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**ARTICLE** *in* ANALYTICAL CHEMISTRY · JULY 2008

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# Amperometric ATP Microbiosensors for the Analysis of Chemosensitivity at Rat Carotid Bodies

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**The physiological application of amperometric adenosine triphosphate (ATP) microbiosensors for characterizing the stimulus-response at rat carotid bodies superfused with high potassium concentrations, during normoxic hypercapnia, and during hypoxia is demonstrated using the peripheral arterial chemoreceptors in the carotid body of rats as a model system. Amperometric microbiosensors based on glucose oxidase (GOD) and hexokinase (HEX) immobilized within a polymer matrix at the surface of Pt disk microelectrodes (diameter: 25 $\mu$m) are positioned at a distance of approximately 100 $\mu$m above the carotid body surface for detecting extracellular ATP. A linear calibration function of ATP microbiosensors in the physiologically relevant concentration range of 0–40 $\mu$M ATP enables quantitative detection of ATP released at the carotid body surface in response to physiological stimuli. It is shown that these stimuli induce extracellular ATP release from the carotid body at levels of 4–10 $\mu$M. Other electroactive neurotransmitters such as, e.g., catecholamines are coreleased by the carotid body at hypercapnic, hypoxic and high-potassium stimulus, are simultaneously detected utilizing a dual-electrode assembly with an ATP microbiosensor and a second bare channel providing a colocalized reference measurement for ATP quantification.**

Miniaturized biosensors are currently emerging as a suitable methodology for investigating neurochemicals.[1] Among the wide variety of transduction technologies, amperometric microbiosensors have attracted particular attention for real-time *in situ* neurotransmitter monitoring with suitable spatial and temporal resolution. Microelectrode-based biosensors have been used in clinical studies to determine the release of acetylcholine,[2] ATP,[3,4] adenosine, [5–7] and lactate.[8,9] However, detection and especially quantification of ATP at a cellular level is a challenging task and has been the focus of research for several decades.

Quantification of physiologically relevant ATP concentrations has been achieved by liquid chromatography,[10–15] fluorescence,[16,17] chemiluminescence,[18–20] bioluminescence,[21–24] and amperometric biosensors.[3,25–27] Among these techniques, amperometric ATP microbiosensors provide the required spatial and temporal resolution for *in situ* monitoring of ATP released at biological tissues.

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10.1021/ac7018969 CCC: $40.75 © 2008 American Chemical Society
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Consequently, clinical applications of microbiosensors for physiologically relevant analytes including the detection of ATP released from the spinal network[3] and from the ventral medulla[28] at hypoxic conditions have recently attracted attention.

Dual-enzyme assays provide a suitable strategy for amperometric ATP detection, e.g., the successive reaction of glycerol and ATP with glycerol kinase and glycerol-3-phosphate oxidase immobilized in a sol–gel matrix.[3] Alternatively, a competition assay based on GOD and HEX entrapped in a polymer matrix at Pt microelectrodes has demonstrated sufficient sensitivity for ATP detection in the biologically relevant range,[26,29] whereby the analytical signal is generated by the reaction of glucose and ATP with hexokinase. Recently, an immobilization scheme based on self-assembled monolayers yielded ATP microbiosensors sensitive to $\mu$M ATP concentrations.[30] Hence, a decrease in the concentration of $H_2O_2$ generated by the GOD catalyzed reaction at a constant glucose concentration is indicative for the presence of ATP.[31] The electroactive molecule $H_2O_2$ generated in the enzymatic conversion is oxidized at the amperometric microbiosensor biased at 650 mV vs Ag/AgCl quasi-reference electrode (AgQREF). This competition enzyme assay format has been used for locally detecting ATP release at superfused carotid body preparations in response to a variety of stimuli presented in these *in vitro* studies (Figure 1).

The carotid body is a chemosensory organ instrumental in monitoring the chemical composition of arterial blood via reflex control of ventilation. In response to hypoxia (decreased $PO_2$), hypercapnia (increased $PCO_2$), or decreased pH, the carotid body releases a cascade of neurotransmitters for regulating these parameters.[32] While there is general agreement on the main inhibitory transmitter system mediating chemosensitivity via dopamine and inhibitory D2-dopamine receptors, the key neurotransmitter system mediating excitatory response remains controversial.[33,34] However, recent evidence from pharmacological[35] and genetic studies[36] strongly suggests that the excitatory release of ATP and binding via postsynaptic P2X2 receptors may be key to the regulatory system in mediating excitatory responses from the carotid body. The carotid body represents an ideal model system for demonstrating the physiological applicability of ATP microbiosensors, as (i) the peripheral arterial chemoreceptors of the carotid body respond to changes in $O_2$ and $CO_2$ tension, pH, potassium levels and glucose concentrations, which are parameters that could also affect the reliability of ATP measurements; (ii) the carotid body contains multiple excitatory and inhibitory neurotransmitters including catecholamines that may interfere with the sensing capabilities; and (iii) the dimensions of the carotid body (approximately 500 $\mu$m) necessitate the application of dimensionally corresponding microbiosensor techniques. Hence, within this study it is proposed that such amperometric microbiosensors could reliably detect ATP released at superfused carotid body preparations in response to relevant physiological stimuli mediating ATP release. The present data demonstrates accurate and reproducible measurements toward a variety of physiological stimuli, which substantiates the potential of amperometric microbiosensors for *in vitro* studies, and establishes broad applicability of amperometric microbiosensors for *in situ* investigations at a wide variety of organ systems.

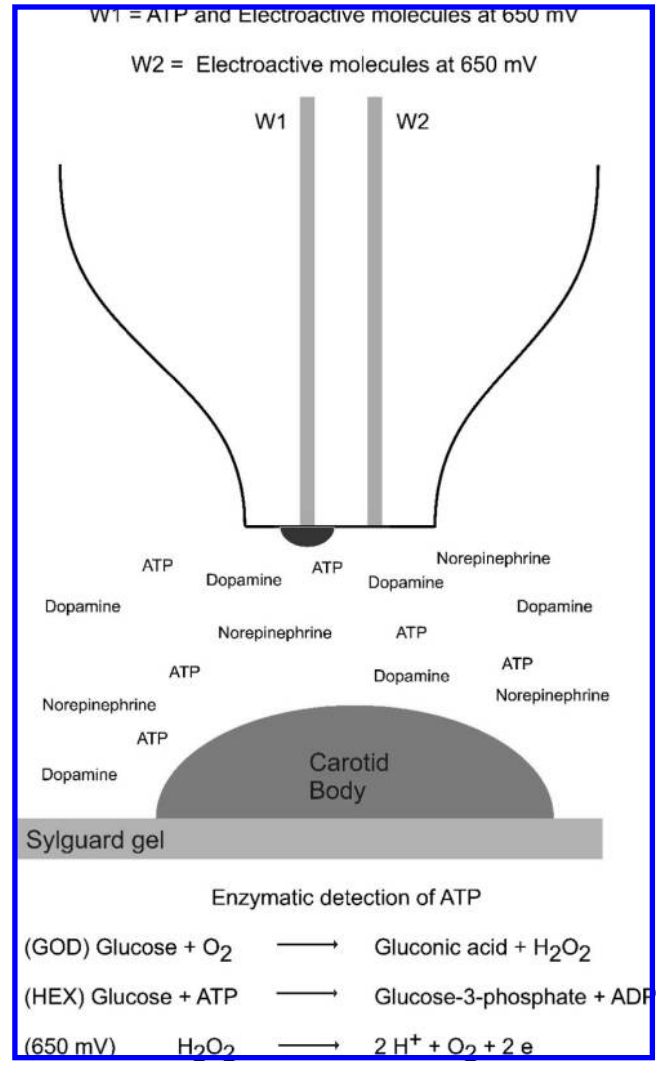


**Figure 1.** Detection principle for amperometric dual-enzyme ATP microbiosensors at the carotid body.

## EXPERIMENTAL SECTION

**Carotid Body Preparations.** Sprague–Dawley rat pups between 16 and 20 days of life were investigated in this study. Rats were anesthetized by inhalation of isoflurane for approximately 1–2 min. Following decapitation, the bifurcation of the artery including the carotid body, the superior cervical ganglion, and the petrosal ganglia was removed *en bloc*. Thereafter, the tissue was treated with a phosphate buffer saline solution containing 0.01% trypsin, and 0.05% of collagenase (Worthington type II) for


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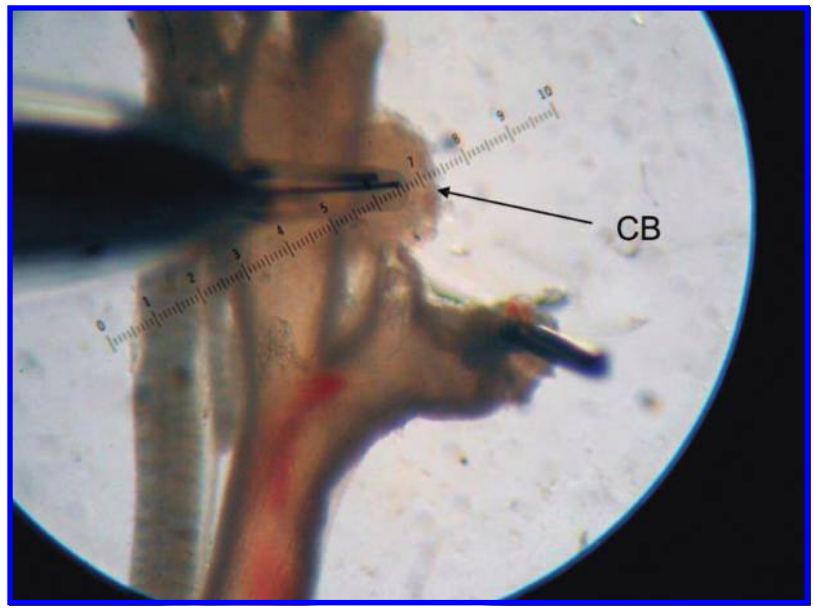


**Figure 2.** Image of a dual-barrel ATP microbiosensor located above a superfused carotid body sample. The carotid body typically has a diameter of approximately 500 $\mu$m diameter in rat pups.

20 min. The ganglia were removed and the surrounding bifurcation was cleared of connective tissue, thereby exposing the carotid body. The carotid body was transferred into a Sylguard-based perfusion cell and superfused with 3 mL/min of Ringer's solution at 37 °C until use, which was typically within 10–15 min. Otherwise, carotid body preparations were stored for a few hours in cold Ringer's solution sparged with a mixture of 95% $O_2$/5% $CO_2$. The composition of Ringer's solution, hypoxic O% $O_2$, and acidotic hypercapnic (10% $CO_2$) experiments was 125 mM NaCl, 5 mM KCl, 1 mM $NaH_2PO_4$, 1 mM $MgSO_4$, 11 mM glucose, 26 mM $NaHCO_3$, and 2 mM $CaCl_2$. Sparging Ringer's solution with 5% $CO_2$ titrates the pH to 7.4 at 37 °C. Test solutions using high potassium were identical to the Ringer's solution with the exception of 60 mM KCl.

**Determination of ATP Released at the Carotid Body.** Amperometric ATP microbiosensors were prepared by immobilizing glucose oxidase (GOD) and hexokinase (HEX) at Pt disk electrodes with a diameter of 25 $\mu$m via pH-shift induced precipitation polymerization, as previously described.[26,29] Within the present experiments, three polymerization cycles were applied. A 25 $\mu$m substrate electrode for ATP microbiosensors significantly improves the sensitivity to ATP compared to the initial studies reported by Kueng et al. utilizing a 10 $\mu$m ATP microbiosensor.[29] For measurements at the carotid body surface, the sensor was positioned at approximately 100 $\mu$m above the carotid body surface utilizing a motorized micromanipulator (Figure 2) and an inverted microscope. A standard calomel electrode (SCE) and a Pt disk (1 mm diameter) counter electrode complemented the electrochemical cell. The amperometric ATP microbiosensor was biased at 650 mV vs SCE with a sampling rate of 10 Hz using a bipotentiostat (CH832A, CH Instruments, Austin, TX). The resulting amperometric $i-t$ curve was processed using Matlab (Matlab 7.0, Mathworks, Natick, MA). Repetitive stimulation of the carotid body during periods of 3 min with 60 mM $K^+$ in Ringer's solution (high-potassium), with 10% $CO_2$ /21% $O_2$/69% $N_2$ in Ringer's solution (normoxic hypercapnia), or with $N_2$ (hypoxia, [$O_2$] < 1 ppm) induces ATP release from the carotid body. The carotid bodies are superfused at 3 mL/min in a perfusion cell with a diameter of 35 mm. A time of 3 min rinsing the cell with control buffer between stimulations was required for restoring the baseline of the sensor enabling accurate quantification. Furthermore, an *in situ* 1-point recalibration of the sensor with 10 $\mu$M ATP was performed accounting for sensor-to-sensor variability. Typically, cycles of 3–4 consecutive measurements for each stimulus were performed with each carotid body. The errors reported for each measurement represent two standard deviations of the mean ATP concentration.

**Self-Referencing Dual-Barrel Electrodes.** In response to hypoxia catecholamines (in particular dopamine and norepinephrine) are released from the carotid body and thus ATP would likely be coreleased with these transmitters. Dopamine and norepinephrine are oxidized at the same working potential (650 mV vs SCE) as hydrogen peroxide produced during the ATP conversion. Thus, the amount of catecholamines released must be independently determined for ensuring accurate ATP quantification. Consequently, dual-barrel electrodes utilizing two Pt disk electrodes with a diameter of 25 $\mu$m each and spaced approximately 100 $\mu$m to avoid cross talk were used for simultaneously sensing ATP at the miniaturized biosensor electrode, and catecholamines at the bare Pt disk electrode. Hence, accurate referencing of the ATP signal in case of catecholamine corelease is ensured. The electrodes are manufactured pulling dual-barrel glass capillary (Hilgenberg, Germany), which resulted in disk electrodes, which are spaced at a distance of approximately 100 $\mu$m. The ratio of the insulating glass radii to the electrode radii (RG value) is approximately 5. A detailed description of dual-barrel electrode fabrication is described elsewhere.[29]

## RESULTS AND DISCUSSION

**Calibration of ATP Microbiosensors.** Calibration of biosensors is among the most critical aspects determining their utility in clinical environments. For *in situ* applications as described here, extensive multipoint sensor calibration within the perfusion chamber would be impractical given the limited lifetime of carotid

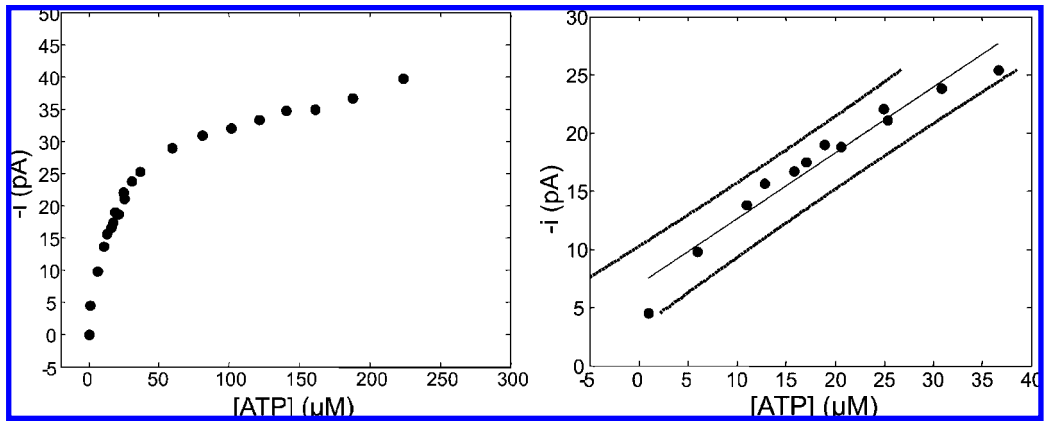


**Figure 3.** (Left) ATP signal from a 25 $\mu$m diameter ATP microbiosensor in 100 mM phosphate buffer at pH 7.4 containing 11 mM glucose and 1 mM $MgCl_2$. (Right) Linear calibration plot of an ATP microbiosensor from 0 to 40 $\mu$M ATP. The dotted lines represent two standard deviations on the linear least-squares regression.

body preparations (approximately 1–2 h), after repetitive excitatory stimuli. Hence, each sensor was thoroughly calibrated prior to *in vitro* analysis, and complemented by several 1-point recalibrations at the carotid body ensuring accurate ATP quantification. Typically, 2–3 measurements of ATP released by the carotid body due to stimulation were followed by recalibration utilizing 10 $\mu$M ATP standards within the perfusion chamber. The amperometric ATP microbiosensors used in this study are characterized by a linear response behavior in the range of 0–40 $\mu$M ATP, thereby justifying the 1-point recalibration strategy (Figure 3). Calibration of ATP microbiosensors prior to *in vitro* studies was performed at the same glucose and $Mg^{2+}$ concentration as present in Ringer's solution used for the carotid body measurements. The sensitivity of these prepared amperometric microbiosensors was determined at 115 ± 18 mA $M^{-1}$ $cm^{-2}$ within a range of 0–40 $\mu$M ATP. The signal is reported as a positive current value facilitating comparison with other sensing techniques, even though the actual signal is recorded as a decrease in current correlated with the amount of ATP present. Data evaluation was performed utilizing a linear least-squares regression model; the dotted lines in Figure 3 and the error for the reported sensitivity represent two standard deviations for the regression and for the sensitivity, respectively. At ATP concentrations exceeding 40 $\mu$M, the sensor response is nonlinear; however, concentrations beyond this level are not expected at the carotid body surface, and could still be evaluated using appropriate functions modeling nonlinear response behavior of the sensors. A linear least-squares regression results in a simple model for *in situ* recalibration of the sensor. Using a Chi-square regression could minimally improve the accuracy of the measured ATP concentration, however, at the expense of requiring more extensive *in situ* recalibration procedures, which is considered a significant drawback in clinical applications. Furthermore, the possible gain in accuracy utilizing a Chi-square regression would not result in enhanced accuracy of the present measurements due to the biological variability of the investigated model system currently limiting the accuracy of the measurement.

ATP concentrations released at the carotid body will be expressed in $\mu$M with the quantification performed by averaging >50 data points from the ATP signal, and subtracting the average baseline current (>50 data points) if only glucose is present. The associated errors are reported as two standard deviations on the mean of multiple measurements at identical conditions. As the variability of the measured signal usually increases at clinical samples, averaging of multiple data points is essential. The reported $n$ value relates to the number of measurements at a particular stimulus; high potassium ($K^+$), normoxic hypercapnia (HC), and severe hypoxia (HP). $n$ is also reported for the number of carotid bodies from different rat pups ($n_{CB}$), and for the number of different litters ($n_L$). These parameters enable the comparison of data determined at individual carotid bodies, within a litter, or within multiple litters.

**Real-Time *In Situ* Studies of ATP Release at Carotid Bodies.** *Biological Significance of Measuring ATP at the Carotid Body.* The carotid body regulates the ventilatory response to changes of arterial $O_2$ and $CO_2$ partial pressures. However, the carotid body in mammals does not mature until weeks after birth.[37] Hence, the body is unable to appropriately respond to changes in arterial $O_2$ and $CO_2$ variations until the carotid body matures. This maturation mechanism is currently not fully understood, and especially the role of ATP chemosensitivity within this mechanism is suspected to represent a key molecular signaling pathway. Consequently, amperometric ATP microbiosensors provide a useful analytical tool aiding understanding of the chemosensitive maturation of the carotid body. The aim of the current study is not to establish the chemosensitive response of the carotid body through developmental stages of young rats, but to validate the developed biosensor methodology for future use of such microbiosensors in clinical studies.

The use of a whole carotid body preparation as an *ex vivo* model system is appropriate, as described by Buttigieg and Nurse.[35] A carotid body preparation remains biologically active for up to a few hours at *ex vivo* conditions, if maintained in a controlled environment, as described in the Experimental Section. The carotid body must always be superfused in an oxygenated Ringer's solution to maintain biological activity, except for short exposure to a stimulus. In this article, experiments to demonstrate ATP response from the carotid body to three different stimuli was performed for validating the methodology of measuring ATP release at the carotid body with amperometric microbiosensors.

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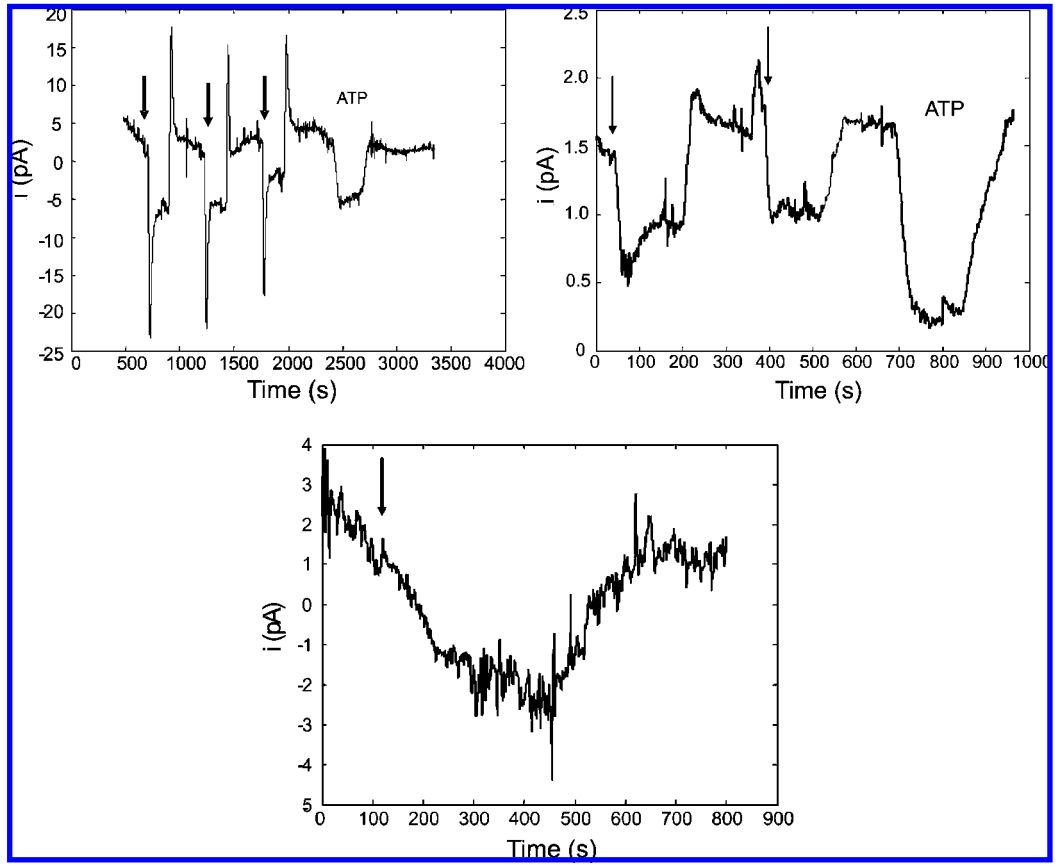


**Figure 4.** (Top left) Stimulation of ATP release at the carotid body with 60 mM potassium. (Top right) ATP release stimulated by normoxic hypercapnia (10% $CO_2$). (Bottom) ATP release stimulated by hypoxia at the carotid body ([$O_2$] < 1 ppm). In each trace the arrows indicate when the stimulus reached the carotid body.

*High Potassium.* ATP release by the carotid body was induced using three stimuli: high potassium, normoxic, acidotic hypercapnia, and severe hypoxia. High potassium is induced by exposing the carotid body to increased $K^+$ concentrations in the buffer solution. Native Ringer's solution contains 5 mM $K^+$; increasing the $K^+$ concentration to a level of 60 mM triggers ATP release at the carotid body surface (Figure 4). The signal in Figure 4 was baseline corrected for removing the slow capacitive current dissipation at the carotid body. Baseline correction is achieved by using a $y = ax^b + c$ mathematical model accurately fitting the baseline. Subtracting the fitted baseline from the raw data generates the baseline-corrected data set. The arrows in Figure 4 indicate when high potassium conditions reach the carotid body. Following three 60 mM $K^+$ stimulus cycles, each with a duration of 3 min, a 10 $\mu$M ATP standard point was recorded ensuring robust calibration of the ATP biosensor. ATP release at the carotid body is evident by a decrease of the Faradaic current following a 60 mM $K^+$ stimulus, which reveals similar characteristics to the bienzymatic sensor response anticipated from ATP standards. The main advantage of the coimmobilized GOD/HEX biosensor concept is the fact that the obtained signal is specific to ATP, corroborated by comparable signal levels between actual measurements and the standards. In addition, extensive tests prior to *in vitro* studies have confirmed that false positive signals at the selected potential (650 mV) are highly unlikely at these clinical conditions. ATP detection based on coimmobilized GOD and HEX leads to a decrease in the measured current; however detectable interferants would yield an increase in the current signal at the oxidative potential of 650 mV. Hence, such interferants are easily discernible from an ATP response. In addition, possible interferences from HEPES buffer have extensively been studied by our research group, and can be avoided by working in Ringer's solution.[38]

Figure 4 shows a rapidly developing burst of ATP within the first 10 s following exposure to 60 mM $K^+$, which stabilizes at a constant and lower ATP concentration released by the carotid body for the remainder of the elevated $K^+$ exposure. It is hypothesized that the rapid burst of ATP results from the difference in ionic molarities between the solution and the cells, which creates a substantial osmotic pressure supporting elevated ATP release during the first few seconds until the cells equilibrate. As this spike is not present during perfusion with 10 $\mu$M ATP standards, it can be excluded that this signal behavior is related to the biosensor behavior. The removal of the high potassium solution coincides with the upward spike (at approximately 1000 s, 1500 s, and 2000 s) at the end of each high potassium stimulation cycle. It was experimentally confirmed that the change in osmolarity causes the spikes at the beginning and the end of the high potassium cycles. The measured extracellular ATP concentration is constant at 8.0 ± 1.8 $\mu$M for a variety of different carotid

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body preparations ($n_{K^+} = 16$, $n_{CB} = 6$, $n_L = 2$). The rat pups investigated during this particular study were from the age groups 16 and 19 days of life. It is important to note that the measurements at both litters were acquired with different sets of ATP microbiosensors, enzymes of different lots immobilized at the respective electrode surfaces, and several months apart during different clinical measurement campaigns, which impressively demonstrates the achievable reproducibility of amperometric microbiosensor technology.

*Normoxic Acidotic Hypercapnia.* While potassium stimulation is commonly used to determine responses during maximum depolarization, it is of substantial clinical relevance to determine the response of the carotid body to both normoxic acidotic hypercapnia and hypoxia. ATP released by the carotid body at these physiological conditions is a key neurotransmitter for the signaling process involved in regulating the respiratory and cardiovascular pathways..[32] However, ATP chemosensitivity of the carotid body at hypercapnic and hypoxic conditions is not yet fully understood. Consequently, the presented approach using amperometric ATP microbiosensors could aid in elucidating the molecular mechanism regulating carotid body chemosensitivity. As shown in Figure 4, extracellular ATP was detected by stimulating the carotid body with normoxic acidotic hypercapnia (10% $CO_2$, 21% $O_2$, and 69% $N_2$). It has to be noted that a small pH decrease by 0.1 to 0.2 will occur during normoxic acidotic hypercapnia, caused by the higher $CO_2$ concentration in Ringer's solution. In contrast to high-potassium stimulation, the carotid body does not show rapid release of ATP when stimulated with normoxic acidotic hypercapnia within the first few seconds. At these conditions, the ATP release remains relatively constant throughout a 3 min exposure. However, during the first stimulation of the carotid body with normoxic acidotic hypercapnia, slightly higher ATP concentrations were detected during the first 60 s, which were not observed for the second and third cycles. Exposure of the carotid body to normoxic acidotic hypercapnia was limited to 3 min per measurement in order to avoid exhaustion of the carotid body chemosensitivity. Furthermore, a 3 min recovery period with normoxic and normcapnic Ringer's solution was optimized ensuring sufficient recovery time for the carotid body, and for obtaining a stable current baseline. From days of life 16 to days of life 20, it was observed that excitatory ATP release at normoxic acidotic hypercapnic conditions reached $5.9 \pm 1.8\ \mu$M at the carotid body ($n_{K^+} = 15$, $n_{CB} = 7$, $n_L = 3$). As previously observed for high-potassium conditions, the variability of the determined ATP concentration released at the carotid body was minimal for repeated measurements at a single carotid body, from carotid bodies within the same litter, and from litter to litter, which again confirms the appropriateness of amperometric ATP microbiosensors to accurately measure extracellular ATP at the carotid body.

*Hypoxia.* A critical factor for operating the presented amperometric ATP microbiosensors at hypoxic conditions is the effect of the $O_2$ level in solution. The GOD catalyzed conversion of glucose consumes $O_2$ as a cosubstrate resulting in the generation of gluconic acid and hydrogen peroxide. Thus, a reduced concentration of $O_2$ in the superfusing buffer solution could potentially yield false positive results by contributing to a decreased amount of hydrogen peroxide. Consequently, prior to these studies the response of the developed amperometric ATP microbiosensor scheme was investigated in a solution containing 9.4 $\mu$M ATP and equilibrated at room air (normoxia: [$O_2$] approximately 6 ppm), which was then continuously sparged with Ar until the [$O_2$] reached approximately 1 ppm (hypoxia). ATP solutions for this study were prepared in 100 mM phosphate buffer with 5 mM glucose and 5 mM $MgCl_2$. The $O_2$ level was determined at 25 °C with a dissolved oxygen probe. The observed current for ATP in a buffer solution equilibrated in room air was $-8.3 \pm 0.4$ pA ($n = 3$), which is not statistically different from $-7.7 \pm 0.5$ pA ($n = 3$) obtained for ATP after sparging the solution to a level of 1 ppm $O_2$. A $t$ test confirmed that there is no statistical difference between ATP measurements at normal $O_2$ concentration, and at depleted $O_2$ conditions: $t = 1.74$ is smaller than $p_{0.05} = 2.78$ (4 degrees of freedom). Therefore, the determination of ATP released from the carotid body at severe hypoxia with the developed amperometric ATP microbiosensors provides reliable data.

Exposure of the carotid body to hypoxic conditions induces extracellular ATP release, as previously observed by Buttigieg and Nurse using an ATP bioluminescence assay.[35] In this study, ATP release was triggered with an $O_2$-depleted Ringer's solution as depicted in Figure 4. Oxygen levels of <1 ppm in Ringer's solution were determined using a dissolved oxygen sensor. Normoxic $O_2$ levels in Ringer's solution are approximately 6 ppm. Hypoxia-induced release events yielded approximately 5 $\mu$M ATP from the carotid body. The ATP levels release from the carotid body during this study compare well to ATP levels released by the ventral medulla during hypoxia in rats using a similar amperometric ATP microbiosensor at approximately 3 $\mu$M.[28]

The detection of ATP with amperometric microbiosensors offers several advantages compared to bioluminescence ATP assays. First, amperometric microbiosensors may be located in close proximity of the carotid body surface using a micropositioner or a scanning electrochemical microscopy (SECM) setup. The microbiosensor can be accurately positioned close to the carotid body by monitoring an approach curve with SECM. Second, the determination of ATP close to the carotid body surface with amperometric microbiosensors minimizes dilution in the bulk buffer solution; thus, the ATP concentration can be measured at the carotid body and hence reflects a closer estimate of the actual ATP concentration released by the carotid body when the microbiosensor is located in close proximity above the carotid body. Furthermore, bioluminescence assays usually provide only bulk concentration.

**Referencing Catecholamines Using Dual-Barrel Electrodes.** At high-potassium conditions, catecholamines, dopamine and norepinephrine, are coreleased at the carotid body. The oxidation of these catecholamines at 650 mV vs SCE will falsify the quantification of ATP via $H_2O_2$ oxidation at the same potential. Consequently, lower ATP concentrations would be determined if the effect of catecholamine oxidation is not referenced. Hence, we have introduced the use of dual-barrel electrodes for these studies, where one electrode bears the dual-enzymatic ATP microbiosensor, and the other Pt disk electrode remains unmodified for direct oxidation of catecholamines.

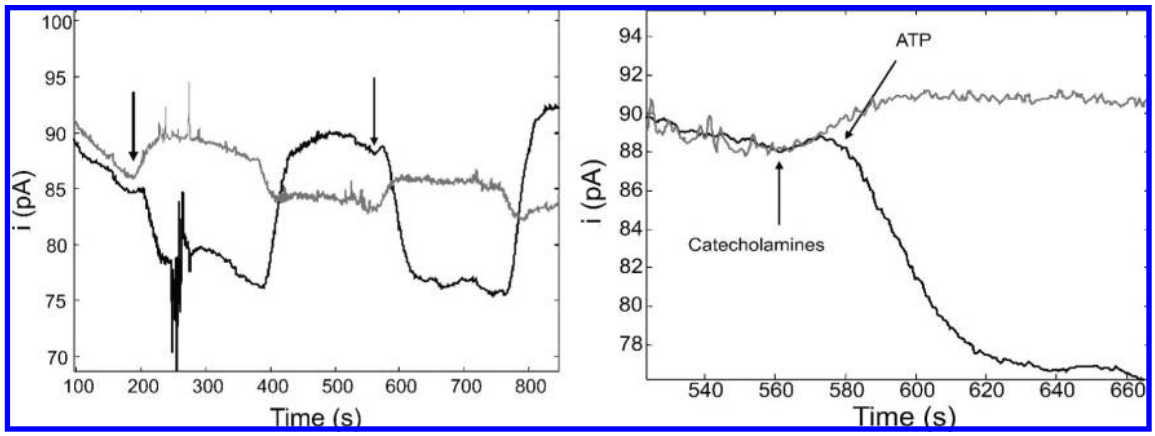


**Figure 5.** Corelease of catecholamines with ATP at the carotid body at high-potassium conditions. Both electrodes of the dual-barrel amperometric ATP microbiosensors/microelectrode assembly identically respond to catecholamines. The light gray amperometric response is the signal of the reference sensor (bare Pt disk microelectrode), while the ATP microbiosensor signal is shown in black. The arrows mark the time at which 60 mM $K^+$ reached the carotid body.

Figure 5 shows a study where ATP and catecholamines were coreleased at the carotid body. The light gray trace shows unequivocally that catecholamines are coreleased with ATP (black trace) by the carotid body at high-potassium stimulus conditions. The advantage of the amperometric ATP microbiosensors is that the current decrease from ATP is easily discernible from the current increase resulting from direct catecholamine oxidation. Nonetheless, for accurate quantification of ATP released by the carotid body, the signal from catecholamines must be subtracted from the ATP signal. Both the ATP and the reference sensor identically responded to catecholamines, thus facilitating signal subtraction of the reference sensor signal from the ATP sensor signal. It should be noted that there is an observable delay in sensor response to ATP vs the catecholamines. As evident, catecholamines are released approximately 20 s prior to ATP by the carotid body. This observation provides new insight on the signaling response behavior of the carotid body at high-potassium conditions. Prior to the presented study using dual-barrel amperometric ATP microbiosensors/microelectrodes, simultaneous determination of both catecholamines and ATP at live carotid body preparations with sufficient temporal resolution for observing differences in the release behavior has not been reported in literature.

Catecholamines released by the carotid body at hypoxic conditions were previously reported by Donnelly.[39] In these studies it was observed that the carotid body releases five times more dopamine than norepinephrine. The total concentration of catecholamines reported for moderate hypoxia was 0.69 ± 0.32 $\mu$M and 1.64 ± 0.43 $\mu$M for severe hypoxia. Using the same ratio of dopamine and norepinephrine released by the carotid body as reported by Donnelly, and calibrating the amperometric response of the microbiosensor to dopamine and norepinephrine, it was observed that rat carotid bodies in the age group 16–20 days of life release approximately 2.4 ± 1.2 $\mu$M catecholamines at high-potassium conditions, and approximately 1.0 ± 0.5 $\mu$M catecholamines at normoxic acidotic hypercapnia. The carotid body can release catecholamine during normoxic acidotic hypercapnia.[40,41] Catecholamine release during hypoxic stimulation of the carotid body has not been observed. Thus, catecholamine levels released by the carotid body during hypoxia may be lower than the detection limit of Pt electrodes.

## CONCLUSIONS

The present study describes localized quantitative detection of ATP released superfused carotid body preparations extracted from young rats at clinically relevant stimulus conditions, and demonstrates the utility of amperometric ATP microbiosensors for *in vitro* applications. ATP concentrations ranging from 4 to 10 $\mu$M were detected as a consequence of stimulating at high-potassium conditions, with normoxic hypercapnia, and with severe hypoxia. Furthermore, it was confirmed that decreased oxygen levels in the buffer solution do not significantly affect the ATP microbiosensor response in the physiologically relevant concentration range. *Ex situ* calibration of the ATP microbiosensor combined with repetitive *in situ* 1-point recalibration for each measurement series ensured adequate accurate quantification of the ATP levels released by the carotid body upon stimulation. Utilizing dual-barrel ATP microbiosensor enables simultaneous detection and correction for catecholamines, which are coreleased with ATP at certain conditions of the investigated clinical samples. The amperometric ATP microbiosensors sensors developed in recent years by our research group confirmed high reproducibility within an individual measurement series, at studies of carotid bodies from different rats, and even across several litters at different time points over 1 year. Consequently, the obtained results corroborate the utility of amperometric ATP microbiosensors in clinical environments with the carotid body providing a highly relevant biological model system. The data presented establishes this technology as an excellent tool for both analytical chemists and medical researchers in elucidating molecular signal-

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ing processes responsible for chemosensitivity and broader application to other clinically relevant studies.

## ACKNOWLEDGMENT

The authors are grateful to Reed Cooper and Ariel Mason at the Johns Hopkins Hospital, and to Jack Smith (undergraduate researcher) at the Georgia Institute of Technology for technical support during these studies. Financial support was generously provided by NIH within Projects R01 HL080725 and R21 HL082860.

Received for review September 9, 2007. Accepted March 1, 2008.

AC7018969