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Use of Microdialysis for In-vivo Monitoring of Hydroxyl Free-radical Generation in the Rat

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Abstract

Free-radicals are reported to cause the tissue-damage associated with some toxins and diseases, yet there is no suitable method for routine in-vivo monitoring of these species. This paper introduces an in-vivo microdialysis technique in which the hydroxyl radical reacts with salicylate to generate dihydroxybenzoic acids (DHBA) which are measured by HPLC with electrochemical detection.

When pargyline, a monoamine oxidase inhibitor, was infused into rat brain, the levels of DHBA increased markedly. When noradrenaline was administered to animals pre-treated with pargyline, DHBA levels increased markedly compared with the group treated with noradrenaline only. When the heart was subjected to 15-min regional ischaemia by occlusion of the left anterior descending coronary artery, levels of DHBA in heart dialysate were unchanged. Electrical stimulation of the stellate ganglion resulted in marked elevation of levels of DHBA the myocardial dialysate. Infusion of Fe^{2+} into rat liver resulted in increased formation of DHBA. When the intestine was rendered ischaemic for 10, 20 and 30 min, the highest DHBA level was obtained after 10-min ischaemia and the lowest after 30 min.

These results confirm that free-radical production might make a major contribution at certain stages in the progression of the injury.

Although free-radical reactions are part of normal metabolism, oxygen-derived species such as the superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) have been implicated as damaging agents in the action of many toxins and in a number of diseases (McCord 1985). O_2^- and H_2O_2 are two oxygen free-radical species, usually present in biological systems only at very low concentrations, which have been implicated as etiological agents in several pathological conditions (Ganote et al 1982). O_2^- itself is somewhat poorly reactive in aqueous solution, but does participate in reactions with iron ions, generating the more damaging hydroxyl radical (·OH) (Halliwell & Gutteridge 1984; Das et al 1989; Pou et al 1989). The highly reactive ·OH species is frequently generated in biological systems (Gerlach et al 1994).

Aromatic hydroxylation was used as early as 1976 to measure ·OH in biochemical systems, the hydroxylated products being quantitated by colorimetry (Halliwell & Ahluwalia 1976; Halliwell 1978). Although improved colorimetric (Richmond et al 1981) and fluorescence-based (Gutteridge 1987; Baker & Gebicki 1986) assays for measurement of hydroxylated products have been described, complete determination of hydroxylated product formation can be achieved only by use of gas chromatography (Richmond et al 1981; Karam & Simic 1988) or high-performance liquid chromatography (HPLC) with UV- or electrochemical (EC) detection, or both (Radzik et al 1983). The hydroxyl radical reacts with salicylate and generates 2,3- and 2,5-dihydroxybenzoic acids (DHBA) (Grootveld & Halliwell 1986; Halliwell et al 1991) which can be measured electrochemically in picomole quantities by HPLC-EC (Chiueh et al 1992; Obata & Chiueh 1992). The formation of DHBA after systemic administration of salicylate has been used as an index of ·OH generation in heart and brain tissues during ischaemia and reperfusion (Cao et al 1988; Powell & Hall 1990). We have focused on the possible use of salicylate hydroxylation for monitoring the time-course of DHBA generation in several organs of the rat by use of an in-vivo microdialysis technique.

Materials and Methods

Microdialysis probe

The microdialysis probe (Fig. 1A) was made from a 22-gauge needle and a microlitre pipette tip. The plastic hub of the needle was removed and its stainless steel shaft was cut to an appropriate length. The ends of this shaft were glued (epoxy resin) to the point cut from a microlitre pipette tip and a length of fused silica tubing (470 μm o.d. × 350 μm i.d.). Pieces of flexible fused silica tubing (150 μm o.d. × 75 μm i.d.; Eicom) were supported by metal tubing. One of the open ends of a piece of tubular dialysis membrane (220 μm o.d., 10 μm wall thickness, 50 000 MW cut-off; hollow cellulose fibre) was sealed with the same resin. The sealed tubular membrane was then passed over the fused silica until the tip of the silica was 0·2 mm or less from the sealed end of the dialysis tubing. Finally, the joint between the membrane and the large silica tubing was sealed with resin under a dissection microscope (Obata et al 1992).

In-vitro relative and absolute recoveries of 2,3- and 2,5-DHBA

A probe with a 3·0-mm length of dialysis tubing was initially tested in-vitro to determine the effect of flow rate on the recovery of standard 2,3- and 2,5-DHBA. A dialysis probe was bathed, at 37°C, in Ringer's solution containing 10^{-6} M 2,3- and 2,5-DHBA; dialysate samples were collected at different perfusion speeds (0·5–4 μL min^{-1}; Fig. 1B) and 2,3- and 2,5-DHBA in the dialysate were measured directly by HPLC with electrochemical (EC) detection.

Brain dialysis

Adult male Wistar rats, 300–400 g, were anaesthetized with chloral hydrate (400 mg kg, i.p.) and prepared for intracranial microdialysis brain perfusion. For detection of ·OH generation and its time-course in the regions of the brain we modified a published procedure for in-vivo intracranial microdialysis (Miyake & Chiueh 1989; Obata & Chiueh 1992; Chiueh et al

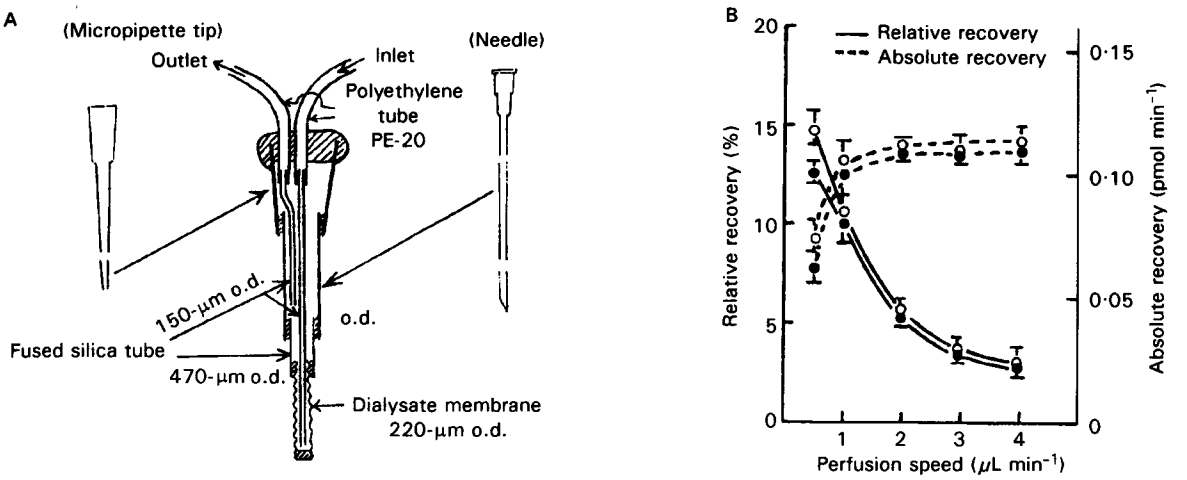


FIG. 1. A. Schematic diagram of the construction of the microdialysis probe (diagonal hatching indicates epoxy resin). B. Effects of perfusion flow-rate on the recovery of standards at 37°C. In-vitro relative and absolute recoveries of (●) 2,3- and (○) 2,5-dihydroxybenzoic acids obtained by perfusing a microdialysis probe in a solution of standards. When perfusion speed was increased, relative recovery rate ([perfusate concn/perfused standard concn] × 100%) decreased. However, absolute recovery rate (amount of standard, in pmol min^{-1}) increased in a non-linear fashion, approaching almost steady-state levels. Each value represents the mean ± s.e.m. of results from three dialysate samples.

1993). The probe was washed with Ringer's solution containing 147 mM NaCl, 2·3 mM $CaCl_2$ and 4 mM KCl (pH 7·4) for at least 30 min before stereotaxical implantation in the striatum (Paxinos & Watson 1982) (stereotaxic co-ordinates; A: 1·0, R/L 2·5, H: −7·0 from the dura matter) and then the flow (1 μL min^{-1}) of the solution was continued for at least 60 min. Thereafter ·OH radicals were trapped by perfusing the striatum with 0·5 mM sodium salicylate (Sigma, St Louis, MO) in Ringer's solution (0·5 nmol μL^{-1} min^{-1}) for 120 min (Floyd et al 1984; Halliwell et al 1991). Brain dialysate was collected every 15 min in tubes containing 15 μL 0·1 M $HClO_4$ and assayed immediately for dihydroxyphenylacetic acid and 2,3- and 2,5-DHBA by HPLC-EC (Obata et al 1994).

Generation of ·OH by monoamine oxidase. The effect of pargyline, a monoamine oxidase (MAO) inhibitor, on the generation of ·OH was investigated by in-vivo perfusion of pargyline (Obata & Yamanaka 1995) and striatal microdialysis. Salicylic acid in Ringer's solution (0·5 nmol μL^{-1} min^{-1}) was passed through the microdialysis probe to detect the generation of ·OH as reflected by the formation of DHBA in the striatum.

Cardiac microdialysis

Wistar rats, 250–350 g, were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.). The level of anaesthesia was maintained by continuous intravenous infusion of chloral hydrate (20 mg kg^{-1} h^{-1}). Artificial ventilation was maintained by constant-volume respiration with room air mixed with oxygen. Heart rate, arterial pressure, and electrocardiogram (ECG) were monitored and recorded continuously.

Microdialysis probe holding system. Akiyama et al (1991) applied a dialysis technique to the heart and attempted to monitor noradrenaline levels in local myocardial interstitial fluid. However, this technique for in-vivo detection of reactive oxygen species in biological systems is quite difficult. Therefore, we designed a system for holding the microdialysis probe (Obata et al 1994) which includes loose fixation of the tube and synchronization of the movement of the probe with that of the heart (Fig. 2). With the animal in the lateral position, the fifth or sixth rib on the left side was partially removed to enable observation of the heart. A small incision was made in the pericardium, and, with a fine guiding needle, the dialysis probe was implanted in the area of the left anterior descending coronary artery (LAD) of the ischaemic zone. Heparin sodium (200 units kg^{-1}) was administered intravenously before probe implantation and 100 units kg^{-1} were then given every hour to prevent blood coagulation. Vibration from the beating movement is accommodated by partial rotation about a stainless-steel wire. The inlet side of the microdialysis probe was connected by a polyethylene tube to a 1-μL syringe which was driven by a micro-injection pump (Carnegie Medicine CMA/100). The outlet side of the probe was connected to the same tube and this was placed into a small collecting tube. The dialysate (1 μL min^{-1}) was collected every 15 min into 15 μL EDTA in 0·1 M $HClO_4$ to prevent amine oxidation and assayed immediately for 2,3- and 2,5-DHBA by HPLC-EC (Chiueh et al 1983). This system is referred to as the 'Obata perfusion system' (O System) (Obata et al 1994).

Hydroxy radical generation associated with sympathetic nerve stimulation. To examine ·OH generation in response to cardiac sympathetic nerve stimulation, the stellate ganglion was electrically stimulated (Obata & Yamanaka 1996). The second rib on the left side was removed, and a shielded bipolar palladium electrode was placed around the two limbs of the left ansa subclavia, which was then stimulated by means of an SEN-3301 digital stimulator (Nihon Kohden, Tokyo, Japan) with a rectangular pulse (1, 2, 3, 4 and 5 V, 6 Hz, 2 ms in duration) during collection of one dialysate sample.

Liver microdialysis

Wistar rats, 250–350 g, were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and prepared for microdialysis liver perfusion. The abdominal cavity was opened through midline incision and the liver was perfused by means of a microdialysis

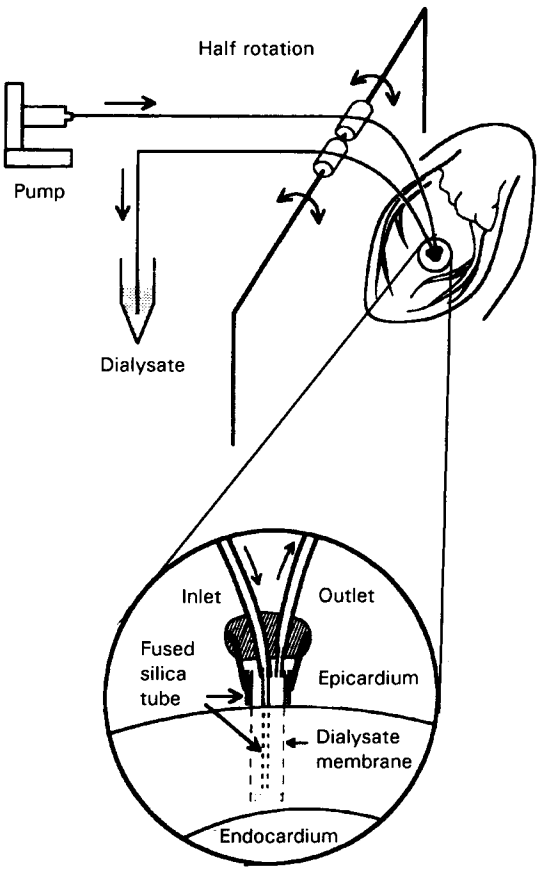


FIG. 2. Microdialysis probe-holding system in heart. The dialysis probe was implanted in the area of the left anterior descending artery (LAD) and perfused with Ringer's solution by means of a micro-injection pump. The microdialysis probe-holding system involves loose fixation of the tube and synchronization of movement of the heart and probe, the probe being supported by a stainless steel axis. This system increased recovery rate without an accompanying increase in tissue damage. The inlet of the microdialysis probe was connected to the micro-injection pump and the outlet to a small collecting tube.

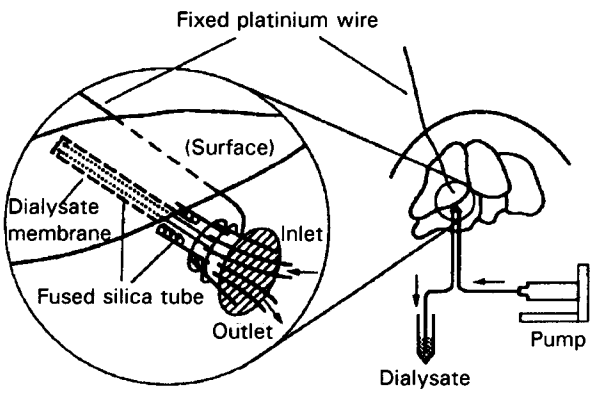


FIG. 3. Method of hepatic infusion. The abdominal cavity was opened through a midline incision. After implantation of a microdialysis probe (3-mm exposure) in the median lobe of the liver through the reverse side, the probe was fixed with a platinum wire. Ringer's solution containing salicylic acid was perfused by means of a micro-injection pump and liver dialysate was collected every 20 min into a small collecting tube containing 20 μL ice-cold 0·1% Na_2EDTA in 0·1 M $HClO_4$ and immediately assayed for 2,3- and 2,5-DHBA by HPLC-EC. (Diagonal hatching indicates epoxy resin.)

probe developed by Obata et al (1992) (Fig. 3). To increase the recovery rate without increasing tissue damage, we designed a liver microdialysis-probe-holding system which can be prepared speedily. After implantation of the microdialysis probe (3-mm exposure) with platinum wire in the median lobe of the liver through the reverse side, the probe was fixed by means of the platinum wire. Ringer's solution containing salicylic acid was perfused by means of a micro-injection pump to collect the basal levels of the formation of 2,3- or 2,5-DHBA during a defined time period. A sample was collected every 20 min into a small collecting tube containing 20 μL ice-cold 0·1% Na_2EDTA in 0·1 M $HClO_4$.

Jejunal microdialysis

Experiments were performed on Wistar rats, 300–400 g. The animals were deprived of food for 24 h before experiments but had free access to water. They were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and the level of anaesthesia was maintained by continuous intravenous infusion of chloral hydrate (20 mg kg^{-1} h^{-1}).

For this study, we used the microdialysis probe developed by Obata et al (1992). After anaesthesia, a 2–3 cm midline incision was performed to expose abdominal viscera. A portion of jejunum was isolated in-situ with intact vascular supply and laid on the abdominal wall. The intestine was punctured with a 23 gauge needle and the probe placed through the hole on the jejunal mucosa. The probe was then raised slightly to provide close contact between the mucosa and the tuner (Fig. 4). The isolated intestine was then subjected to ischaemia for 10, 20 or 30 min by occlusion of the mesenteric arteries. Heparin sodium (200 units kg^{-1}) was administered intravenously before probe implantation and then given every 30 min (100 units kg^{-1}) to prevent blood coagulation. The inlet side of the microdialysis probe was connected by a polyethylene tube and a 1-mL syringe to a micro-injection pump (Carnegie Medicine CNlA/100). The outlet side of the probe was connected to the same tube and placed into a small collecting tube.

Results and Discussion

In-vitro relative and absolute recoveries of 2,3- and 2,5-DHBA

Attack of ·OH radicals, generated by a Fenton system, upon salicylate produces 2,3- and 2,5-dihydroxybenzoic acids as major products and catechol as a minor product (Moorhouse et al 1985; Grootveld & Halliwell 1986; Halliwell et al 1991) (Scheme 1).

The relative recovery rate ([perfusate concentration × 100]/perfused standard concentration; in %) of 2,3- or 2,5-DHBA declined exponentially as the flow rate was increased from 0·5 to 4 μL min^{-1}. Conversely, absolute recovery (the amount of standard in pmol sample min^{-1}) increased as the perfusion flow rate was increased from 0·5 to $\approx$ 1 μL min^{-1} and levelled off at that flow rate. The mean relative recovery rates of 2,3- and 2,5-DHBA were 10·1 ± 0·8% and 10·5 ± 0·9%, respectively, at a flow rate of 1 μL min^{-1}. Using this perfusion speed (1 μL min^{-1}) in-vitro we obtained almost uniform relative recovery rates with different concentrations of 2,3- or 2,5-DHBA in the testing solution. On the basis of these results we chose a perfusion speed of 1 μL min^{-1} for in-vivo experiments.

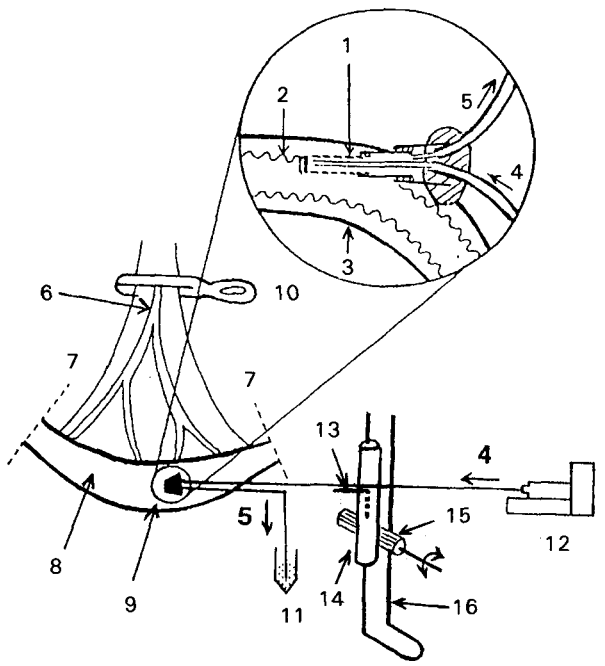
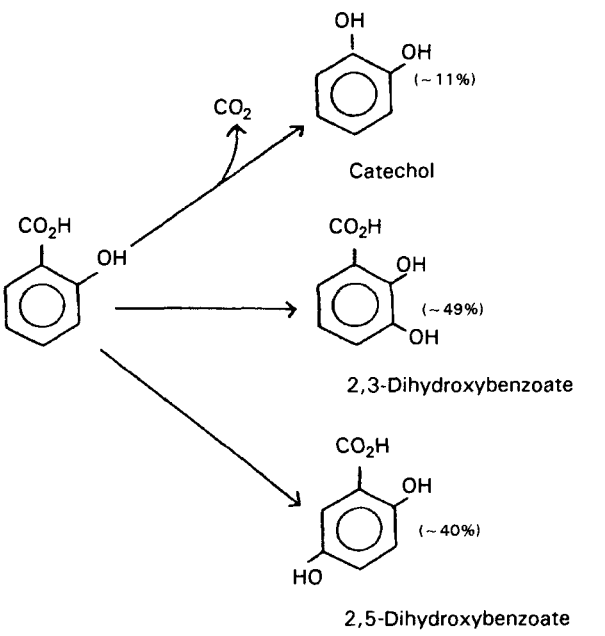


FIG. 4. Microdialysis probe-holding-system in intestine. After opening of the abdominal wall through a midline incision the jejunum was cut and the dialysis probe was placed on the jejunal mucosa and raised slightly to provide a close contact with its surface. The probe was then perfused with Ringer's solution by connecting its inlet to a microinjection pump. The outlet of the probe was connected to a small collecting tube. 1, Dialysis membrane; 2, mucosa; 3, serosa; 4, tube inlet; 5, tube outlet; 6, mesenteric vessels; 7, cutting end; 8, jejunal portion; 9, dialysis probe; 10 clamp; 11, dialysate; 12, pump; 13, wire supporter; 14, glass tube which can move freely by rotation on another silicon tube (tuner); 15, tuner; 16, fixed wire.



SCHEME 1. Products of the attrack by ·OH radicals on the salicylate molecule.

Brain dialysis

When pargyline (100 nmol μL^{-1} min^{-1}) was infused into rat brain, the level of 3,4-dihydroxyphenylacetic acid (DOPAC) gradually decreased in a time-dependent manner and a marked elevation of DHBA was observed (Fig. 5).

O_2^- has an extremely short half-life (Halliwell 1989) and rapidly undergoes dismutation yielding H_2O_2. H_2O_2 then undergoes a Fenton-type reaction in the presence of iron yielding the highly cytotoxic ·OH. ·OH can also arise from reaction between H_2O_2 and O_2^- (Haber–Weiss reaction). Theoretically, ·OH might be formed in-vivo during non-enzymatic oxidation (Graham et al 1978; Graham 1984; Fornstedt et al 1989; Ben-Shachar et al 1991) or by enzymatic oxidation of dopamine (Fig. 6), especially in the regions of the brain (putamen, caudate nucleus, and substantia nigra zona compacta) where levels of dopamine are high. Neuromelanin is formed in the brains of man and other mammals and is located in catecholaminergic neurons in the substantia nigra and other brainstem nuclei (Bogerts 1981). Normally, Fe^{3+}, is bound to

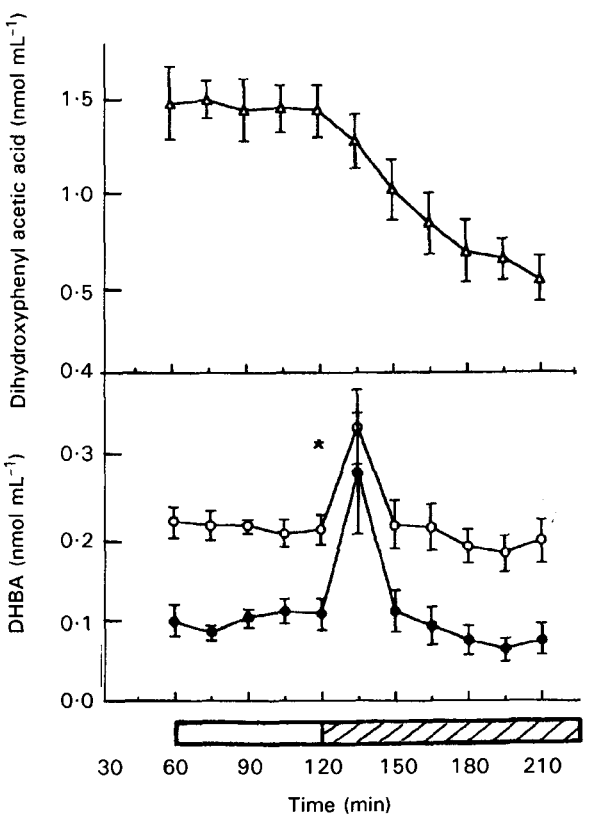


FIG. 5. Relationship between dihydroxyphenylacetic acid and ·OH generation after pargyline treatment. Time course of dialysate dihydroxyphenylacetic acid (top; △) and in-vivo trapping of highly reactive ·OH (bottom; ○ 2,3-DHBA, ● 2,5-DHBA) in the extracellular fluid of the caudate nucleus were investigated by infusing salicylic acid (0·5 nmol μL^{-1} min^{-1}) in Ringer's solution through an intracranial microdialysis probe placed in the rat striatum for 150 min. Pargyline (100 nmol μL^{-1} min^{-1}) added to the salicylic acid solution was infused directly into the rat brain for 90 min through a microdialysis probe. Thereafter dialysate samples were collected at 15-min intervals, starting at 120 min, and immediately assayed for dopamine and 2,3- and 2,5-DHBA by use of HPLC-EC. Differences between the time-courses for 2,3- and 2,5-DHBA levels were statistically studied by use of the Mann–Whitney U-test. Values are expressed as means ± s.e.m. of results from six rats. $*P < 0.05$ compared with levels at 105–120 min. Abscissa: infusion of salicylic acid was started after 60-min washout.

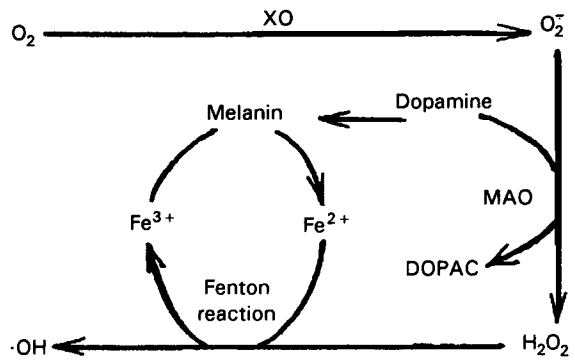


FIG. 6. Reaction pathway in rat brain illustrating the non-enzymatic and enzymatic oxidation of striatal dopamine.

endogenous chelators such as ADP and in the absence of a significant amount of ferritin in the substantia nigra could be chelated by melanin. Increased concentrations of dopamine could serve as the catalyst for the conversion to Fe^{3+} to Fe^{2+} by melanin and, in the presence of H_2O_2, result in further formation of ·OH, depending on the environmental conditions (Pilas et al 1988). The production of ·OH in the presence of melanin is significantly greater when Fe^{3+} is predominant (Pilas et al 1988), and is further demonstrated by the greater lipid peroxidation of rat cerebral cortex in the presence of Fe^{2+} and higher dopamine melanin concentrations (Ben-Shachar et al 1991).

Cardiac microdialysis

Noradrenaline and ·OH generation as a result of myocardial ischaemic injury. The heart was subjected to myocardiac ischaemia for 15 min by occlusion of the rat LAD and typical changes in the electrocardiogram were observed. When the time-course of control dialysate noradrenaline levels was observed over a period of 240 min the concentration of noradrenaline decreased over the first 120 min and then reached an almost steady-state level 150 min after probe implantation. This time-course was similar to that seen in brain dialysis monitoring of putative neurotransmitters (Imperato & Chiara 1984; Chiara 1990) and in heart-dialysis monitoring of noradrenaline (Akiyama et al 1991). Some investigators have reported that in brain (Benveniste 1989) or heart (Akiyama et al 1991) dialysis the relative in-vivo recovery rate of the dialysis probe decreased and then reached an almost steady-state level. However, when the heart was reperfused, the levels of noradrenaline and 2,3- and 2,5-DHBA were elevated (Fig. 7). We examined the effect of pargyline, an MAO inhibitor, on ·OH generation in the heart. When noradrenaline was administered to animals pre-treated with pargyline a marked elevation in the levels of 2,3- and 2,5-DHBA formation was obtained, compared with the group treated with noradrenaline only. Noradrenaline clearly caused an increase in ·OH formation (Obata & Yamanaka 1997). The sustained elevation of noradrenaline elicited by pargyline in the extracellular fluid can be autooxidized, which in turn leads (possibly by an indirect mechanism) to the formation of cytotoxic ·OH free-radicals.

Hydroxy radical generation during ischaemia. When the rat heart was subjected to 15-min regional ischaemia by occlusion of the LAD the levels of 2,3- and 2,5-DHBA in the heart dialysate (150–165 min after probe implantation) were unchanged. Changes in the ECG and blood pressure were recorded throughout the experiment. When premature ventricular contraction occurred at almost 25-s intervals, a marked elevation of the levels of 2,3- and 2,5-DHBA was observed in the heart dialysate after 30-min ischaemia (Obata et al 1995b).

Hydroxy-radical generation associated with sympathetic nerve stimulation. Electrical stimulation of the stellate ganglion resulted in marked elevation in the levels of the 2,3- and 2,5-DHBA in the myocardial dialysate. After stimulation, dialysate noradrenaline concentration returned almost to the pre-stimulation level. It is known that sympathetic nerve stimulation increases myocardial interstitial noradrenaline levels (Graham 1984; Riederer et al 1989). MAO is one of the heart enzymes playing a role in the metabolism of noradrenaline. When pargyline (100 nmol μL^{-1} min^{-1}), an MAO inhibitor, was infused into the rat heart, the level of noradrenaline gradually increased and a marked elevation in the levels of 2,3- and 2,5-DHBA was also observed. These findings suggest that the noradrenaline accumulating in the extracellular fluid as a result of the action of pargyline can be autooxidized. Sympathetic-nerve stimulation increased the

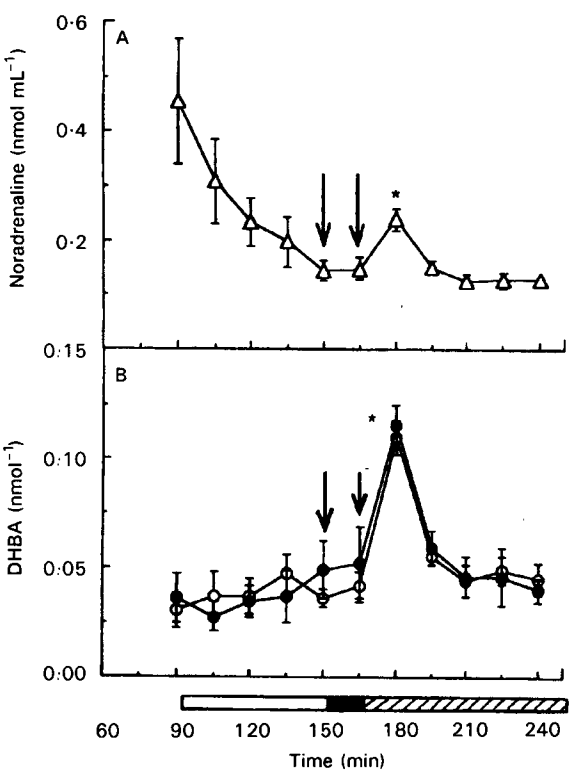


FIG. 7. Relationship between noradrenaline and free-radical generation in ischaemic injury. Time course of dialysate noradrenaline (A) and in-vivo trapping of highly reactive hydroxyl radicals (B) in extracellular fluid of the myocardium was investigated by infusing salicylic acid in Ringer's solution (0·5 nmol μL^{-1} min^{-1}) for 150 min through a myocardial microdialysis probe placed in a rat heart. Dialysate of perfusion-ischaemia-reperfusion was collected at 15-min intervals and immediately assayed for noradrenaline and 2,3- and 2,5-dihydroxybenzoic acid (DHBA) by HPLC-EC. Values are means ± s.e.m. of results from six rats. *$P < 0·01$ compared with levels at 150–165 min (steady-state levels). Abscissa: infusion of salicylic acid was started after 90 min of washout. First arrow = occlusion, second arrow = reperfusion.

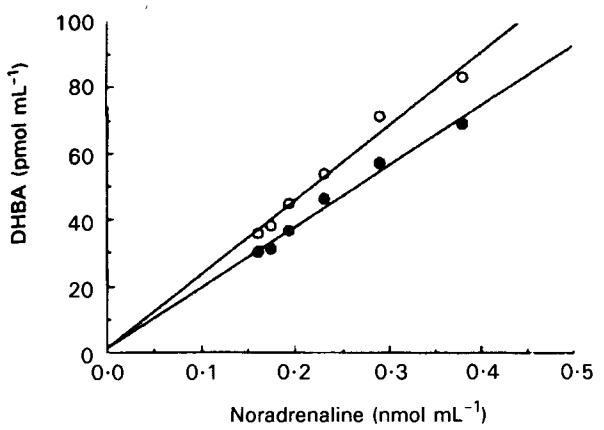


FIG. 8. Linear correlation between the formation of DHBA (·OH adducts of salicylate) and the efflux/oxidation of noradrenaline elicited by cardiac stimulation. Electrical stimulation (1, 2, 3, 4 and 5 V, 6 Hz, and 2 ms in duration) of the left stellate ganglion increased the concentration of noradrenaline in the dialysate. HPLC was used to assay noradrenaline levels and the formation of 2,3- (●) and 2,5-DHBA (○) in the dialysate. The linear correlation coefficients for 2,3- and 2,5-DHBA were $r^2 = 0{\cdot}982$ and $r^2 = 0{\cdot}976$, respectively.

release of noradrenaline and the formation of DHBA. A positive linear correlation was observed (Fig. 8) between release of noradrenaline and the formation of 2,3-DHBA ($r^2 = 0{\cdot}982$) or 2,5-DHBA ($r^2 = 0{\cdot}976$). These data indicate that the sustained elevation of noradrenaline in extracellular fluid can lead to autooxidation, which in turn leads (possibly by an indirect mechanism) to the formation of cytotoxic ·OH free-radicals. Non-enzymatic or enzymatic oxidation of the noradrenaline released by sympathetic nerve stimulation in the extracellular fluid might play a key role in electrical stimulation-enhanced generation of ·OH free-radicals in the heart.

Liver microdialysis

Determination of the effect of Fe^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} on the formation of ·OH. When the metal ion was infused through the dialysis probe, Fe^{2+} but not Fe^{3+}, Cu^{2+} or Zn^{2+} caused an increase in the formation of DHBA in rat liver. When, to eliminate binding of protein with Fe^{2+}, Ringer's solution containing 10 μmol kg^{-1} Fe^{2+} was injected into the penile vein, the levels of 2,3- and 2,5-DHBA increased by approximately 60% and 40%, respectively (Obata et al 1993). Free-radical-formation processes might contribute to in-vivo free-radical formation induced by Fe^{2+}. Fe^{2+} can be oxidized to Fe^{3+} by H_2O_2 resulting in ·OH formation by the iron-catalysed Haber–Weiss reaction (Fenton reaction). This study predicts that the process of liver disorders, such as Wilson's disease, and cases of iron overload in systemic organs might be treated by the use of iron chelators.

Jejunal microdialysis

Generation of ·OH as a result of jejunal ischaemic injury. Recent studies (Das et al 1989; Nilsson et al 1993) have conclusively demonstrated the presence of ·OH in the rat ischaemic intestine and reperfused feline intestine, and it has been reported that levels of ·OH during early reperfusion in ischaemia-reperfused tissue decreased as reperfusion progressed (Das et al 1989). The results of our previous study were in accordance with these findings. When the intestine was rendered ischaemic for 10, 20 or 30 min, progressive elevation of the level of 2,3-DHBA was observed; the maximum was recorded after 20 min of reperfusion and the level gradually decreased to baseline after 60 min (Hirata et al 1996). In the current study, the highest 2,3-DHBA level was obtained after 10-min ischaemia and the lowest after 30 min. However, the reading after 30 min was not significantly different from the baseline level. In this study, because the oxidative flux, at least of hydroxyl, was reduced, progressive injury of jejunal mucosa was observed after severe ischaemia-reperfusion.

Ischaemia-reperfusion of rat intestine resulted in morphological changes. When the morphological changes were classified into six grades according to the system of Chiu et al (1970), the most severe damage of the mucosal epithelium and villi was observed in the 30-min ischaemia reperfusion group. We have previously reported that the destruction of blood capillaries can change the blood-flow pattern in the course of jejunal ischaemic injury (Obata et al 1995a). However, when the jejunum was reperfused, the highest level of 2,3-DHBA was found after 10-min ischaemia and the lowest level after 30-min ischaemia. Thus, because the apical villus epithelium was lifted and peeled away comparatively early during the ischaemic period, it is entirely possible that much of the capacity for generating radicals has been removed after 30-min ischaemia. Therefore, the destruction of villi might be the reason for the decrease in ·OH production after 30 min of ischaemia. However, further investigation is necessary to elucidate the correlation between the duration of ischaemia-reperfusion, radical formation and tissue damage in the intestine. Dialysis using this microdialysis-probe-holding system provides the possibility of monitoring the generation of ·OH in ischaemic-reperfused jejunal mucosa. The technique might have versatile applications and enable new possibilities in in-vivo studies of gastroenterology or transplantation.

Conclusions

Although there are certainly several aspects of clinical ischaemic and reperfusion injury, it appears likely that free-radical production might be of major importance at certain stages of the progression of the injury (McCord 1985). The overproduction of reactive oxygen, such as O_2^-, H_2O_2 and ·OH might cause cellular injury. Owing to the extremely short half-life of oxygen free-radicals, demonstration of the generation of highly reactive oxidants has previously been limited to in-vitro studies. Free radicals from in-vitro generation of reactive oxygen species can be trapped and displayed unequivocally by electron paramagnetic resonance (EPR) spin-trapping procedures. However, the practical application of EPR spectroscopy for in-vivo detection of reactive oxygen species in biological systems is quite difficult and there remains much room for improvement. Therefore, we have employed salicylate hydroxylation to detect the formation of ·OH in-vivo. In-vivo microdialysis techniques enable monitoring of the generation of free-radicals in various tissues and could answer fundamental questions about the clinical implications of reactive oxygen species.

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