porcine kidney tissues, and typical results are illustrated in Figure 2b–d. Under no circumstances was any change in the measured NO level observed when R2 was added to the bathing solution (at a concentration to yield the equivalent of 1 mM total L-arginine). In contrast, the addition of both R6 or R10 (with total final L-arginine monomer levels equivalent to 1 mM) enhanced NO production in all experiments. The measured surface NO levels showed a more rapid and much greater increase in concentration upon the addition of R6 or R10 compared to that observed upon the addition of L-arginine monomer (compare, for example, Figure 2a–d).

In the case of R6 and R10 additions, the increased NO concentrations usually reached maximum values within 30 min after their addition and then started to slowly decrease. Such a dynamic NO increase upon addition of the peptides is likely related to a combination of the rate of cellular uptake or enzymatic cleavage, NOS activity, and the size of sensor, which can affect the access/mass transport of the peptides into the cells within the kidney slices (i.e., blocking effect, see below).

Since the total number of L-arginine molecules was kept identical in all experiments, the differences in the detected NO concentrations must be dependent on the characteristics of the cellular uptake of the various L-arginine species. No change in NO production upon the addition of R2 suggests that R2 cannot be transported into kidney cells. In contrast, the rapid and consistent increase in measured NO after the addition of R6 or R10 indicates the extremely rapid cellular uptake of these peptides. The transport mechanism for R10 is different from the L-arginine monomer transport mechanism (basic amino acid transporter) since the addition of R10 induced the increase in NO in every experiment, though the percent increase varied in the different trials (see Table 1). Indeed, these experimental observations are in accord with a previous report that polymers containing more than five residues of L-arginine efficiently translocate the membranes of biological cells independently of the membrane basic amino acid transport system.[16] Figure 2 and Table 1 clearly show that the observed NO enhancement is greater in the case of R10 than that of R6. This can possibly be ascribed to the higher cellular uptake rate of R10 compared to R6. In fact, it has been reported that the greatest uptake rate of L-arginine polymers is obtained using oligomers composed of between 7 and 20 arginine residues.[16] The reason for the occasional failure of the R6 to increase NO production significantly (see Table 1) is not clear but is likely related to its slower rate of translocation and, hence, its inability to consistently increase steady-state L-arginine levels intracellularly.

Interestingly, the addition of naturally occurring protamine (a polycationic peptide often used to neutralize the anticoagulant activity of heparin) also induces a significant increase in NO generation (see Figure 2e). These increases with protamine were all statistically significant compared to all the other agents tested,

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Table 1. Percentage Increase in NO Concentration Determined 10 μm above the Renal Medulla Region (Compared to Initial Basal Levels) for Various L-Arginine Species Added to Solution Bathing Kidney Slices in a Series of Separate Experiments[a,b]

expt no.	L-arginine 1 mM	R2 0.5 mM	R6 0.167 mM	R10 0.1 mM	protamine 0.1 mM	protamine (normalized)[c]
1	47 (1)	0 (1)	0 (2)	179 (2)	89 (3)	47
2	0 (2)	0 (3)	9 (4.5)	55 (3)	67 (4)	35
3	0 (3)	0 (4)	23 (5)	133 (4)	82 (5.5)	43
4	16 (3)	0 (5)	41 (6.5)	58 (5)	47 (7)	25
5	21 (4)	0 (9.5)	26 (9)	62 (6.5)	70 (8)	37
6	0 (4.5)	0 (19)	0 (12)	82 (7.5)	54 (8.5)	44
7	33 (6)	0 (24)	48 (22.5)	91 (24)	83 (18)	23
8	7 (8)	0 (32)	0 (23.5)	65 (31)	43 (19.5)	43
9	0 (27.5)		37 (23.5)		82 (22)	28
10	11 (30)					
average	14	0	20	91	69	36
Std Dev	16	0	19	44	17	9

[a] Except for protamine, equivalent numbers of arginine residues (to achieve 1 mM L-arginine in test solutions as a final concentration) were added. [b] Numbers in parentheses represent time in hours after harvesting kidneys. [c] Percentage increase in NO concentration compared to initial basal levels when normalized for a concentration of L-arginine residues of 1 mM.

except for the R10 peptide. The pattern of NO increase was similar to that observed upon the addition of R6 or R10, i.e., a rapid increase directly after the addition, with a maximum NO level reached within 30 min. Because the concentration of protamine in the test bathing solution was 0.1 mM, the final concentration of L-arginine residues in the solution was estimated as 1.9 mM (1.9 times more than the case of L-arginine, R2, R6, and R10), if one assumes protamine's composition to be 67% arginine (in terms of the number of arginine residues).[17] When normalized for a concentration of L-arginine residues of 1 mM, the induced NO increase by the addition of protamine is between those achieved with R6 and R10, as shown in Figure 2e (also see Table 1). This suggests that the translocation of protamine into kidney cells may be less efficient than R10 while more efficient than R6. This observation is consistent with the previous report cited above, regarding the cellular uptake of homopolymers of L-arginine, with the most effective uptake occurring for polymers of lengths between 7 and 20 residues. Indeed, it has been demonstrated that longer polymers (RN when $N > 20$) of L-arginine are less effectively transported into cells.[16] Because protamine is a longer peptide, composed of 30−33 amino acids, one would expect less efficient cellular uptake of protamine compared to R10. Even though the degree of NO production increase per arginine residue is somewhat smaller in the case of protamine than R10, the use of protamine to enhance NO generation intracellularly is appealing since this species is readily available at low cost.

Three subsequent experiments with an adjusted concentration of protamine (i.e., 1 mM total L-arginine residues) instead of 0.1 mM protamine (i.e., 1.9 mM total L-arginine residues) yielded 31, 39, and 45% increases in NO concentration, respectively. The average of these three values is a 38 (±7)% increase, which is close to the average percentage increase (36 ± 9% increase) determined by normalization of the data obtained with 0.1 mM protamine (1.9 mM L-arginine) (see Table 1). These experimental results support a linear relationship between protamine concentration and NO generation as assumed for the normalization of the data obtained with 0.1 mM protaime shown in Table 1.

To summarize the effects of the different peptides, the average percent increase from the basal NO level (Δ[NO]) was calculated

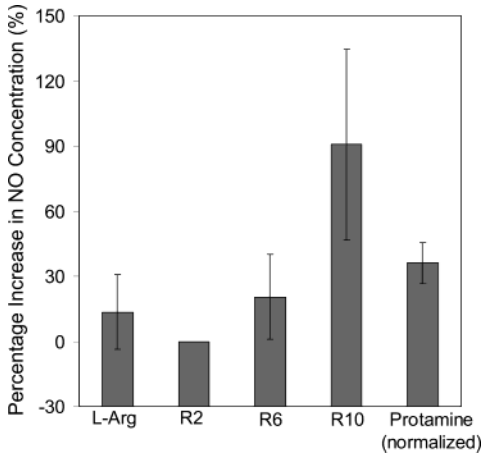

Figure 3. Percentage increase in NO concentration (average ± standard deviation in Table 1) determined 10 μm over the renal medulla region of kidney tissue slices induced by the additions of various L-arginine species to the solution bathing kidney slices.

for the addition of each L-arginine species to the bathing solution. The results are summarized in Table 1 and Figure 3. The calculated standard deviations were large for each of the different species tested. The reason for this variability might be due to differences in the cold storage time of the individual kidney slices, physiologic factors associated with the donor swine, or differences in the slice thickness, as well as technical factors that limit the ability to reproducibly place the planar sensor at the exact same distance above the tissue slices for each separate experiment. However, no apparent correlation between the cold storage time and the generated NO concentration of kidney slices is observed (see Table 1); therefore, factors other than storage time are likely the major reasons for the data variability. Discrepancies in the initial basal levels resulted in the different relative increases in NO reported (as percent of basal level). Though the calculated Δ[NO] has a wide range for each L-arginine species examined, the effect on NO generation for the various peptides tested clearly reveals the following trend: R10 > protamine ≥ R6 ≥ L-arginine > R2 = 0. Using a paired Student's *t*-test (with a significance level

of $p < 0.05$, normalized values for a concentration of L-arginine residue of 1 mM were used for protamine), the differences between the following pairs of data in Table 1 were determined to be significant: L-arginine/R2 ($p = 0.0399$); L-arginine/R10 ($p = 0.0016$); L-arginine/protamine ($p = 0.0035$); R2/R6 ($p = 0.0276$); R2/R10 ($p = 0.006$); R2/protamine ($p < 0.0001$); R6/R10 ($p = 0.0045$); R10/protamine ($p = 0.0065$) (i.e., all pairs other than L-arginine/R6 and R6/protamine). In addition, one-way analysis of variance (ANOVA statistics)[28] was used for all data sets. It was found that the same data sets were statistically different (based on $p < 0.05$) using this approach. However, the R6/protamine pair is also statistically different using this ANOVA method of data analysis.

Sensor Size Effect. We have shown previously, both experimentally and theoretically, that the level of NO detected by the new planar amperometric sensor in proximity to the surface of a NO-emitting material is dependent on the size (diameter) of the sensing probe (see part 1).[24] That is, there is a NO trapping effect when a large-diameter sensor with a planar geometry is moved to within 10−70 μm of a NO-emitting surface.[24] This trapping effect will yield a falsely elevated surface concentration of NO. Although a smaller diameter sensor results in more accurate concentration values, the analyte trapping effect under the larger diameter sensor is actually an advantage for probing "relative changes" in NO emission rates from samples in which the NO flux is quite low (such as the experiments reported herein). In effect, the larger diameter sensor provides a more sensitive measurement tool, since even low NO fluxes give rise to a greater amperometric sensor signal (falsely elevated due to the analyte trapping effect) than would be detected by a sensor with a smaller diameter distal sensing tip.

To examine the effect of sensor size on the basal NO level detected from the kidney slices, as well as on the observed increases in NO generation upon adding the L-arginine species, planar amperometric NO gas sensors possessing either a 0.15- or 1-mm o.d. were examined. The center Pt electrode diameter for each type of working sensor was fixed at 76 μm, regardless of the size of insulator. Nitric oxide generated from kidney slices was measured as a function of time with the 0.15-mm-o.d. sensor upon treatment with L-arginine or R10. Typical data for the NO concentration profiles using this smaller o.d. sensor are shown in Figure 4. It can be seen that the initial basal level of NO before the addition of the L-arginine species is significantly less than that observed when using the 1-mm-o.d. sensors (see Figure 2). The larger diameter insulator sheath traps more NO emitted from the surface of the kidney slices, inhibiting free diffusion of the NO from the thin gap between the sensor and the tissue. This effect results in an elevated local NO concentration for a given flux of NO. Even though the observed basal NO levels exhibited a wide range of values (depending mainly on the condition of the kidneys), the basal values obtained with the small sensor (0.15-mm diameter) were always much lower than the value range obtained with the larger sensor (1-mm diameter).

In addition to the different basal NO levels, the degree of enhancement in NO induced by the various L-arginine species can also be affected by the sensor size. If the sensor has a large

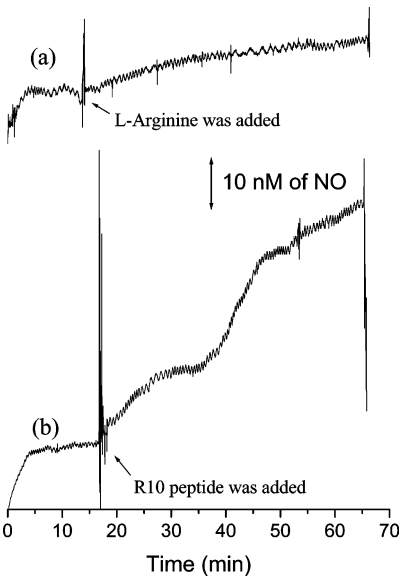

Figure 4. Representative NO release profiles from the medulla region of porcine kidney slices as a function of time obtained with a sensor possessing a 0.15-mm o.d. upon addition of 1 mM L-arginine (a) and 0.1 mM R10 decamer (b). Both sensors were positioned 10 μm over the surface of the kidney slice. (Note: traces are offset for clarity, but concentration scale is the same for both).

outer insulator sheath, yet is positioned tightly near the surface of the cells, this positioning potentially blocks access of the L-arginine species to the cells that the sensor (i.e., the centered electroactive platinized Pt area of the sensor) is monitoring. In the case of the sensor with a smaller outer diameter, access may not be as restricted. Thus, when the same amount and type of L-arginine species is added to the test solution, a more enhanced increase in NO generation (% change) may be observed using the sensor with a smaller outer diameter. Figure 4 illustrates this effect. The small sensor (0.15-mm diameter) exhibits a much lower initial basal level but a much larger increase in NO release (relative to the initial basal NO amount) following addition of either L-arginine or R10 as compared to the large sensor (1-mm diameter, see Figure 2).

Another distinct difference in NO measurements with the different size sensors was the dynamics of the observed NO increase. Indeed, the NO concentration profiles obtained with the 1-mm-o.d. sensor exhibited a relatively fast increase in NO surface levels after the addition of the L-arginine species, followed by a gradual decrease in the measured NO. In contrast, surface NO levels detected with the 0.15-mm-o.d. sensor increased more slowly over a longer time period and never actually decreases when monitored for a similar time period as in experiments using the 1-mm-o.d. sensor. This difference can be attributed to the real NO concentration at the surface of the kidney slices. Nitric oxide is converted to nitrite through its autoxidation reaction (second order in NO concentration and first order in oxygen concentration) with oxygen in aqueous solution. Because of the NO trapping effect with the larger diameter sensor, there is a higher concentration of NO in the sensing region (between the sensor and the tissue slice) and addition of the L-arginine species causes the NO levels in this area to become even higher. A high concentration of NO decays more rapidly through the autoxidation reaction with oxygen. Therefore, the lifetime of the enhanced NO under the

(28) Clarke, G. M.; Cooke, D. *A Basic Course in Statistics*, 4th ed.; Arnold: London, 1998; Chapter 22.

larger area sensor is likely to be shorter and this may account for the observed decrease in NO after a certain period of time (10−30 min after the addition of L-arginine species) when using the larger sensor to monitor surface NO levels.

CONCLUSIONS

Real-time measurements of NO generated from porcine kidney tissue slices were achieved using a new amperometric planar NO gas sensor. The flat geometry of the sensor is well suited to measure NO directly near the surface of kidney cells that continually release NO due to intracellular NOS activity. The monitored initial NO profiles as a function of time show relatively stable and steady NO levels from both the renal medulla and pelvis regions, but the renal medulla area generates much greater steady-state levels of NO. The NO generated from the renal medulla was markedly enhanced by the administration of select L-arginine species, in particular R10 and protamine. Further, the outer diameter of the planar amperometric NO sensor was found to play an important role in enabling NO measurements to be made near the surface of kidney slices. Larger diameter sensors yield higher basal NO values owing to a greater NO trapping effect. However, as a result, there was less relative enhancement in NO generation upon the addition of the L-arginine species. Smaller diameter NO sensors produce lower steady-state basal levels of NO but larger relative changes upon addition of the L-arginine species.

The significant increase in NO generation from kidney cells observed with R10 and protamine suggests that these species may be good candidates to increase local concentrations of NO within kidney cells. Indeed, the presence of these species to increase NO production during storage and transport of organs may find benefit to help ameliorate the deleterious effects of ischemia−reperfusion injury when organs (such as kidneys and livers) are used for transplantation. Further animal studies to examine the potential benefit of such additives for kidney and liver storage are in progress.

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