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On-Line in Vivo Monitoring of Endogenous Quinones Using Microdialysis Coupled with Electrochemical Detection

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The continuous on-line monitoring of endogenous quinones has been realized for the first time in an animal model of brain ischemia induced by a vasoconstrictor peptide, endothelin-1. A microdialysis probe, implanted in the striatum of a freely moving rat, was coupled on-line with an amperometric thin-layer cross-flow detector with a glassy carbon working electrode operating at −200 mV vs Ag/AgCl. The instrumental setup comprised a syringe pump pulse-damper consisting of an air bubble and a silica capillary, which permitted considerable reduction of background current fluctuations and allowed improved detection limits. This original configuration allowed the quantitation of micromolar amounts of total quinones, generated from dopamine during the reperfusion period, to be readily monitored. Several operational parameters have been investigated: flow rate, presence of oxygen in the perfusion fluid, and the working potential. The selectivity of the assay toward quinones was confirmed by studying possible interfering species such as ascorbate, hydrogen peroxide, riboflavin, and thiols. The results on freely moving rats have shown that the endogenous quinone amount was directly related to dopamine concentrations. The latter was determined by HPLC from dialysate samples collected at the outlet of the on-line system. HPLC studies showed that the primary quinone, generated from dopamine by bulk electrolysis, was also found in dialysates from ischemic brain.

During brain ischemia, high amounts of neurotransmitters are released from nerve terminals into the extracellular space.[1,2] During reperfusion (i.e., restoring of blood circulation), released catecholic species such as dopamine (DA), norepinephrine, epinephrine, and their metabolites, e.g., 3,4-dihydroxyphenylacetic acid (DOPAC), may be oxidized enzymatically or nonenzymatically by oxygen present in the reperfusing fluid.

The enzymatic oxidation of DA by monoamine oxidase (MAO) leading to aldehyde and hydrogen peroxide seems to be a minor process because MAO is located only in the mitochondrial membrane. Yet aldehydes and H_2O_2 are highly damaging substances, and the inhibition of monoamine oxidase (MAO) reduces neuronal necrosis.[3] The *O*-methoxylation of DA by catechol *O*-methyltransferase, leading to 3-methoxytyramine, is a more rapid process because this enzyme is present in the extracellular fluid, in contrast to MAO.[1,4]

The nonenzymatic oxidation of catecholamines (*o*-hydroquinone derivatives) by molecular oxygen produces *o*-quinones which are very reactive and thus toxic. The mechanism of their formation probably consists of a chain of radical reactions, which may also be a source of semiquinones and of reactive oxygen species (e.g., H_2O_2, $^{\bullet}OH$, $O_2^{\bullet-}$).[5] The lifetime of these quinones is very short in vivo because they may undergo further reaction, such as nucleophilic substitution, particularly at position 6 of the aromatic ring.[6]

With respect to dopaquinone (DOQ), the fastest nucleophilic reaction is observed with thiols such as L-glutathione, L-cysteine, etc., which are present in relatively high concentration in the extracellular fluid. The rate constant (first-order for DOQ) is 480 s^{-1}.[6] This reaction pathway may give rise to neuromelanin formation.[7] It has been reported that the concentration of glutathione decreases during reperfusion, probably also because of the nucleophilic addition described above.[8,9] Another reaction of DOQ, ∼1800× slower, is nucleophilic attack by the amino group of DOQ, leading to internal cyclization with formation of aminochrome (rate constant 0.26 s^{-1}).[6] The reaction of DOQ with another amino group, e.g., from a free amino acid or a protein, is slower than the internal cyclization (rate constant 0.033 s^{-1}).[6] The products of the nucleophilic attack are more easily oxidizable than DA itself, and, since the oxygen is in excess during the reperfusion, they may be further oxidized to secondary quinones. Since the resulting quinones are as electroactive as the primary DOQ, they can also be monitored electrochemically.[10]

Experimental setups for on-line in vivo measurements usually consist of a syringe or a peristaltic pump, an implanted microdialysis probe, and an amperometric or fluorometric detector. This technique has been successfully applied for monitoring several endogenous compounds at millimolar levels such as subcutaneous

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glucose,[11−17] lactate,[16,18] and brain glutamate.[19,20] The determination of *o*-quinones which are unstable species because of risks of polymerization[21] and reduction[22] and present at micromolar levels requires the implementation of an on-line sampling closely connected to a sensitive detection system.

Quinones can be reduced electrochemically at low negative potentials of ∼−200 mV vs Ag/AgCl on carbon electrodes.[23−27] Yet a sensitive and reproducible on-line EC detection requires a very stable flow rate. This was technically achieved by a previously described setup which comprises an original syringe pump pulse-damper.[28] It is the aim of the present study to develop an analytical method for the on-line in vivo monitoring of the sum of quinones generated during the reperfusion period, following a brain ischemia, in an animal model of stroke. 1,4-Benzoquinone (1,4-BQ) was used as model compound and for calibration. The use of 1,2-benzoquinone was avoided because of its high instability. Several experimental parameters have been optimized, such as flow rate, presence of oxygen in the perfusion fluid, and selectivity toward interferences (H_2O_2, ascorbate, riboflavin). The flow pattern in the electrochemical cell and the relative recovery as a function of the flow rate were investigated, and data were fitted to a theoretical model.

Ischemia in the brain of a freely moving rat was induced by administration of the vasoconstrictor peptide endothelin-1[29] near the middle cerebral artery.

The identification of the endogenous quinones was achieved by comparing the primary quinones generated by bulk electrolysis of DA and DOPAC at +600 mV with those present in in vivo samples. This was realized by HPLC using a PEEK-lined[22] analytical column.

EXPERIMENTAL SECTION

Reagents. Endothelin-1 (ET-1) and 1,4-benzoquinone (99%) were obtained from Sigma Chemicals (St. Louis, MO). Hydrogen peroxide 35% (w/w) (Janssen Chimica, Beerse, Belgium), ascorbic acid (Roche, Basel, Switzerland), riboflavin 99% (Merck, Darmstadt, Germany), and 2-mercaptoethanol 98% (Janssen Chimica) were used as obtained. The perfusion fluid for in vitro studies contained 147 mM NaCl, 4 mM KCl, and 1 mM Na_2HPO_4 to ensure a physiological pH value of 7.30, which was adjusted by HCl. The perfusion solution for in vivo experiments contained 147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$. The pH of this solution was ∼6.4−6.6 (pK_a of carbonates), but it raised up to 7.3 when reaching the living brain, probably because of buffering by free amino acids. Phosphate buffers were not used due to incompatibility with calcium. Standards were prepared using the perfusion solution to avoid pH effects at the detector.

The mobile phase for HPLC contained 20 mM citrate buffer, 0.2 mM sodium octanesulfonate, and 0.2 mM tetramethylammonium hydroxide. It was free of strong nucleophilic agents (amines, chloride, ...), which might react with *o*-quinones. The pH was adjusted to 3.80 by NaOH.

All reagents were of analytical grade from Merck. The perfusion solution and mobile phase were filtered (BAS filter, pore size 0.2 μm) before use. Water was purified by a Seralpur Pro 90 CN (Belgolabo, Brussels, Belgium) system, allowing an output conductivity of ∼50 nS/cm.

Equipment. A CMA 12 microdialysis probe (cylindrical membrane, 0.5 mm o.d., length 3 mm, active area A_M = 2.356 mm^2; CMA Microdialysis AB, Stockholm, Sweden) was employed for all the experiments. The probe was implanted in the striatum, i.e., a brain area with a large dopaminergic innervation, and the in vivo coordinates relative to bregma were $x = +2.4$, $y = +1.2$, and $z = -5.8$ mm.[30] The flow rate was controlled by a CMA/100 microinjection pump and by a BAS Bee syringe pump (Bioanalytical Systems, W. Lafayette, IN), both with 1 mL GASTIGHT syringes (Hamilton, Reno, NV). To suppress short time fluctuations of the flow rate, the previously designed damping system was used.[28] The UniSwitch syringe selector (BAS) was used to select the input for the on-line detector.

The electrochemical detector consisted of a thin-layer cross-flow three-electrode cell (UniCell, BAS) with a glassy carbon working electrode (GCE, 2 mm i.d.), a stainless steel auxiliary electrode, and a Ag/AgCl reference electrode. It should be mentioned that this reference electrode has no salt bridge and that its potential is governed by the concentration of free chloride ions in the perfusion fluid. Here it is shifted by ∼+100 mV compared to the widely used Ag/AgCl, 3 M KCl. The thickness of the cross-gasket (BAS) imposed the thickness of the flow channel to 51 μm. The cell was electrically connected to an LC-3C amperometric detector (BAS), coupled to a $y-t$ recorder (BAS). Teflon tubing (120 μm i.d. BAS) with a special mechanically resistive connector was used for tubing connections. The distance from the probe to the detector was 50 cm (overall volume 5.6 μL, delay time 168 s at a flow rate of 2 μL/min). The pH was controlled by a standard pH meter with a classical glass electrode, or by an ISFET solid state pH meter (pH Boy-P2, Shindengen Electric, Tokyo, Japan) for volumes of ∼10 μL.

The administration of ET-1 in vivo was performed into the piriform cortex ($x = +5.6$, $y = +0.3$, and $z = -4.0$ mm)[30] using a BAS Bee syringe pump with a 500 μL syringe (BAS) coupled to an implanted microdialysis probe, from which the membrane was

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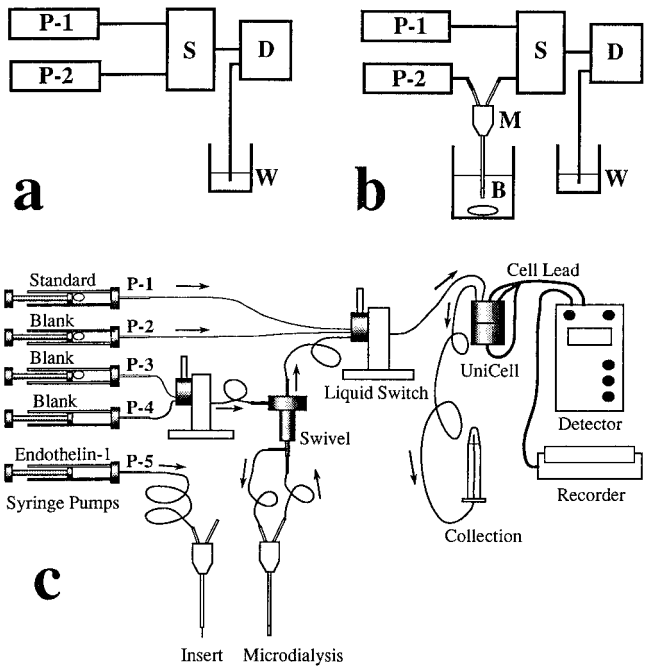

Figure 1. (a) Experimental setup for studying the behavior of the amperometric thin-layer cross-flow cell (P-1 and P-2, syringe pumps containing blank and standard solutions, respectively; S, syringe selector; D, detection cell; and W, waste). (b) Scheme of setup used for in vitro studies (P-1, P-2, blank; M, microdialysis probe; and B, batch with stirrer and standard solution). (c) Detailed view of system used for in vivo experiments (P-1, P-2, pumps for electrode calibration; P-3, analytical pump; P-4, stand-by pumps; P-5, endothelin-1 pump).

removed. The entire experimental setup is schematically depicted in Figure 1.

The analysis of dopamine in samples collected from the output of the on-line quinone monitoring system was realized by reversed-phase microbore liquid chromatography, described elsewhere.[31]

The HPLC setup for the identification study was achieved with BAS equipment and consisted of a PM-80 isocratic pump coupled to a PEEK-lined analytical column, 100 mm × 2 mm, packed with 3 μm C18 stationary phase. The thin-layer cross-flow electrochemical detection cell CC-5, comprising two parallel glassy carbon electrodes (3 mm i.d.), with Ag/AgCl (3 M NaCl) reference electrode and a gasket of 16 μm, was coupled to LC-4C and LC-3C amperometric detectors. The latter were connected to a dual-pen $y-t$ recorder. The mobile phase was degassed on-line by an LC-26 vacuum degasser in order to remove oxygen.

Procedures. Optimization of the parameters influencing the electrochemical response was performed with the setup shown in Figure 1a. One pump contained the blank perfusion fluid and the second one the same perfusion fluid plus 1,4-benzoquinone (1,4-BQ). A third pump was added when necessary. The selection of the appropriate pump was performed by the liquid switch (see Figure 1c). The steady state signal corresponding to 1,4-BQ was recorded.

The batch setup (Figure 1b) was employed for validation of the on-line system in vitro and consisted of a 5 mL beaker with a magnetic stirrer and a holder for the microdialysis probe. Experiments were performed in well-stirred (stirring rate ~500 rpm) and in quiescent media. In the study of the effect of the flow rate (see Figure 2), the system was switched from P-1 (blank) to P-2 (1,4-BQ standard solution). The current was constantly recorded throughout the experiment. At the end of the experiment it was checked, by switching from P-2 to P-1, that the blank value remained constant.

When necessary, the perfusion fluid was deoxygenated by nitrogen gas (purity > 99.99% v/v) and degassed for 10 min in an ultrasonic bath.

In vivo experiments were performed on rats weighing 200−250 g. The animals were first anesthetized with a mixture of ketamine:diazepam (50:5 mg/kg) and mounted on a stereotaxic frame. Intracerebral guides for the probe and for the ET-1 administrator were implanted, and the continuously perfused probe was inserted. The animals were then allowed to recover for 24 h, and the ET-1 administrator was inserted. The microdialysis experiment was carried out on freely moving rats, having free access to food and water.

In vivo experiments were made with the setup shown in Figure 1c. Three Bee pumps (BAS) were used: P-1 and P-2 served for calibrating the electrode (with blank and standard solutions of 1,4-BQ, respectively) and P-5 for delivering ET-1. The calibration of the electrode for the in vivo experiment was performed by three standard solutions: 1, 2, and 5 μM 1,4-benzoquinone in the buffered perfusion fluid. Two CMA/100 pumps were used: P-3 served as a well-damped analytical pump and P-4 as a stand-by pump. These two pumps were connected to the on-line setup via the syringe selector and via the swivel to the probe. The output of the electrochemical cell was collected every 10 min into PE vials (250 μL with cap, Elkay, Shrewsbury, MA). Care must be taken to ensure adequate removal of the static charge during vial exchange (picoamperes are recorded during in vivo analysis!). The content of dopamine in these collections was analyzed by HPLC.

The working and reference auxiliary blocks of the UniCell were polished daily using alumina (BAS). Residual polishing particles were removed by ultrasonication. The reference silver electrode was subsequently recoated by AgCl. The gasket was also sonicated before the cell was completed. Before analysis, the cell was left to equilibrate at the chosen flow rate using pump P-2 and the applied potential of −200 mV (Figure 1c) for 30 min. Subsequently, the P-3 circuit was selected (output from probe) and the system equilibrated for ~1 h. After the initial system had stabilized, the in vivo assay was carried out. After completion of the experiment, the electrode was calibrated using P-1 and P-2 pumps. All experiments were carried out at room temperature.

Before the experiments, all stainless steel tubings were passivated using 6 M HNO_3 to avoid leaking of iron ions, which are known to catalyze the dopamine oxidation. Also, bare stainless steel metal may act as a reducing agent for quinones.[22]

The single-drop bulk oxidative electrolysis was performed by placing a drop of 10 μL of phosphate buffer (pH 7.30, 0.1 M) and 100 μL of 5 μM DA (or DOPAC) on the surface of a GCE (6 mm i.d.). The reference and auxiliary (separated by a glass frit) electrodes were immersed in the drop. The electrolysis was performed at +600 mV in unstirred solution during about 2 h. Five microliters was diluted to 10 μM and sequentially injected into the HPLC during the electrolysis.

RESULTS AND DISCUSSION

In Vitro Validation of the Experimental Setup. *Effect of Flow Rate on the Amperometric Cell (Figure 1a).* The Faradaic

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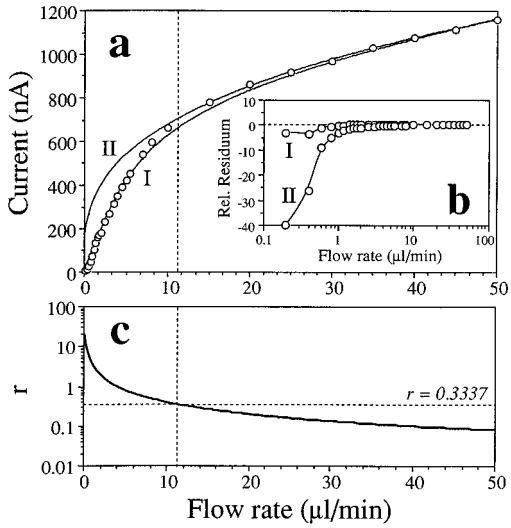

Figure 2. (a) Steady state response of 100 μM 1,4-benzoquinone at the GCE polarized at −200 mV vs Ag/AgCl in the thin-layer cross-flow cell as a function of the flow rate. Two models, I and II, were used to fit the experimental data. (b) The residual plot for each model. (c) Parameter *r*, obtained from model I, shown as a function of the flow rate.

steady state current of a thin-layer cross-flow amperometric cell[32] has been described by Weber and Purdy[33] as

$$i_F = k_1 nF \frac{W_E}{d} U_V (1 - 0.3992\ e^{2.505(0.3337-r)}) C_0 \qquad (1)$$

where k is a constant, n is the number of electrons transferred per molecule, F is Faraday's constant, W_E is the working electrode width (mm), d is the width of the channel (mm), U_V is the flow rate (μL/min), and C_0 is the bulk concentration of analyte (mol/L). The dimensionless parameter $r = DdL/(U_V h)$ determines if the electrochemical cell is operating under either amperometric or coulometric conditions. For $r \ll 0.3337$, i.e., amperometric mode, the expression above reduces to

$$i_F = k_2 nFA_E (D/h)^{2/3} (U_V/d)^{1/3} C_0 \qquad (2)$$

where A_E is the working electrode area (mm^2), D is the diffusion coefficient (mm^2 min^{-1}), L is the electrode length (mm), and h is the thickness of the flow channel (mm). In Figure 2a is shown the evolution of the limiting current as a function of flow rate under the present experimental conditions (empty circles). The data were fitted by a regression model (solid line) resulting either from eq 1, i.e.,

$$i_F = P_1 U_V (1 - 0.3992\ e^{2.505(0.3337-P_2/U_V)}) C_0$$
regression model I

or from eq 2,

$$i_F = P_1 U_V^{1/3} C_0$$
regression model II

P_1 and P_2 are parameters of model I and were calculated by least-squares nonlinear regression to be 0.96 ± 0.08 nA (μL/min)$^{-1}$ μM^{-1} and 3.9 ± 0.5 μL/min (n = 28), respectively. The parameter P_1 of model II appears to be valid only for flow rates above 8 μL/min (Figure 2a) and was found to be ~3.14 nA (μL/min)$^{-1/3}$ μM^{-1}. As can be seen from Figure 2a, model I fits the experimental data better, especially at low flow rates. This can be more clearly seen from Figure 2b, where relative residuals obtained as $(i_{exp} - i_{pred})/i_{exp}$ are shown. Not only the expected deviation from the model II (below 8 μL/min) but also a deviation from the model I at low flow rates (below 0.8 μL/min) were observed. The deviation from the simplified model II can be explained by plotting the parameter r, given as P_2/U_V, as a function of the flow rate (Figure 2c). Model II is valid when $r \ll 0.3337$, i.e., amperometric mode. When the values of r are above this limit (i.e., flow rates lower than 10 μL/min), a fouling of model II (see Figure 2a,c) was observed.

The slight deviation of the more rigorous model I at flow rates below 0.8 μL/min cannot be explained by changes in the hydrodynamic pattern because the flow was laminar at all flow rates, even for the highest value of 50 μL/min. Laminarity flow pattern can be inferred from the value of the Reynolds number (for 50 μL/min, Re = 0.54; if Re < 2300, then the flow is laminar).[34] The model I was applied for further investigations of the on-line system.

Effect of Flow Rate on the Microdialysis (Figure 1b). The relation between the outer concentration of analyte C_x measured in the external medium and the output concentration C_0 of the microdialysis probe was previously described by Jacobson et al.[35] as

$$C_0/C_x = (1 - e^{-K_0 A_M/U_V}) \qquad (3)$$

where K_0 is the average mass transfer coefficient (mm min^{-1}) and A_M is the microdialysis membrane area (mm^2). The C_0/C_x ratio is called relative recovery (RR, dialysate extraction fraction). The detailed mathematical analysis given by Bungay et al.[36] showed that the factor K_0A_M represents the overall probe−external medium permeability, given as $1/(R_d + R_m + R_{ext})$. R_d, R_m, and R_{ext} are resistances-to-mass flow (min mm^{-3}, in analogy with Ohm's law) of the analyte for the dialysate, membrane, and external medium, respectively. As R_d and R_m are constant for the membrane and perfusion medium, R_{ext} characterizes the surrounding medium. The prediction of this parameter was given for a well-stirred solution ($R_{ext} = 0$), a quiescent solution, and the tissue.[36]

The relative recovery of the probe is shown in Figure 3a. It was calculated for each flow rate as the ratio of the current from the on-line setup (Figure 3b) to the steady state current (Figure 2a). Using linearization of eq 3, leading to $\ln(1 - RR) = -P_3(1/U_V)$ (Figure 3c), the values of P_3 for 1,4-BQ were found to be 1.43 μL/min (K_0 = 0.61 mm/min) and 1.14 μL/min (K_0 = 0.48 mm/min) in well-stirred and quiescent media, respectively. The calculated resistances-to-mass flow were $R_d + R_m$ = 0.70 min/μL, and R_{ext} of the quiescent medium was found to be 0.18 min/μL.

Effect of Flow Rate on the Combined On-Line System (Figure 1b). When the microdialysis probe is coupled on-line with the

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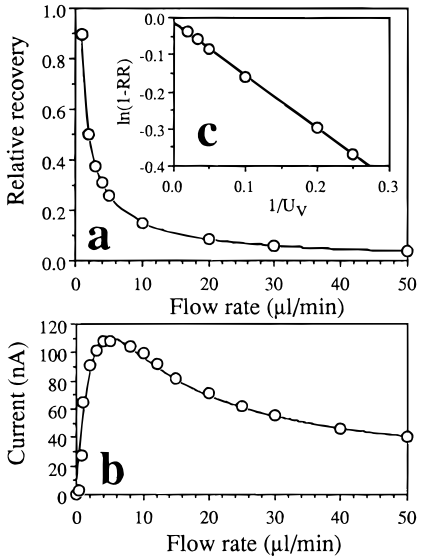

Figure 3. (a) The relative recovery (RR) calculated as a division of the graph from Figure 3b by the graph from Figure 2a. (b) The response of the on-line setup to 100 μM 1,4-benzoquinone in the well-stirred batch. The line is the predicted nonlinear regression model. (c) The linearization of data obtained from (a).

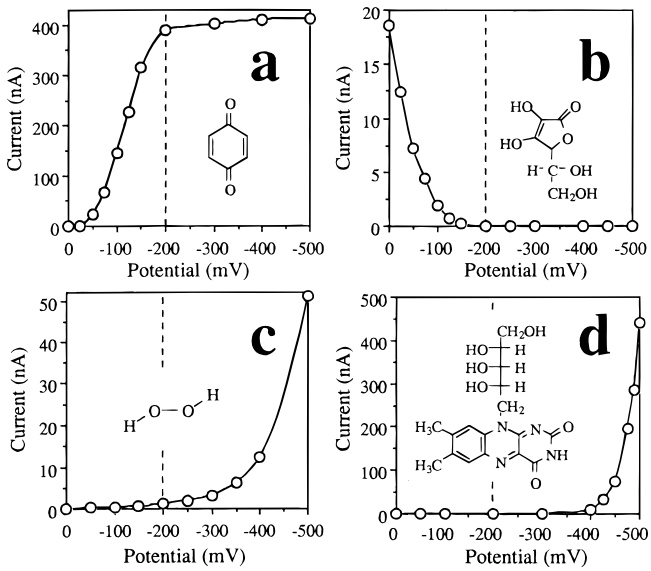

Figure 4. Hydrodynamic voltammograms of 100 μM 1,4-benzoquinone (a), 1 mM ascorbate (b), 10 mM hydrogen peroxide (c), and 100 μM riboflavin (d), GCE, flow rate 5.00 μL/min, pH 7.30.

cross-flow thin-layer amperometric cell, the system can be described by a combination of eqs 1 and 3, which leads to

$$i_F = k_1 nF\frac{W_E}{d}U_V(1 - 0.3992\ e^{2.505(0.3337-r)})(1 - e^{-K_0 A_M/U_V})C_x \quad (4)$$

For our in vitro studies, the current was measured as a function of the flow rate, which was varied between 0.20 and 50.00 μL/min. Other variables were kept constant during the experiment. The microdialysis probe was dipped into a batch system containing 100 μM 1,4-benzoquinone. As can be inferred from the equations above, the electrode signal increases upon raising U_V (eq 1, Figure 2a), while the relative recovery decreases following eq 3 (Figure 3a). The signal profile given by eq 4 (Figure 3b) should thus exhibit a maximum. In the beginning of the graph, the signal is limited by the rate of the electrochemical reaction, and after reaching a maximum the signal decreases, because limitation by the diffusion across the dialysis membrane becomes significant. Actually, the increase in the flow rate cannot compensate for the recorded current due to the decrease in 1,4-BQ concentration (C_0). The behavior of a well-stirred solution is depicted in Figure 3a. Data obtained in quiescent medium are not shown because they are very similar. The data were analyzed by nonlinear regression using the following model obtained from eq 4:

$$i_F = P_1 U_V(1 - 0.3992\ e^{2.505(0.3337-P_2/U_V)})(1 - e^{-P_3/U_V}) \quad (5)$$

We may note the good matching of experimental data (empty circles) with the curve obtained by regression by eq 5 (Figure 3b, solid line). Parameters P_1, P_2, and P_3 were calculated. The first two characterize the electrochemical process, while the third one describes the diffusion process across the microdialysis membrane. The values calculated by nonlinear regression (α = 0.05) were as follows: $P_1 = 0.55 \pm 0.20$, $P_2 = 4 \pm 2$, $P_3 = 3 \pm 2$ ($n = 16$) in well-stirred solution and $P_1 = 0.72 \pm 0.96$, $P_2 = 7 \pm 5$, $P_3 = 1 \pm 2$ ($n = 8$) (mean ± 95% confidence interval) in quiescent solution.

By comparing the results of the nonlinear regression for the on-line system to the results obtained separately for the amperometric cell (depicted in Figure 1a) and for the microdialysis (see Figure 1b), no significant differences were observed due to the high dispersion of the results obtained by nonlinear regression of eq 5. This was expected, because the regression model with three parameters requires more data points compared to an evaluation of the parameters individually. The method detailed above, based on the separation of electrochemical and membrane diffusion behaviors, allows one to clearly identify the deviation of the models from experimental data.

Linearity. Despite the fact that the flow behavior of the cell deviated slightly from the models, the electrode response versus C_0 (i.e., calibration) should be linear in the range studied (0.1−100 μM), in accordance with the model (eqs 1 and 2), for each value of the flow rate studied (0.20−50.00 μL/min). The sensitivity of the cell to 1,4-BQ (i.e., ratio i_F/C_0) as a function of the flow rate is given by the parameters P_1 and P_2, following regression model I. However, the typical sensitivity of the cell to 1,4-BQ under the conditions used in vivo (U_V = 2.00 μL/min) at a concentration below 10 μM is between 0.020 and 0.036 nA/μM, in contrast to the sensitivity at the concentration of 100 μM, which is almost 100× higher. The problem with 1,4-BQ is that the rate of its degradation is higher at low concentrations. Fluctuations in sensitivity from experiment to experiment are attributed to differences in handling of the cell, polishing, recoating, etc., but sensitivity remained unchanged within one experiment.

Selectivity. The hydrodynamic voltammogram of 1,4-BQ at the GCE is shown in Figure 4a. The plateau begins at ∼−200 mV. This potential corresponds to the limiting current of quinones and is usually applied at carbon electrodes for the detection of quinones generated by enzymatic reaction.[24−27] This low negative potential offered high selectivity for quinones over other compounds potentially present in the extracellular fluid.

Ascorbate at physiological concentration (∼1 mM) did not interfere under our experimental conditions. As can be seen from

Figure 4b, the oxidation of ascorbate started at ~−150 mV. The oxidation potentials of other readily oxidizable molecules, such as urate and acetaminophen, were shifted to even more positive values.[37,38]

In the reperfused ischemic brain tissue, a high amount of hydrogen peroxide may be present.[2,39] The electroreduction of H_2O_2 at a GCE is a very slow process, because this electrode material exhibits no catalytic effect (in contrast to platinum). A high concentration of 10 mM H_2O_2 was required in order to obtain a measurable signal (Figure 4c). It should be recalled that the stainless steel tubing of the microdialysis probe and the connectors can catalyze the degradation of H_2O_2 and induce quinone reduction as well.[22]

The flavino group representing the active center of many oxidoreductases can also be electrochemically reduced. The voltammogram of riboflavin (vitamin B_2) at the GCE (Figure 4d) shows that the reduction started at ~−400 mV.

These results demonstrated about a 10 000× and 40 000× higher sensitivity of 1,4-BQ detection over riboflavin and H_2O_2, respectively. Any possible passivation effect of thiols on the working electrode was also studied. Amino acids (L-cysteine) and short-chain peptides (L-glutathione) containing SH groups are present in the extracellular fluid and can easily permeate through the microdialysis membrane to the perfusion fluid. A standard solution of 100 μM 1,4-BQ was perfused through the detector, yielding the initial signal (100%). Subsequently, a solution of 10 mM 2-mercaptoethanol in the perfusion fluid was passed through the cell during 10 min. The cell was then rinsed with the blank perfusion solution for 5 min, and finally the standard of quinone was reinserted. This procedure was repeated three times. After the first input of mercaptoethanol, the quinone signal decreased to 87% of its original value and remained constant after the next repeated thiol perfusing. This behavior can be explained by adsorption of thiols and saturation of the GCE surface. The value of 87% can thus be considered as the limit of the passivation effect. However, as thiols are present in vivo in much lower concentrations (micromolar level), no significant passivation effect was observed under our experimental conditions.

Oxygen in the Perfusion Fluid. The nonenzymatic oxidation of DA to DOQ occurs not only in the brain tissue but also possibly in the tubing, if oxygen and DA are present together during the time elapsed from the microdialysis sampling to the detection cell. The latter, if it occurs, could be a significant limitation of this work. To point out any oxidation of DA under our experimental conditions, an in vitro study in the batch configuration was performed (Figure 1b). A well-stirred solution of 100 μM DA maintained under inert N_2 atmosphere was investigated. Two perfusion fluids were used: one was deoxygenated and the other air-saturated. After switching from the first one to the other, no quinone formation was detected. Actually, the Teflon tubing thickness should minimize oxygen diffusion.

When using a deoxygenated perfusion fluid in vivo, Parry et al.[40] observed local tissue hypoxia after 1 h of use. This was also observed during our in vivo investigations, as inferred from the nonreproducible DA concentrations. They also reported that the use of an air-saturated perfusion solution may raise the local oxygen concentration to ~200% of the basal value.

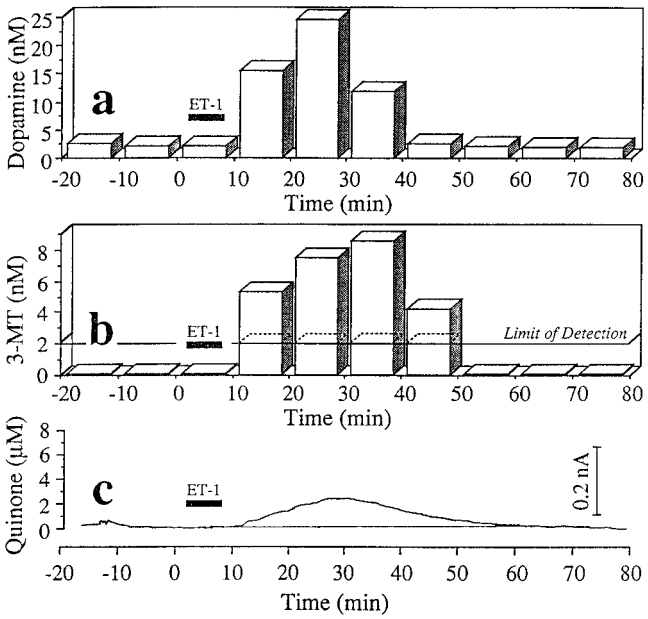

Figure 5. In vivo experiment on a freely moving rat using the on-line setup. (a) Extracellular dopamine and (b) 3-methoxytyramine (3-MT) concentrations from samples collected from the on-line setup output, $\Delta t = 10$ min. (c) The on-line record of quinones, $E_{app} = -200$ mV vs Ag/AgCl. Flow rate 2.00 μL/min, nondeoxygenated perfusion fluid. Endothelin-1 120 pmol in 6 μL given during 6 min. Pulse-damper used to suppress short-term fluctuations of flow rate.

Since a deoxygenated perfusion fluid may cause in vivo problems (hypoxia) and since no formation of quinones was observed in the tubing when using the air-saturated fluid, the latter was selected.

In Vivo Assay on Freely Moving Rats. The microdialysis experiment was carried out by collection of the microdialysis output $C_0(t)$, during a time period Δt, into the vial containing a fixed volume of an antioxidant solution (V_A). The mode of this detection setup can be regarded as an integrator,[41] for which the resulting "mean" concentration of the analyte in the ith collection (C_{ci}, for $i = 1, 2, 3, \ldots$) can be described as

$$C_{ci} = q\frac{1}{\Delta t}\int_{(i-1)\Delta t}^{i\Delta t} C_0(t)\, \mathrm{d}t \qquad (6)$$

where q is a dimensionless dilution factor equal to $V_0/(V_A + V_0)$. V_0 is the volume collected during the period Δt. Assuming that the flow rate is constant during the experiment, then $V_0 = U_V \Delta t$.

The value of $C_0(t)$ is also a function of the flow rate, as can be seen from eq 3. The solution of the integral is given by the knowledge of the time dependency of the extracellular in vivo analyte concentration $C_x(t)$, which is reflected by the microdialysis output $C_0(t)$. The approximate solution of this problem for first-order kinetics has been described by Ståhle.[42] However, this unknown time function $C_0(t)$ can be easily measured by any on-line technique, such as the electrochemical detection system described in this study.

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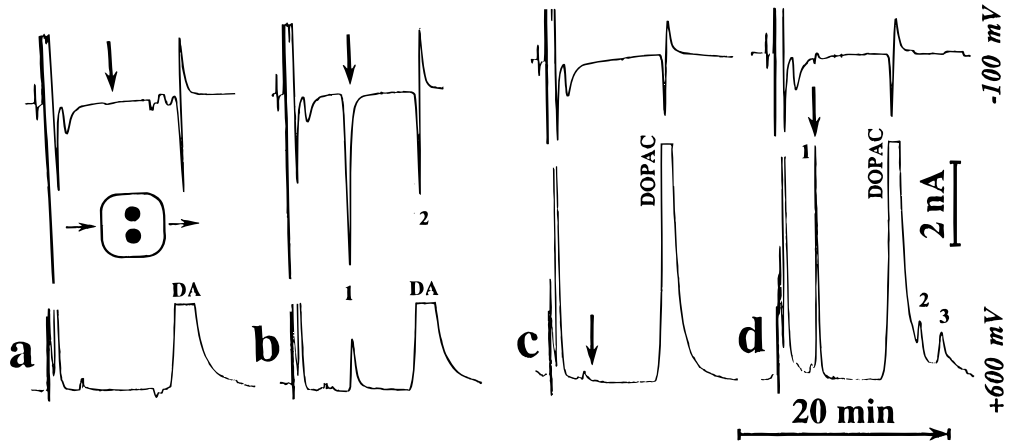

Figure 6. Chromatograms of (a) DA, (b) DA oxidized by bulk electrolysis for 60 min, (c) DOPAC, and (d) DOPAC after 60 min of electrolysis. Bulk electrolysis: GCE at +600 mV vs Ag/AgCl. HPLC conditions: column, PEEK 2 mm i.d.; stationary phase, C18 3 μm; flow rate, 300 μL/min; 20 μL of 10 μM compound injected. Detector: dual parallel GCEs at +600 and −100 mV, gasket 16 μm.

It was of interest to point out any relationship between quinones, measured on-line, and dopamine, sampled as described above and analysed by HPLC. As can be seen from eq 6, a ratio of C_{0max} (quinone maximum concentration) to C_{cmax} (DA maximum concentration) may be used as a criterion for validation of the on-line method for quinones with respect to the well-established method for dopamine, provided that all the other parameters are kept constant (C_{cmax} calculated for $V_A = 0$). Actually, if the dopamine and quinone concentrations follow the same trend, this ratio will be quasi-constant in a wide range of ET-1 doses. It was observed that this ratio varied from one animal to another between 30 and 80 in our experiments ($n = 6$, $U_V = 2.00$ μL/min, $\Delta t = 10$ min). A relative constancy of this ratio per animal, for repeated ET-1 administration, was observed, and this may indicate that the signal measured by the on-line technique is effectively attributed to endogenous quinones. The observed variation of this ratio between different animals could be explained by the different rate of oxygen reestablishment during reperfusion because of differences in the intensity of the ischemia. The critical factor affecting the degree of ischemia, and thus the increase of dopamine and quinones and the rate of reperfusion in our experiments, was found to be the distance of the ET-1 administrator from the middle cerebral artery (MCA) (see Figure 1c).

The in vivo correlation between the dopamine, 3-methoxytyramine, and quinones concentration is shown in Figure 5. A similar profile with quasi-identical lag time and rise time was obtained (Figure 5). As predicted, the increase of quinones and 3-MT follows the trend of dopamine. Finally, this study showed also that the reperfusion period of ischemia can be monitored by the on-line detection of quinones. Interestingly, the behavioral change of the animal due to the ischemia usually occurred around the maximum of quinone detection, i.e., 30−40 min after ET-1 administration.

Quinone Identification. To identify the major quinones present in dialysates, DA and DOPAC were electrochemically oxidized in two ways. In the first method, the oxidation of the catechols was performed by bulk electrolysis. The resulting products were injected into the HPLC system. Chromatograms of DA and electrolyzed DA are shown in Figure 6a,b, respectively. Peak 1 (capacity factor $k = 4$) in Figure 6b at −100 mV corresponds to the DOQ, while the origin of peak 1 at +600 mV is not clear. Xu et al.[22] suggested that this peak, coeluting with

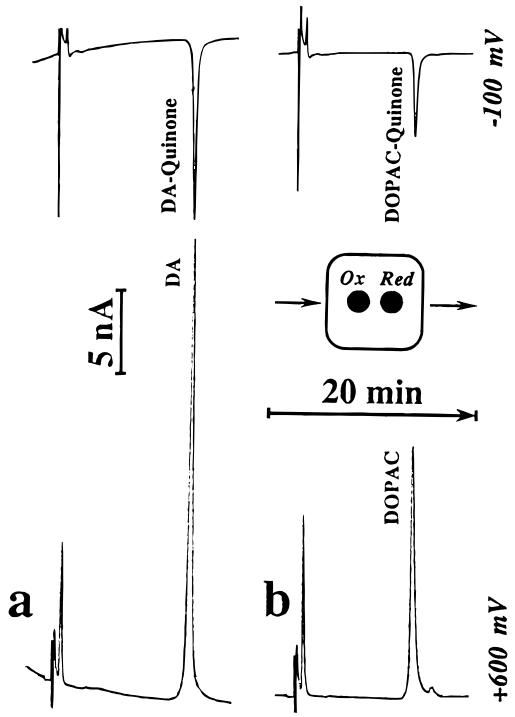

Figure 7. Generation of *o*-quinones in the detector (serial electrodes) of the HPLC setup (at +600 mV) with their rapid subsequent detection (at −100 mV). (a) DA and (b) DOPAC injected, each of 2 μM. Other conditions as in Figure 6.

quinone, is a product of the exposure of the quinone to stainless steel columns. However, in our setup, no stainless steel column was used, and the only steel material which was in contact with the solution in the injection syringe needle was passivated with HNO_3. Taking into account this observations and literature data on dopamine oxidation, we may conclude that this coeluting substance might rather be the reduced aminochrome, a product of internal cyclization of DOQ. The double spiking peaks 2 at −100 mV in Figure 6b are likely of capacitive origin because of the significant changes in ionic strength during the elution of DA (also DOPAC, see Figure 6). This type of double peak was also observed in other chromatograms (for DOPAC and 5-hydroxyindoleacetic acid (5HIAA), see below).

In the case of DOPAC, the electrolysis generated a product showing a peak 1 ($k = 2.3$, Figure 6d) at the detection GCE

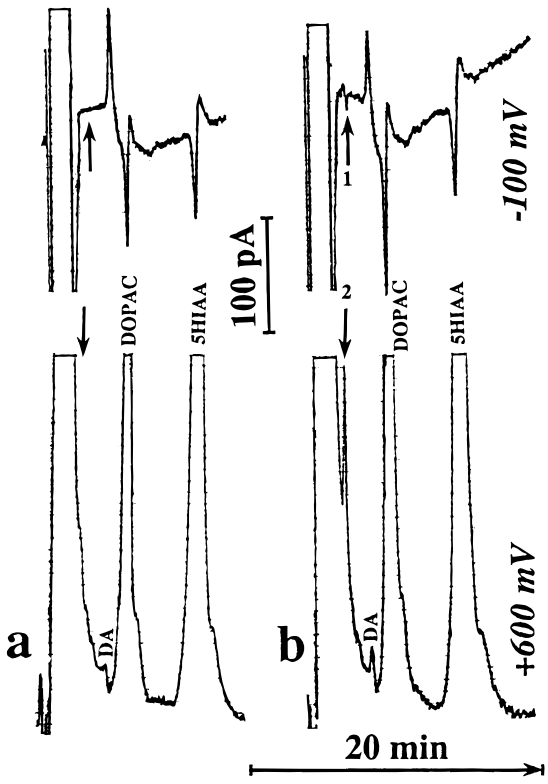

Figure 8. Chromatograms of brain in vivo dialysates: (a) basal conditions and (b) 20 min after ET-1 (240 pmol) administration. Collection of 2 μL/min during 20 min. HPLC conditions: flow rate, 600 μL/min; dual parallel detection. Other conditions as in Figure 6.

polarized at +600 mV. This peak increased as a function of the time of electrolysis. No quinone reduction peak was detected at −100 mV. A capacity peak was detected because of DOPAC elution only. Other compounds, peaks 2 and 3, were formed.

The results of the electrolytical oxidation of DA and DOPAC were similar to those obtained by chemical oxidation with KIO_4. However, neither the electrolytical nor the chemical oxidation generated DOPAC−quinone. Actually, because of the high reactivity of DOPAC−quinone, this species was lost between its formation and injection into the HPLC column.

To clarify the existence of DOPAC−quinone, the following study was performed. The *o*-quinone was generated in the electrochemical detector installed in serial configuration (Figure 7) at the electrode *Ox* (+600 mV) and rapidly detected (delay of ~10 ms) downstream on the electrode *Red* (−100 mV). The resulting chromatograms are shown in Figure 7a for DA and in Figure 7b for DOPAC. The ratios between the peaks of the oxidized and the reduced compound are similar for DA and DOPAC. From these data we can conclude that both quinones rapidly undergo side reactions, and that DOQ is more stable in the solution than DOPAC−quinone, which was undetectable a few minutes after its formation. Full identification of these peaks requires HPLC-MS instrumentation.

Comparing the results in Figure 6b and d with chromatograms obtained from in vivo dialysates (Figure 8), we can see in the dialysate from the ischemic brain (Figure 8b) a small peak 1 at −100 mV, corresponding to DOQ (see Figure 6b, peak 1), and a peak 2 at +600 mV, corresponding to the product of oxidation of DOPAC (see Figure 6d, peak 1). Also, the capacitive peaks of DOPAC and 5HIAA as well as the elevation of DA concentration, as a result of ischemia, were observed.

CONCLUSION

The method presented has been found to be suitable for continuous on-line endogenous quinone monitoring in the investigation of the kinetics of action of endothelin-1. The results were possibly overestimated by the use of 1,4-benzoquinone as a standard for the calibration of the electrode. The presence of oxygen in the perfusion fluid had no significant effect on the observed increase of quinones. The applied potential and working electrode material eliminated interferences of hydrogen peroxide, riboflavin, and easily oxidizable species. The increase of endogenous quinones was directly related to the increase in dopamine.

Finally, the identification study of the primary oxidation products has confirmed that both DA and DOPAC were oxidized during the ischemic injury, but the signal obtained by the on-line technique was probably related to DOQ, which has been found to be more stable than DOPAC−quinone. To confirm these data, implanted carbon fiber microelectrodes could be used in vivo as a complementary tool by applying fast-scan voltammetry.[43]

This on-line experimental configuration is potentially of interest in studies dealing with selective and sensitive in vivo detection of various physiologiccal compounds and drugs by the use of appropriate modified electrodes.[44]

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