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Original Contribution

ELEVATED EXTRACELLULAR GLUTAMATE LEVELS INCREASED THE FORMATION OF HYDROXYL RADICAL IN THE STRIATUM OF ANESTHETIZED RAT

CHUNG-SHI YANG, PI-JU TSAI, NAI-NU LIN, LIN LIU, and JON-SON KUO

Department of Medical Research, Taichung Veterans General Hospital, Taichung, Taiwan, Republic of China

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Abstract—Results from various in vitro experiments have indicated that excitotoxicity and oxidative stress are two interrelated major mechanisms in causing neuronal damage in brain disorders such as cerebral ischemia/reperfusion. Thus, we have conducted experiments to investigate whether in the striatum of anesthetized rats the elevated brain extracellular concentrations of glutamate could increase the formation of hydroxyl radical. Elevation of glutamate levels and trapping of hydroxyl radical were accomplished by perfusing Ringer solutions containing both glutamate and salicylic acid through microdialysis probes implanted in rat striatum. The formation of hydroxyl radical was quantitated as the increased amounts of 2,3 and 2,5 dihydroxybenzoic acid (DHBA) which were the hydroxylative products of salicylic acid. Eluted microdialysates were directly injected onto high performance liquid chromatography (HPLC) with electrochemical detector via an on-line automatic injector. This method was authenticated by in vitro studies employing Fenton-type hydroxyl radicals generation system. Our results indicated that elevated glutamate concentrations (15 mM, 1.5 mM, and 150 μM glutamate in perfusing solutions) would significantly increase both the concentrations of 2,3 and 2,5 DHBA. In conclusion, we have obtained direct evidence showing that the elevated glutamate concentrations in brain extracellular space would increase the formation of hydroxyl radical, and these results implied that oxidative stress occurring in brain disorders might be induced by excitotoxicity.

Keywords—Free radicals, Excitotoxicity, Oxidative stress, Rat striatum, Glutamate, Hydroxyl radical, Microdialysis

INTRODUCTION

Cerebral ischemia has been known to cause elevation of extracellular excitatory amino acids in the brain,[1–3] and to increase formation of reactive oxygen species (ROS).[4,5] Prolonged presence of excitatory amino acids and ROS can induce excitotoxicity and oxidative stress, respectively, and these are two of the major mechanisms responsible for neuronal damage in cerebral ischemia/reperfusion.[6,7] Several pieces of evidence have pointed out that excitotoxicity and oxidative stress are related in that excitatory events may stimulate the formation of ROS, and ROS could also lead to the release of excitatory amino acids.[8–13] For example, significant release of excitatory amino acids occurs when slices of rat hippocampus are incubated with enzymes and substrates (such as xanthine/xanthine oxidase) known to generate ROS.[9,10] On the other hand, glutamate receptor agonists are able to induce the formation of ROS.[11,12] Although there is evidence supporting the bidirectional relationship between excitotoxicity and oxidative stress, whether one of these events give rise to the second is not yet well established.[13] Additionally, most of the evidence suggesting the interrelation between excitotoxicity and oxidative stress is from in vitro studies. Therefore, results from in vivo experiments using anesthetized animals might be helpful in clarifying the relationship between excitotoxicity and oxidative stress.

Salicylic acid is a commonly used trapping reagent for hydroxyl radical. Intracerebral microdialysis is a useful technique for continuous monitoring of metabolite concentrations in extracellular space. A microdialysis perfusing technique, in which salicylic acid was used as a trapping reagent, has been used to determine the formation of hydroxyl radical from dopamine autooxidation.[14,15] Thus, we used in vivo microdialysis perfusion of glutamate solution containing salicylic acid to investigate whether the elevated brain extracellular

Address correspondence to: Chung-Shi Yang, Department of Medical Research, Taichung Veterans General Hospital, Taichung, Taiwan, R.O.C. 40705.

glutamate concentrations would increase the formation of hydroxyl radical. High performance liquid chromatography (HPLC) with electrochemical detector was combined with the microdialysis perfusion system via an on-line automatic injector to shorten the analysis time and avoid possible analyte degradation.

MATERIALS AND METHODS

General procedure for microdialysis

The microdialysis system, obtained from Carnegie Medicine Associates (CMA, Stockholm, Sweden), was perfused with Ringer solution at a flow rate of 2 μl/min. The microdialysis probes were purchased from CMA (CMA/10, membrane length 2 mm). The probe was perfused in corresponding outer medium for 30 to 60 min before starting the measurement to avoid the changes of relative recovery with time.

Hydroxyl radical generating system in microdialysis perfusing

Biological Fenton-type system was utilized to generate the hydroxyl free radical according to published procedure, with modifications.[16,17] Briefly, the reaction was carried out as follows: perfusion solutions containing 500 μM salicylic acid, 2 mM ADP, and 0.003% hydrogen peroxide (H_2O_2) were perfused (2 μl/min) through microdialysis probes implanted in solutions containing various concentrations of Fe^{2+}. Microdialysates were collected in a 20 μl loading loop of a CMA-160 on-line injector for direct and automatic injection onto an HPLC system.

Animal preparations

Male Sprague–Dawley rats (280–350 gm) were used. The animals were anesthetized with urethane (1.2 g/kg, IP), and body temperature was maintained at 37°C with a heating pad. Polyethylene catheters were inserted into the femoral artery for monitoring the systemic arterial blood pressure (SAP) with a Gould pressure processor. The rat's head was mounted on a stereotaxic apparatus (Davis Kopf Instruments, Tujunga, CA, USA) with the nose bar positioned 3.3 mm below the horizontal. Following a midline incision, the skull was exposed and one burr hole was drilled on the skull for inserting a dialysis probe. The microdialysis probe was implanted into the striatum (1.2 mm anterior and 2.5 mm lateral to the bregma, and 6 mm from the brain surface). The microdialysates were directly loaded and injected onto the HPLC system through a CMA 160 on-line injector. Sample loop for the on-line injector was 20 μl, and the injecting time length was 8 s. Probe recoveries for 2,3 and 2,5 DHBA were obtained from the injections of microdialysates perfused from standard 2,3 and 2,5 solutions after each experiment.

HPLC system for determination of 2,3 *and* 2,5 *DHBA*

The HPLC system consisted of a Hewlett Packard 1050 series quarternary pump, a 1050 series on-line degasser, and the Bioanalytical System LC-4C electrochemical detector (Bioanalytical Systems, Lafayette, IN, USA). Chromatographic conditions were followed by published procedures[9] except 5 μm Econosphere C18 reversed-phase cartridge columns (4.6 mm × 150 mm) (Alltech Associates Inc., Deerfield, IL, USA) were used for separation. Detection mode was amperometric with a potential set at 0.6 V, and maximum sensitivity was 50 nA.

HPLC system for glutamate analysis

The HPLC system used in glutamate analysis was ICI automated amino acid analysis system (aminomate) (ICI Instruments, Australia). The system included an LC 1150 Quaternary HPLC Pump controlled by an ICI Instruments DP900 Chromatography Management System, a TC 1900 Column Oven, and a LC 1250 Fluorescence Detector (excitation wavelength was 270 nm, and emission wavelength was 316 nm). Alltech 5 μm Econosphere C18 reversed-phase cartridge columns (4.6 mm × 150 mm) were used for separation. Glutamate was analyzed as its derivative with 9-fluorenylmethyl chloroformate, and sample preparation procedure and chromatographic conditions were followed by published procedures.[18]

RESULTS

On-line determination of 2,3 *and* 2,5 *DHBA resulting from* Fe^{2+}*-induced hydroxyl radical*

To test the operational condition of this on-line injection-analyzing system for hydroxyl radical, microdialysates from perfusing solution containing ADP, H_2O_2, and salicylic acid through probes implanted in Fe^{2+} solutions of various concentrations were analyzed. The results are shown in Figure 1. When the ADP and H_2O_2 concentrations were kept constant, the amounts of both 2,3 and 2,5 DHBA were linearly correlated with the Fe^{2+} concentrations. These results demonstrated that the microdialysis perfusing solution containing salicylic acid was able to trap the hydroxyl free radical generated from reaction of Fe^{2+} with H_2O_2 and ADP.

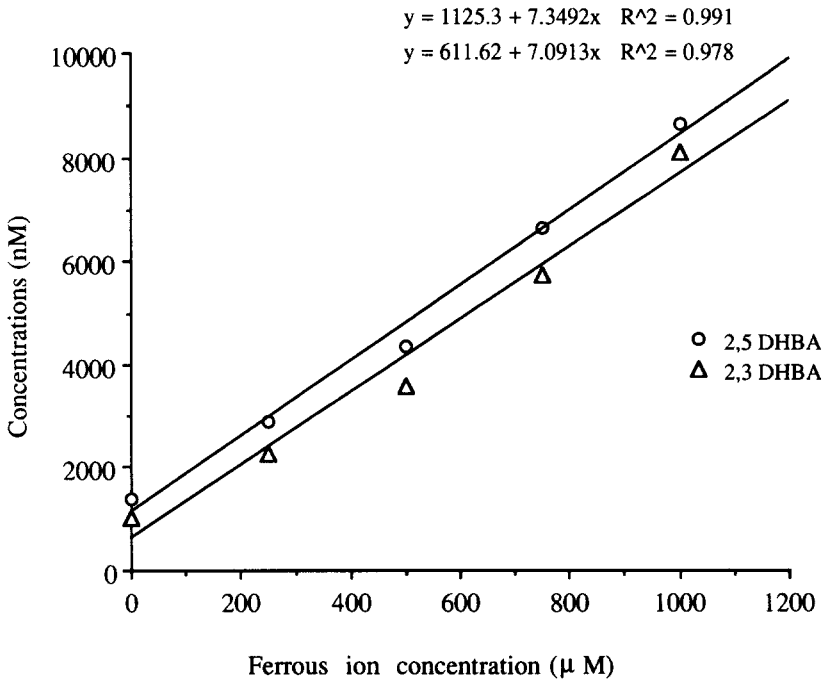

Fig. 1. Effect of Fe^{2+} concentrations on the hydroxyl radical formation represented as the amounts of 2,3 and 2,5 DHBA formation trapped by salicylic acid through microdialysis perfusion.

Determination of glutamate concentrations

Recoveries for glutamate through microdialysis probes (CMA/10 membrane length 2 mm) used in this study was determined before and after the animal experiments. Four microdialysis probes were tested. The recoveries were found to be in the range from 12 to 20% (mean ± SEM 17.2% ± 2.3%). We further determined the basal extracellular glutamate concentrations in rat striatum. From the average of eight different rats, the extracellular glutamate levels in rat striatum varied in the range from 6 μM to 88 μM (mean ± SEM 24.74 ± 11.13 μM).

Effect of elevated glutamate concentrations on the increased formation of hydroxyl radical

Ringer solutions containing both glutamate and salicylic acid were perfused through the rat striatum to investigate the formation of hydroxyl radical. Three different concentrations (15 mM, 1.5 mM, and 150 μM) of glutamate solutions containing salicylic acid were perfused consecutively through the microdialysis probes implanted in rat striatum. In this perfusion process, there was a 30-min period for the perfusion of Ringer solutions containing no glutamate in between the perfusions of glutamate solutions. Formation of hydroxyl radical was represented as the amounts of 2,3 and 2,5 DHBA. Results from the experiments of five rats are summarized in Figures 2 and 3. Although no obvious dose-related increase of hydroxyl radical with glutamate levels was observed, the amounts of both 2,5 and 2,3 DHBA were dramatically increased after the perfusion of glutamate solutions at these concentrations.

In another set of experiments, perfusing sequence for the three glutamate concentrations were reversed (0.15 mM, 1.5 mM, and 15 mM) to compare its effect with the previous perfusing sequence on hydroxyl radical formation. These results are shown in Figures 4 and 5. Interestingly, the increase of hydroxyl radical was less dramatic compared with the hydroxyl radical formation when the perfusing sequence glutamate solutions were reversed. However, the increase of 2,3 and 2,5 DHBA levels after the perfusion of glutamate solutions was still evident.

To clarify whether the increased formation of 2,3 and 2,5 DHBA induced by elevated glutamate content was indeed resulting from the excitatory effect of glutamate, a nonexcitatory amino acid (tryptophan) was perfused with salicylic acid to examine the effect on extracellular hydroxyl radical formation. Tryptophan was perfused either before or after the perfusion of glutamate to the same animal. The results from eight different rats are shown in Figures 6 and 7. At the

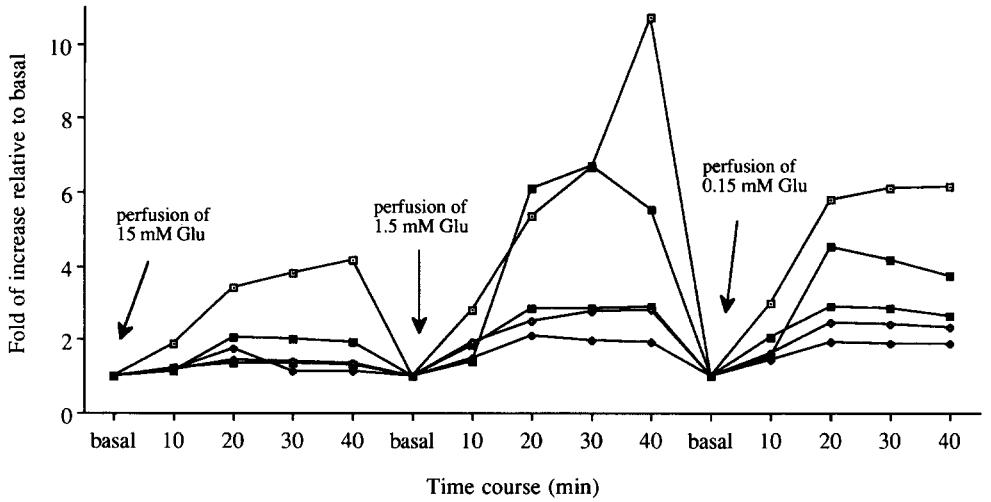

Fig. 2. Formation of hydroxyl radical as represented by the formation of 2,3 DHBA in rat striatum extracellular space after infusion of various concentrations (15 mM, 1.5 mM, and 0.15 mM) of glutamate solutions. Absolute values for basal (100%) 2,3 DHBA ranged from 0.28 to 3.20 μM.

concentration of 1.5 mM in the perfusing solution, tryptophan had no significant effect on the extracellular hydroxyl radical formation, while glutamate at the same concentration could indeed induce the increased formation of 2,3 and 2,5 DHBA.

DISCUSSION

Various reports have documented the interrelation between excitotoxicity and oxidative stress.[8-13] Most of the results were obtained from experiments in which cultured brain slices from hippocampus and striatum were used. With the use of microdialysis perfusion in which the glutamate infusion and trapping of hydroxyl radical could be achieved simultaneously, we were able to observe the direct effect of glutamate elevation on formation of hydroxyl radical in anesthetized rat brain. Additionally, the use of an on-line collector for microdialysates not only minimized the time period before the injection but also automated the injection. The results clearly indicated that elevated intracerebral glutamate concentrations could induce the formation of hydroxyl radical. We used three different glutamate concentrations for microdialysis perfusion (15 mM, 1.5 mM, and 0.15 mM); selection of these concentrations were based on

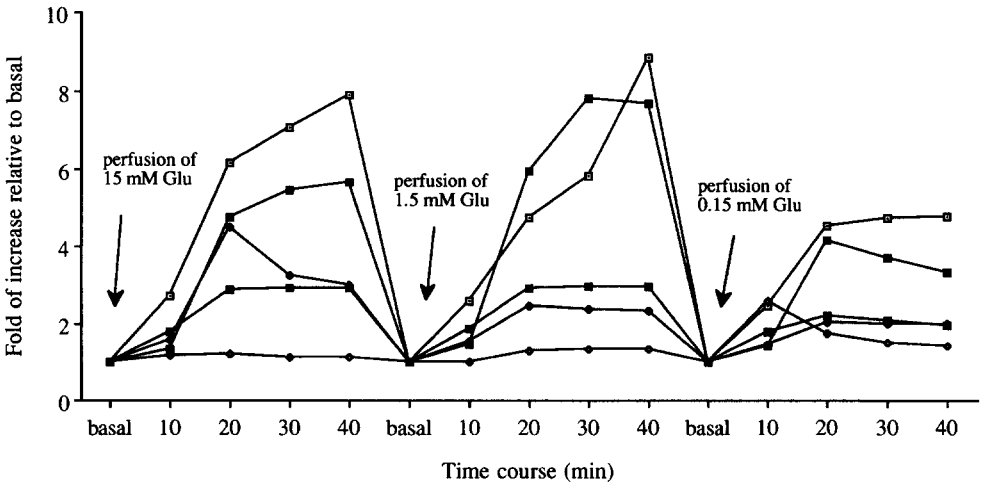

Fig. 3. Formation of hydroxyl radical as represented by the formation of 2,5 DHBA in rat striatum extracellular space after infusion of various concentrations (15 mM, 1.5 mM, and 0.15 mM) of glutamate solution. Absolute values for basal (100%) 2,5 DHBA ranged from 0.45 to 2.31 μM.

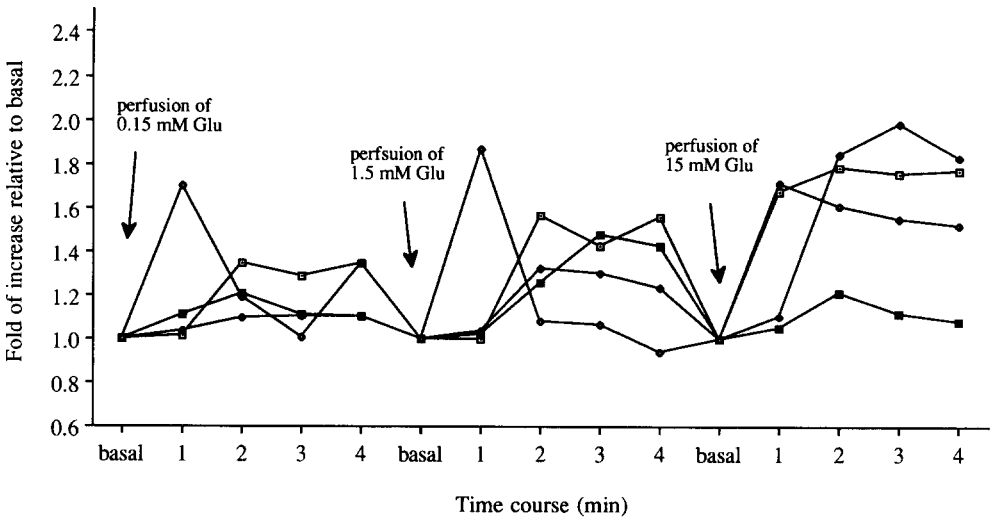

Fig. 4. Formation of hydroxyl radical as represented by the formation of 2,3 DHBA in rat striatum extracellular space after infusion of various concentrations (15 mM, 1.5 mM, and 0.15 mM) of glutamate solutions. Absolute values for basal (100%) 2,3 DHBA ranged from 0.15 to 0.34 μM.

the probe recovery (12 to 20%) obtained from in vitro experiments, and on the fact that after cerebral ischemia striatum extracellular glutamate levels were in the order of 0.01 to 0.1 mM according to published results[19,20] as well as our own data. Extracellular glutamate levels at these concentrations are documented to induce excitotoxicity. It is interesting that when the glutamate solutions were perfused from low concentration (0.15 mM) to high concentration (15 mM), the increased formation of hydroxyl radical was less dramatic than it was when glutamate solutions were perfused from large (15 mM) to small (0.15 mM) concentrations. This interesting phenomenon is probably due to the fact that high concentrations of glutamate sensitized the rat brain to glutamate, but more studies are needed to further clarify the difference in the scale of increase for hydroxyl radical formation when the high and low concentrations of glutamate were perfused reversely. However, regardless of the sequence in perfusing different concentrations of glutamate solutions, it is obvious the increased extracellular glutamate could induce the formation of hydroxyl radical. Additionally, radical adducts have been observed to occur in cerebral ischemia with the

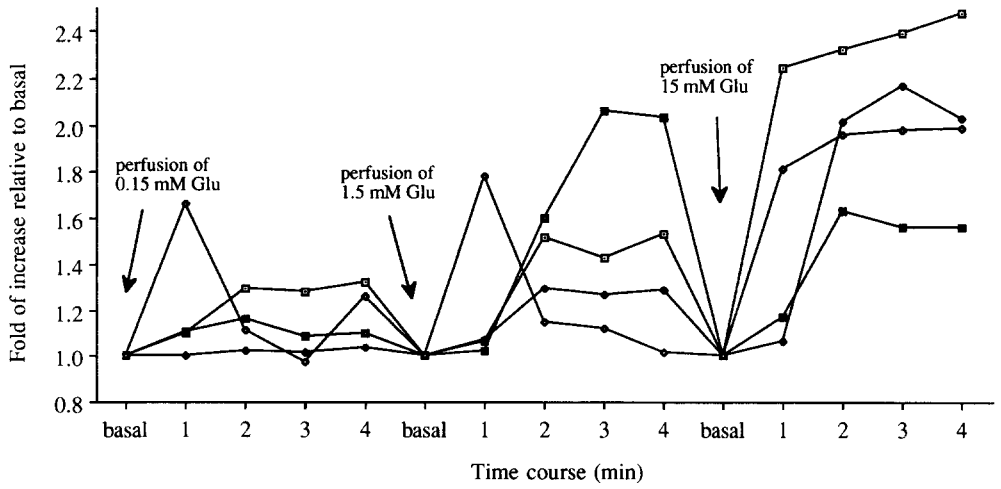

Fig. 5. Formation of hydroxyl radical as represented by the formation of 2,5 DHBA in rat striatum extracellular space after infusion of various concentrations (15 mM, 1.5 mM, and 0.15 mM) of glutamate solution. Absolute values for basal (100%) 2,5 DHBA ranged from 0.18 to 0.31 μM.

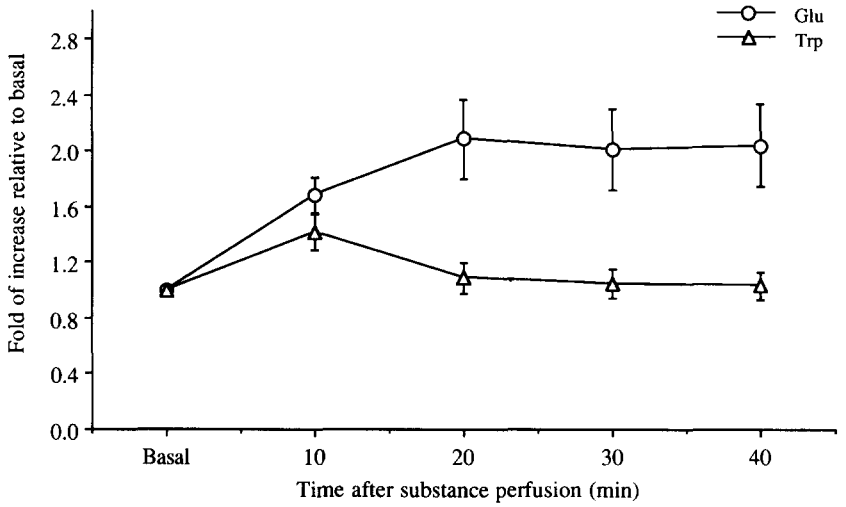

Fig. 6. Comparison of the effect of glutamate and tryptophan on 2,3 DHBA formation in the extracellular space of rat striatum. Absolute values for 2,3 DHBA ranged from 0.16 to 0.60 μM.

use of microdialysis perfusing and spin trapping reagent.[15] Appearance of radical adducts in cerebral ischemia with microdialysis techniques corresponds with our results, in that we have found that increased extracellular glutamate concentrations as often observed in cerebral ischemia, could induce the formation of hydroxyl radical.

Pellegrini-Giampietro et al.[9] hypothesized a vicious cycle illustrating the possible mutual cooperation between free radical formation and EAA release. In the cycle the authors suggested that during ischemia, free radicals were formed and subsequently caused the release of EAA, which could further increase the formation of free radicals. Our results corresponded with the hypothesis in that elevated glutamate content could indeed induce the increased formation of hydroxyl radical. There could be several possibilities for this increased hydroxyl radical formation. One possibility is that the glutamate might interact with glutamate receptors to trigger a series of events, such as calcium ion influx with subsequent activation of phospholipase C and A_2. Subsequently these events might lead to the formation of hydroxyl radical.

In conclusion, we have connected an on-line automatic injection system for continuous monitoring of extracellular hydroxyl radical formation in anesthetized rat brains, and with this method we have found that elevated extra-

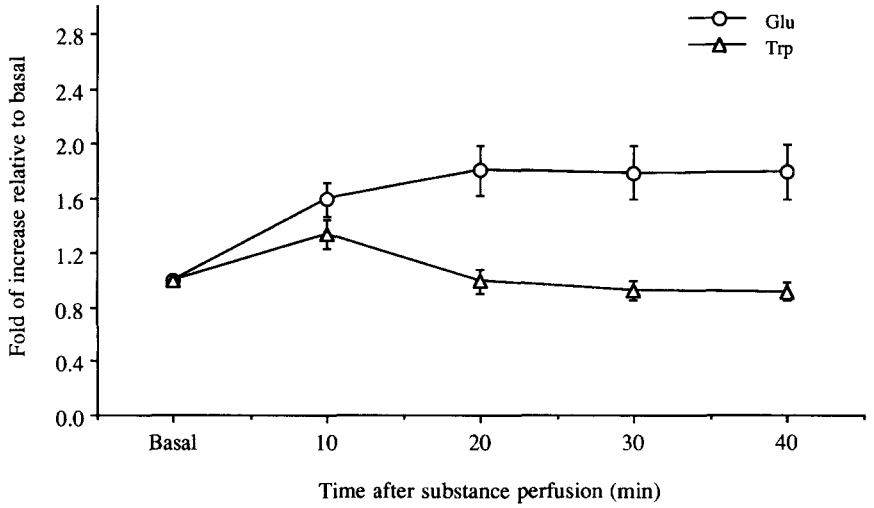

Fig. 7. Comparison of the effect of glutamate and tryptophan on 2,5 DHBA formation in the extracellular space of rat striatum. Absolute values for 2,5 DHBA ranged from 0.18 to 0.43 μM.

cellular glutamate concentrations could indeed increase the formation of hydroxyl radical.

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