Superoxide Production in Rat Hippocampal Neurons: Selective Imaging with Hydroethidine

Vytautas P. Bindokas, Joaquín Jordán, Chong C. Lee, and Richard J. Miller

Department of Pharmacological and Physiological Sciences, The University of Chicago, Chicago, Illinois 60637

Digital-imaging microfluorimetry of the oxidation of hydroethidine (HET) to ethidium can be used to monitor superoxide (O_2^-) production selectively within individual rat hippocampal pyramidal neurons in culture and in brain slices. Under assay conditions, oxidation was not accomplished by hydroxyl radical, singlet O_2 , H_2O_2 , or nitrogen radicals. Neuronal O_2^- production varied with metabolic activity and age. O_2^- generation increased after treatment with AMPA, kainic acid, and NMDA, and the mitochondrial uncoupler carbonylcyanoide p-(trifluoromethoxy)phenyl hydrazone, but usually not after depolarization (50 mM K^+). O_2^- concentrations were

sensitive to scavengers and nitric oxide. HET oxidation was higher in Ca^{2+} -containing versus Ca^{2+} -free saline. However, Ca^{2+} ionophores did not increase oxidation greatly. H_2O_2 application produced a secondary increase in O_2^- . The major source of O_2^- under basal and stimulated conditions appeared to be the mitochondria. Consistent with this, ethidium staining in dendrites was punctate, colocalized with mitochondria, and blocked by CN^- .

Key words: reactive oxygen species; free radicals; excitotoxicity; H_2O_2 ; microfluorimetry; NMDA; mitochondria; NOS; dihydroethidium

Neurons generate reactive oxygen species (ROS) by virtue of oxidative metabolism and increased ROS production during excitotoxic damage, hypoxia, reperfusion, etc. (Ikeda and Long, 1990; Coyle and Puttfarcken, 1993; Lafon-Cazal et al., 1993; Culcasi et al., 1994). ROS are implicated in the neurodegeneration associated with aging, Alzheimer's disease, and acute states such as cerebral ischemia (Halliwell, 1989; Siesjö et al., 1989; Traystman et al., 1991; Coyle and Puttfarcken, 1993). However, the precise contribution of different oxygen radicals to various diseases is unclear. Some radicals also have important functions as second messengers (Ignarro, 1990; Lipton et al., 1993; Maines, 1993; Dawson and Snyder, 1994). Thus, these molecules play a central role in the life and death of neurons.

Neuronal ROS include superoxide (O_2^-), hydroxyl radical ($\cdot OH$), singlet O_2 , and hydrogen peroxide (H_2O_2) (cf. Halliwell and Gutteridge, 1984; Siesjö et al., 1989). H_2O_2 and NO are freely permeable. O_2^- may pass through membranes only via anion channels (Lynch and Fridovich, 1978), is not a particularly reactive species, acts as an electrophile or nucleophile, and potentially forms more reactive species via secondary reactions (cf. Halliwell and Gutteridge, 1984). It inhibits enzymes including glutathione peroxidase, catalase, and creatine kinase. Sources of O_2^- include the following: nitric oxide synthase (NOS) (Pou et al., 1992; Culcasi et al., 1994); NADPH oxidase (Rossi, 1986); mono-oxygenases and NADH dehydrogenase (cf. Turrens et al., 1985); dihydro-orotate dehydrogenase (Forman and Kennedy, 1976); cyclo-oxygenase, lipoxygenases, and xanthine oxidase (XO) (cf.

Siesjö et al., 1989); and components of the electron transport chain (Nohl and Hegner, 1978; Turrens et al., 1985; Nohl, 1990). O_2^- is converted to H_2O_2 spontaneously or by action of superoxide dismutase (SOD). NO reacts with O_2^- to form the powerful oxidant peroxynitrite. Reaction of H_2O_2 and peroxynitrite can give rise to highly reactive $\cdot OH$ (Cadenas, 1989; Hogg et al., 1992). Enzymatic (SODs, catalase, etc.) and scavenging (glutathione, ascorbate, tocopherol, etc.) pathways (cf. Cadenas, 1989) remove most ROS.

Current ROS probes include colorimetric as well as luminescence assays (Nakano, 1990; Tanaka et al., 1994). The former include reduction of cytochrome *c* or analogs, nitroblue tetrazolium, diaminobenzidine- Mn^{2+} , and the hydroxylammonium chloride/sulfanilic acid assay (Eltner and Heupel, 1976; Key et al., 1990; Steinbeck et al., 1993). Other methods use luminol quench by O_2^- (Lissi et al., 1994) and a recently developed O_2^- electrode (Fabian et al., 1995). A drawback of most of these methods is the requirement that ROS exit cells for detection. This hampers detection of highly reactive species and those, like O_2^- , that pass poorly across membranes. Dichlorofluorescein (DCFH) and similar fluorescent derivatives commonly are believed to be selective for H_2O_2 , but other effective oxidants include lipid peroxides and $\cdot OH$, but not O_2^- (Bass et al., 1983; Cathcart et al., 1983). DCFH is a high-affinity substrate for XO (Zhu et al., 1994). Further drawbacks of fluorescein-based derivatives are high rates of auto-oxidation, photoconversion, photobleaching, pH sensitivity, and poor retention in cells (Tsien, 1989) (our unpublished observations). Dihydrorhodamine 123 (DHR123) may be a selective indicator for peroxynitrite (Koody et al., 1994) and recently has been used to show that neurons generate ROS (Dugan et al., 1994). DHR123 is oxidized to rhodamine 123 (R123), a dye that stains mitochondria selectively in accordance with their typically highly negative transmembrane potential (Emaus et al., 1986). R123 may not be well retained under conditions in which ROS are being generated actively, conditions in which cells and mitochondria are depolarized.

Received June 23, 1995; revised Nov. 1, 1995; accepted Nov. 30, 1995.

This work was supported by National Institutes of Health Grants DA02121, MH40165, DA02575, and by Bayer. J.J. was supported by the Spanish Ministry of Education and Science and a NATO Fellowship. C.C.L. was supported by University of Chicago, Pharmacol Sci Res Training Grant 5-T32-GM07151-21. We thank Maria F. Galindo and Charles J. Marcuccilli for preparing hippocampal neurons and secondary glia cultures.

Correspondence should be addressed to Richard J. Miller, Wm. Mabee Professor of Neuroscience, Department of Pharmacological and Physiological Sciences, University of Chicago, 947 East 58th Street, Chicago, IL 60637.

Copyright © 1996 Society for Neuroscience 0270-6474/96/161324-13\$05.00/0

Figure 1. Fundamental properties of HET. *A*, Structures of HET (Hydroethidine, left) and Et (Ethidium, right). Oxidation of HET is accompanied by a hydrogen loss from the heterocyclic ring, resulting in a charged structure with red fluorescence. *B*, Auto-oxidation of HET to Et was slow under normal data collection methods. Each data point is the mean fluorescence ($Ex = 510$ nm, $Em > 590$ nm) for images (average of 16 frames) taken every 10 sec. Here, the mean slope was 0.02 Fl.U./min and was unchanged after addition of a high concentration of H_2O_2 (arrow, 175 mM). Note high gain of ordinate. *C*, The auto-oxidation rate also was low in the presence of 1 $\mu g/ml$ psoralen. The 38 sec illumination of psoralen with 340 nm light was expected to generate singlet O_2 . The resulting increase in fluorescence was small and similar to that in the absence of psoralen (data not shown). The sag in fluorescence probably was attributable to diffusion of Et away from the local site of UV illumination. Similar results were seen after a 54 sec illumination with 340 nm UV. Larger jumps in fluorescence occurred for longer exposures (75 and 142 sec) to full spectrum Xe arc (1.5 ND filter). The dye appears not to be oxidized by singlet oxygen, a result confirmed by lack of effect of singlet O_2 generated by H_2O_2 plus hypochlorite (data not shown). *D*, Microfluorimetry was set to give a linear response to free ethidium concentrations between 10 nM and 20 μM ($R^2 = 0.999$). The fluorescence at 30 μM EtBr saturated the camera. Different optics and camera settings produced various ranges of linear response (data not shown). *E*, HET was oxidized by O_2^- produced by decomposition of KO_2 . Three applications of KO_2 -saturated DMSO are indicated by arrows. Each produced a stepwise increase in Et fluorescence.

Hydroethidine (HET) (also known as dihydroethidium) first was used as a redox probe by Gallop et al. (1984). HET is a freely permeable, blue fluorescent dye until oxidized to the common polynucleotide stain ethidium (Et) (Fig. 1*A*). Gallop et al. (1984) suggested that the dye was converted enzymatically to Et in the presence of $NADP^+$, but provided no experimental data. Subsequently, selective HET oxidation by O_2^- has been proposed (Bucana et al., 1986; Rothe and Valet, 1990). HET has been used to study respiratory burst in immunocytes (Rothe and Valet, 1990; Perticarari et al., 1991; Shi et al., 1991) and redox state in tumor cells (Olive, 1989). The intercalation of Et into DNA and ability to be fixed into tissue (Bucana et al., 1986) made the probe an attractive candidate for studying ROS generation in neuronal tissues.

To establish the roles of specific radical species in different cellular phenomena, it would be helpful to have selective methods for their rapid measurement in functioning cells. To this end, we have investigated the selectivity of HET for O_2^- and whether it is amenable for imaging O_2^- production within individual neurons. Our results demonstrate the use of HET as an O_2^- indicator in various neuronal preparations and implicate mitochondria as a major source of O_2^- production under basal conditions as well as during several potential disease states.

MATERIALS AND METHODS

HET was obtained from Molecular Probes (Eugene, OR). Carbonylcyanide p-(trifluoromethoxy)phenyl hydrazone (FCCP; Sigma, St. Louis, MO) was made as a 1 mM stock in 95% EtOH and diluted to 1 μM final concentration in saline. S-nitrosocysteine (SNOC) was made as a 100 mM stock just before use and was kept on ice until used (Lei et al., 1992). Final concentrations of 300 and 100 μM were used. These concentrations did not affect the pH of the final solution. Sodium nitroprusside (SNP), L-arginine (L-Arg), L-nitroarginine (LNA), and NaCN (CN) (all from Sigma) were mixed in regular saline. R123 and ethidium bromide (EtBr) were obtained from Molecular Probes; KO_2 was obtained from Aldrich (Milwaukee, WI).

Preparation of HET. HET was prepared as a 10 $\mu g/\mu l$ stock in dry, N_2 -sparged dimethylsulfoxide (DMSO), packed under N_2 , and stored at $-80^\circ C$. Working stocks consisted of 1 $\mu g/\mu l$ dilutions also made in sparged DMSO and had a very pale pink color. Although sealed stocks were stable at room temperature for up to a few weeks, a fresh aliquot was used for each experiment. Stocks in open containers slowly turned dark red. Drugs and test solutions usually were made in regular saline; all solutions contained 3.2 μM HET and 0.1% DMSO (14 mM). Fresh working solutions were made daily. Regular 2 Ca^{2+}/Na^+ -based saline contained (in mM): NaCl 144, HEPES 10, $CaCl_2$ 2, $MgCl_2$ 1, KCl 5, and D-glucose 10 (312 mOsm, pH 7.4, adjusted with NaOH). Saline with 50 mM KCl was made by substituting an equivalent amount of NaCl. Saline with 0 Ca^{2+} was made by substituting an equivalent amount of Mg^{2+} and adding 100 μM EGTA. Saline with 0 Ca^{2+} and 0 Mg^{2+} contained 100 μM EGTA, and osmolality was corrected with sucrose. Na^+ -free saline was made by substituting *N*-methyl-D-glucamine chloride (NMDG) for NaCl.

Fluorescence assays. All assays were conducted on the stage of a Nikon Diaphot epifluorescence microscope with illumination from a 150 W Xe arc [attenuated by a neutral density (ND) 1.5, ultraviolet (UV)-grade filter; Omega Optical, Brattleboro, VT] and through standard rhodamine optics (excitation (Ex) 510–560 nm; dichroic mirror (DM) 580 nm; (emission) (Em) > 590 nm) (Nikon, Melville, NY). Most images were formed using a 40× Fluor NA 0.85 objective (Nikon) and collected on a Hamamatsu ICCD (sensitivity set at 7.0). Images were 8-bit (256 intensity levels). Typically, 16 frames were averaged every 10 sec. Background subtraction was made using the first image obtained when HET solution was added and, in addition, from a cell-free region of the field to track subsequent changes in background. Data acquisition was controlled by Fluor or IMAGE1 software (Universal Imaging, West Chester, PA), and average intensity over 1–35 regions of interest were logged to hard disk and displayed in real time. Linear regressions were fit to data to quantify HET oxidation under different treatments. Slopes were compared using ANOVA and post hoc contrasts. Phase-contrast or higher-resolution fluorescence images were averaged (256 frames) to improve signal-to-noise ratio. High-magnification images were made using a Leitz 100×, oil-immersion [numerical aperture (NA) 1.3] objective (Leica, Malvern, PA). All assays were conducted at room temperature (20.5–22.5°C).

The Plexiglas sample chamber contained 1 ml of solution and consisted of a 15 mm, #1 coverslip (cell-free or plated with cells) forming the bottom of a well that was fit with ports for solution changes. The coverslip was held in place by high-vacuum silicone grease and stainless steel clips.

Calibration was made using EtBr as a standard. EtBr was dissolved in deionized water (1 mM) and diluted serially. Intensity measurements were made using the same optics and camera settings as for experiments with cultured neurons.

Cell-free assays. O_2^- was generated using xanthine (X)/XO in phosphate buffer at pH 7.8 (Eltner and Heupel, 1976). Other experiments used $XO + X$ in regular saline and without hydroxylammonium chloride (HAC). HET oxidation was faster without HAC. A saturated KO_2 solution was made by mixing KO_2 reagent in degassed DMSO and allowing excess reagent to settle out (Lokesh and Cunningham, 1986). In some experiments, dry KO_2 was added directly to the observation chamber.

Singlet O_2 was generated by adding $H_2O_2 + NaOCl$ to HET solution. It also was generated by UV excitation of psoralen (cf. Halliwell and Gutteridge, 1984; Bensasson et al., 1993).

Cell culture and assay. Hippocampal pyramidal neurons were prepared from stage E17 animals as described previously (Abele et al., 1990). Cells were maintained in N2.1 serum-free medium above a layer of feeder astrocytes. For comparison, we also used secondary (replated) astrocytes grown in medium containing horse serum. Cells were rinsed twice in regular saline, transferred to the observation chamber, and treated with HET solution. Solution changes were made by removing most of the chamber contents while adding 2.5–3 ml of the new solution directly to the chamber. Mitochondria were stained by applying 10 μ g/ml (26 nM) R123 in saline for 2 min. Staining of the mitochondria was very bright compared with Et, camera gain was reduced, and the contribution of Et to the R123 signal was small.

Hippocampal slices and assay. Hippocampal slices were obtained from 2- to 3-week-old rat pups. Animals were anesthetized deeply and decapitated. The brain was dissected out rapidly in ice-cold bicarbonate-buffered saline bubbled with 95% O_2 /5% CO_2 . Vibratome slices (150–250 μ m thick) were transferred to a chamber containing oxygenated saline (32°C) for 1 hr. Slices were treated with HET and drugs for 20 min in 35 mm plastic culture dishes in an oxygenated chamber at room temperature. HET and drugs were made in regular HEPES-buffered saline and bubbled with medical-grade O_2 . Slices then were fixed in 4% paraformaldehyde and imaged using a 4× NA 0.12 fluorescence objective (Leitz) or a 10× NA 0.5 Fluor objective (Nikon). Shading correction was made using a reference image of 100 μ M EtBr solution. Average fluorescence intensity of the slices was calculated using IMAGE1 software. Intensities were compared using ANOVA and Dunn contrasts.

RESULTS

Basic properties of HET and Et

HET is a cell-permeant, blue-fluorescent compound (Excitation_{max} 365 nm, Emission_{max} 420 nm) until oxidized to Et (Excitation_{max} = 470 nm, Emission_{max} = 590 nm) (Fig. 1A). The reduced as well as oxidized compounds exhibit good photostability under normal illumination conditions (Fig. 1B) [auto-oxidation

rate in this example was 0.2 fluorescence intensity unit (FI.U)/min]. This rate was not increased by the addition of 175 mM H_2O_2 (Fig. 1B). However, photoconversion of HET to Et by short-wavelength illumination has been reported (Swannell et al., 1992). Brief illuminations at 340 nm caused small stepwise increases in fluorescence (Fig. 1C), and long exposures to full-spectrum UV illumination produced larger increases (arrow). The photoconversion was enhanced neither by psoralen, a generator of singlet O_2 (Halliwell and Gutteridge, 1984; Bensasson et al., 1993), nor by singlet O_2 generated with hypochlorite + H_2O_2 (Bensasson et al., 1993) (data not shown). Although HET displayed some propensity for photoconversion, it was more stable than DCFH or DHR123 (slopes 10-fold greater under similar conditions; data not shown).

The imaging system under standardized settings was able to detect free Et as low as 10 nM and gave a linear response up to 20 μ M Et ($R^2 = 0.999$; Fig. 1D). Under standard conditions, each arbitrary FI.U corresponded to ~100 nmol of free Et. The binding of Et to polynucleotides also increases the quantum efficiency by ~20-fold (LePecq and Paoletti, 1967). This property, along with the nonratiometric nature of the HET, complicates quantitation within intact cells and results in semiquantitative estimates of O_2^- production.

HET is oxidized by O_2^-

As reported previously (Rothe and Valet, 1990; Carter et al., 1994), HET is oxidized to Et by O_2^- . KO_2 -saturated DMSO produces a burst of O_2^- when added to aqueous solutions (Lokesh and Cunningham, 1986). The addition of KO_2 solution (Fig. 1E, arrows) to a solution containing 1 μ g/ml HET (3.2 μ M) caused a stepwise increase in fluorescence consistent with the oxidation of HET to Et. Addition of very high concentrations of dry KO_2 reagent resulted in loss of fluorescence, suggesting that Et could be oxidized further to nonfluorescent forms under extreme conditions (data not shown).

We also generated O_2^- enzymatically using X/XO (Fig. 2, $XO + X$). In the experiment shown in Figure 2A, the auto-oxidation rate in X (~0.5 FI.U/min) was increased to 4.1 ± 0.4 FI.U/min after the addition of XO (52 mU/ml) (expected O_2^- generation of 52 nmol·min⁻¹·ml⁻¹). The initial increase in Et fluorescence was linear, or nearly so. The addition of Cu/Zn-SOD (9 U/ml) to the reaction mixture produced an oxidation rate not significantly different from XO alone (0.83 ± 0.02 FI.U/min; $p = 0.2$). The X/XO reaction also is known to produce $\cdot OH$ (Kuppusamy and Zweier, 1989). The assay conditions use a 4300-fold excess of DMSO (a very efficient $\cdot OH$ scavenger) (Traystman et al., 1991; Scaduto, 1995) over HET. Under these conditions, $\cdot OH$ radicals most likely will be scavenged by DMSO before reacting with HET. Additional evidence is the lack of HET oxidation by Fe/ascorbate/ H_2O_2 (Fig. 2B). H_2O_2 and iron-catalyzed $\cdot OH$ production was not able to convert HET to Et under assay conditions. $\cdot OH$ sensitivity was observed when dimethylformamide (not a $\cdot OH$ scavenger) was used as the solvent for HET (data not shown).

We tested agents later used to characterize neuronal O_2^- production for possible interactions with the HET assay. In the presence of ascorbate, SNP generates NO radicals (Feelisch, 1991; Lipton and Stamler, 1994), which by themselves are unable to oxidize HET (Fig. 2B) but do inhibit the X/XO-mediated oxidation HET. It is likely that the inhibitory action was attributable to the formation of peroxynitrite by the rapid reaction of NO with O_2^- (reaction rate = 6.7×10^9 M⁻¹·s⁻¹) (Huie and Padmaja, 1993) that apparently out-competed the oxidation of HET by O_2^- . The ineffectiveness of peroxynitrite itself (actually peroxynitrous acid

Figure 2. Cell-free oxidation assays. *A*, The rate of HET oxidation was enhanced by the action of X and XO in a cell-free system. The rate observed for HET plus 1.9 mM X (initial slope = 0.48 ± 0.11 FI.U/min) was similar to auto-oxidation of HET. Initial rates of HET oxidation were slightly higher in the presence of 3 mU of XO (1.2 ± 0.4 FI.U/min). This may indicate that HET was a weak substrate for XO, similar to the case reported for 2',7'-DCFH (Zhu et al., 1994). Dye conversion in the presence of XO + X was significantly faster (4.1 ± 0.4 FI.U/min) than for either component alone. Addition of excess SOD (9 U/ml; curve XO + X + SOD) produced a rate of rise (0.84 ± 0.02 FI.U/min) similar to XO alone ($p = 0.2$). Data are the mean $\pm$ SEM of three replicates. Only one SEM bar is shown per trace to decrease clutter. *B*, HET was not oxidized by nitrogen radicals or $\cdot\text{OH}$. SNP (1 mM) did not support oxidation of HET (rate = 0.02 ± 0.01 FI.U/min; $n = 3$). A similar result was obtained with SINOC (300 μM ; rate = 0.04 ± 0.01 FI.U/min) (data not shown). HET oxidation did not occur in H_2O_2 (176 mM) plus $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (30 μM) and ascorbic acid (30 μM ; rate = 0.02 ± 0.01 FI.U/min), conditions that favored generation of $\cdot\text{OH}$. XO + X produced a mean rate of 0.7 ± 0.01 FI.U/min ($n = 3$). This was reduced to 0.14 ± 0.02 FI.U/min by the addition of 1 mM SNP. SIN-1 generates both O_2^- and NO, thus forming peroxynitrite. SIN-1 (1 mM) produced a low rate of HET oxidation (0.13 ± 0.04 FI.U/min), possibly because of O_2^- that escaped peroxynitrite formation. *C*, Oxidation of HET by O_2^- was reduced by 53% with the addition of 3 mM NaN_3 (XO + X rate = 1.2 ± 0.6 , XO + X + N_3 = 0.6 ± 0.4 FI.U/min; $n = 3$ and 4, respectively). This inhibition may have been attributable to NO generation by N_3 and loss of O_2^- to peroxynitrite. *D*, Cyanide (3 mM) did not affect X/XO-mediated HET oxidation (XO + X rate = 1.6 ± 0.07 , XO + X + CN rate = 1.7 ± 0.1 FI.U/min; $n = 4$; $p > 0.05$). Addition of the iron chelator desferrioxamine (10 μM) increased the oxidation rate significantly (2.4 ± 0.1 FI.U/min; $p < 0.05$), suggesting that O_2^- was consumed by trace iron present in solutions.

at assay pH) in oxidizing HET also was shown by the low oxidation rate in the presence of 3-morpholinosydnonimine [Fig. 2*B*, SIN-1 (1 mM)], a compound that generates both O_2^- and NO (Feelisch et al., 1989; Hogg et al., 1992). The inhibitory effects of NO on X/XO-mediated HET oxidation also were observed in the presence of SINOC (300 μM ; data not shown). The oxidation was not inhibited by equivalent concentrations of L-cysteine/HCl (L-cys; data not shown) (see also Fig. 6*D,E*). The X/XO oxidation of HET was reduced significantly (53%, $p < 0.001$) by NaN_3 (3 mM; Fig. 2*C*). Although N_3 is a scavenger of singlet O_2 (Bensasson et al., 1993), the inhibitory action may be attributable to the ability of azide to generate NO (Smith and Wilcox, 1994), shown in Figure 2*B* to out-compete HET for O_2^- . It is unlikely that the lower oxidation rate was attributable to N_3 scavenging singlet O_2 , because singlet O_2 was unable to oxidize HET under our assay conditions (see above and Fig. 1). Cyanide (3 mM) did not affect X/XO-mediated HET oxidation (Fig. 2*D*), but the addition of desferrioxamine (10 μM), an iron chelator, significantly increased the initial rate 1.5-fold ($p < 0.05$; Fig. 2*D*). A possible explanation for this is that chelation of trace iron contaminants may have prolonged the lifetime of O_2^- by eliminating Fenton-like reactions, protecting the enzyme, thus preserving a higher O_2^- concentra-

tion. L-Arg and LNA did not affect the rate of HET oxidation by X/XO (data not shown).

O_2^- generation in cultured rat neurons and glia

When neurons were incubated in saline containing HET, the basal rate of fluorescence increase was much higher than that observed in cell-free assays (Fig. 3*B*). Oxidation rates higher than auto-oxidation were observed in numerous types of rat neurons including cerebellar Purkinje neurons and superior cervical ganglion sympathetic neurons, as well as in secondary cortical astrocytes, PC12 cells, and endothelial cells (data not shown). The initial sites of Et fluorescence were nonuniform, mostly evident in the soma (Fig. 3*A*), but also prominent within the neurites (see Fig. 3*A,C*). The punctate Et fluorescence was colocalized with mitochondria when subsequently stained with R123, although not all mitochondria had detectable Et fluorescence (Fig. 3*C*). This suggested that mitochondria were one source of O_2^- . If HET was washed out, then the bright punctata appeared to destain rapidly, suggesting that the bright regions were sites of Et generation rather than its sequestration. The apparent staining of only a subset of mitochondria suggested further that Et was not accumulated within mitochondria much like other cationic dyes like R123. Application of

Figure 3. Responses of hippocampal neurons. *A*, Incubation of hippocampal neurons (11 d *in vitro*) with HEt (3.2 μ M) produced an increase in fluorescence first evident in cell somas. The initial staining of nuclei of dead cells (arrows) evident at time 0 ($t=0$) occurred as a result of Et formed by auto-oxidation of HEt solution. Mean fluorescence intensity was determined by setting regions of interest over the somas (in phase-contrast image) and after subtraction of the fluorescence image at time = 0. At time = +10 min ($t=+10$ min), cells were stained to different extents. The amount and rate (see *B*) of HEt oxidation increased after 10.9 min of treatment with 30 μ M NMDA [NMDA (+10.9 min)] (in 0 Mg saline + 10 μ M glycine), and after 1 μ M FCCCP. Cells appeared swollen after these treatments. All fluorescence panels have been gamma-corrected digitally by a constant to aid illustration of faintly stained structures. The width of each panel originally was 324 μ m. *B*, Continuous record of fluorescence for neuronal somas for cells shown in *A*. The increase in O_2^- production was delayed to varying degrees ranging from nearly immediate (curve *) to 7 min after application (open squares). O_2^- production, on average, was not increased additionally by FCCCP. This implies that mitochondria were fully depolarized by the NMDA treatment. Signal from one neuron approaches camera saturation (arrow). *C*, High-magnification image of initial Et staining pattern revealed distinct punctata (arrows). Left image was taken ~3 min after application of HEt solution. Right image pair was taken after 2 min staining with 10 μ g/ml (26 nM) R123 and at lower camera gain. Numerous brightly stained mitochondria are evident, and some were colocalized with Et-stained regions (arrows). Thus, all Et-stained regions colocalized with mitochondria, but the converse was not true. Scale bar, 10 μ m.

the glutamate receptor agonist NMDA (100 μ M) increased the rate of O_2^- production in most neurons (Fig. 3*A*). The bulk of the Et was in the soma consistent with the bulk of the R123-stained mitochondria located there (the nucleus later becoming brightly stained, especially the nucleolus), but also was present in dendritic swellings that formed during NMDA exposure. Other glutamate receptor agonists also increased O_2^- production (see below). Application of FCCCP (1 μ M) uncoupled mitochondria and increased the rate of HEt oxidation (see below), but did not increase the

rate of O_2^- production over that produced by a robust NMDA response (Fig. 3*A,B*). FCCCP did not increase the rate of HEt auto-oxidation in cell-free assays ($n = 4$). This again suggested that mitochondria were a major source of O_2^- . Quantitation of O_2^- production in this example is shown in Figure 3*B*. The mean basal rate of O_2^- production in hippocampal neurons varied over a wide range (e.g., range 0.6–2.6 Fl.U./min for 9 DIV neurons, $n = 8$ preparations; in this example, $n = 11$ cells, 11 DIV, range 0.23–10.7 Fl.U./min, mean = 2.7 ± 0.8 Fl.U./min). Neurons in poor

Figure 4. Cultured neurons produce linear rates of rise under basal conditions. *A*, Long control record of increase in Et fluorescence remained constant until cells were challenged with FCCP (1 μ M). HET solution was replaced with fresh saline as indicated by arrows. Trace is the average of five neurons. *B*, Basal rate of HET oxidation tended to increase with age in cultured hippocampal neurons. The dotted line is a linear regression (linearity, $p = 0.01$; $R^2 = 0.42$) fit to mean rate per DIV ($n = 1$ –8 experiments per DIV). *C*, Basal rate usually was not increased by application of 50 K. Basal rate is shown by the dashed line. In some cells, the rate was lower during 50K application (see Fig. 5*D* and Results). The HET oxidation rate increased during application of saline with 0 mM Mg^{2+} (0 Mg), perhaps because of augmentation of NMDA ionophore activity. *D*, Mean basal rate increased 3.1-fold after application of KA (200 μ M) (basal rate = 1.8 ± 0.02 ; KA rate = 5.5 ± 0.07 FI.U/min; $n = 11$).

health (i.e., those possessing vacuoles, granular cytoplasm, beaded neurites) had especially high “basal” rates (data not shown). Rates of HET oxidation typically were lower in glial cells in which FCCP treatment also often failed to increase rates. The rates in neurons increased ~ 2.5 -fold during NMDA treatment (6.8 ± 1.1 FI.U/min; Fig. 3) with a time lag ranging from nearly immediate onset (asterisk indicates curve) to delays of up to 7 min in the example shown (open squares). O_2^- production could be monitored under standard conditions until the Et fluorescence intensity saturated the camera (intensity = 255 – background). One such cell is seen in this example (Fig. 3*B*, arrow). The mean rate of rise on subsequent FCCP treatment was similar (6.6 ± 0.8 FI.U/min) to that in NMDA, suggesting that the mitochondria already were fully uncoupled by the NMDA treatment. Exceptions to this were observed in immature (≤ 8 DIV) hippocampal neurons that lacked substantial NMDA currents (our unpublished observations) or in cells that lacked NMDA receptors (data not shown). FCCP usually doubled O_2^- production in these cases (data not shown).

The basal rate of O_2^- production remained constant over ~ 50 min of observation (Fig. 4*A*). Fresh HET saline was added as indicated (arrows) as a control for bath exchanges. Application of FCCP increased the rate of O_2^- production by 2.2-fold ($n = 6$ neurons) over the basal rate in this example. This increase was not caused by a direct catalysis of HET oxidation by FCCP (data not shown).

The basal rate of O_2^- production also varied with the age of the cultured neurons (Fig. 4*B*). The rate increased by ~ 0.3 FI.U $\cdot$ min $^{-1}\cdot$ d $^{-1}$ in culture for neurons cultured 5–24 d (R^2 for linear regression 0.42; $p = 0.008$). The main source of variability was primarily associated with different cell preparations rather than between samples within a preparation.

Properties of O_2^- production in rat hippocampal pyramidal neurons

We further investigated which treatments increased O_2^- production in cultured hippocampal pyramidal neurons. Depolarization with 50 mM K^+ (50K) saline did not increase the basal rate in 68 of 69 cells. An example is shown in Figure 4*C*. In 30 of 69 cells, however, the rate of O_2^- production during application of 50K

decreased slightly (mean = 56% of basal rate; Fig. 6*B*). This decrease may have been attributable to swelling of the soma. We did observe an increase in O_2^- production, however, if 50K was applied in Na^+ -free saline (data not shown). This may have occurred because of elimination of swelling and/or Na–Ca exchange and the resultant higher intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) attained (see below). As shown in Figure 4*C*, enhancement of endogenous electrical activity by removal of extracellular Mg^{2+} increased the rate of O_2^- production, probably via action on NMDA receptors (Abele et al., 1990). O_2^- production was increased by concentrations of kainic acid (KA; 200 μ M) expected to activate both AMPA and KA receptors (Fig. 4*D*). Treatment with AMPA (10 μ M) also increased the rate of O_2^- production (data not shown).

The increase in O_2^- production by glutamate receptor agonists appeared to require entry of Ca^{2+} , because both KA and NMDA failed to greatly increase the slope ($p > 0.05$, repeated-measures ANOVA) when applied in Ca^{2+} -free saline (Fig. 5*A,B*). The occasional slight increase in slope may be an indication that intracellular Ca^{2+} sources may be involved or that O_2^- production also can occur by $[Ca^{2+}]_i$ -independent mechanisms. Note that the slope increased abruptly on return to regular Ca^{2+} -containing saline for both KA and NMDA ($p < 0.05$). Increased O_2^- production by KA and NMDA was suppressed by treatment with CN^- (3 mM; Fig. 5*A,B*). Inhibition was complete in most cells but was partial in others (see Fig. 5*A*).

CN was effective in completely blocking the basal production of O_2^- (Fig. 5*C*). Azide (3 mM) also was able to suppress the basal O_2^- production (data not shown), but it is uncertain whether this action was attributable to mitochondrial inhibition and/or its ability to produce NO (see above). It should be noted that both agents inhibit Cu/Zn-SOD (Iqbal and Whitney, 1991; Smith and Wilcox, 1994), an action that actually would be expected to raise O_2^- levels. Oligomycin did not alter the rate of O_2^- production (data not shown). Surprisingly, FCCP was able to rescue O_2^- production in CN-treated neurons (Fig. 5*C*, arrow). Because basal O_2^- production was entirely sensitive to mitochondrial poisons, the main source of basal O_2^- appeared to be the respiratory chain.

Figure 5. The increase in O_2^- production has a Ca^{2+} requirement and is sensitive to mitochondrial state. **A**, KA (200 μM) failed to increase O_2^- production in Ca^{2+} -free saline. A break in slope (arrow) was produced on return to normal saline during continuous KA application. The mean basal rate was 0.7 ± 0.02 , 0.7 ± 0.15 in Ca^{2+} -free saline, 1.1 ± 0.15 in $0 Ca/KA$, and 2.5 ± 0.15 FI.U/min in normal saline + KA ($n = 5$ neurons). Cyanide (3 mM) blocked this rate increase (CN + KA rate = 0.7 ± 0.25 FI.U/min). The degree of suppression of O_2^- production by CN ranged from complete to partial (data not shown). **B**, NMDA also increased the rate of O_2^- production in a Ca^{2+} -sensitive manner. Mean basal rate ($n = 11$) was 1.1 ± 0.03 , and rate in Ca^{2+}/Mg^{2+} -free saline ($0Ca, 0Mg$) was 1.9 ± 0.06 , 2.5 ± 0.12 after addition of NMDA (300 μM) and doubled on return to NMDA in normal saline (5.9 ± 0.11 FI.U/min). CN blocked the NMDA-mediated increase in rate (slope = 1.3 ± 0.11 FI.U/min). **C**, The major source of O_2^- is sensitive to mitochondrial state. Basal HET oxidation was blocked by CN (3 mM) and was restored by FCCP (1 μM) (arrow). Removal of CN may not have been complete during the final 6 min of the experiment. **D**, Application of the Ca^{2+} ionophore 4-Br-A23187 (8 μM) failed to elicit a large change in O_2^- production. A small departure from the basal slope (1.49 ± 0.51 FI.U/min) (dotted line) is evident at the end of 5 min in this example (mean slope = 2.2 ± 0.5 FI.U/min; $n = 7$). NMDA (300 μM) increased the slope 2.9-fold to 6.42 ± 1.9 FI.U/min. **E**, The enhancement of O_2^- production by different treatments was nonadditive. This example shows that the rate of O_2^- production was saturable and equivalent for stimulation by FCCP and H_2O_2 (H_2O_2). **F**, KA applied in Na^+ -free saline (NMDG) increased O_2^- production. Fluorescence continued to increase on return to regular saline; however, the rate of rise slowed after 5 min, perhaps because of swelling of the cells. Rates resumed on final return to Na^+ -free saline. Data are the mean of five neurons. NMDA had similar effects (data not shown).

We investigated further the ionic dependence of O_2^- production. Application of the Ca^{2+} ionophore 4-Br-A23187 (8 μM) increased $[Ca^{2+}]_i$ (cf. Prehn et al., 1994), but failed to elicit a large change in O_2^- production (Fig. 5D,E). Only a small increase over the basal slope (Fig. 5D, dotted line) was evident at the end of 5 min (slopes = 2.0 ± 0.5 vs 1.5 ± 0.5 FI.U/min; $n = 7$; $p > 0.05$, repeated-measures ANOVA) in this example. In the same example, NMDA (300 μM) increased the slope threefold to 6.3 ± 1.9 FI.U/min (significantly greater than basal, $p < 0.05$). A similar result is seen in Figure 5E. In addition, the slope increase produced by NMDA was not enhanced further by FCCP or H_2O_2 . Na^+ entry via glutamate-gated ionophores appeared unnecessary for the increase in O_2^- production as shown for KA application in Na^+ -free saline (Fig. 5F). Similarly, application of NMDA in Na^+ -free saline increased O_2^- production (data not shown).

The rate of O_2^- production was saturable and equivalent for NMDA, FCCP, and H_2O_2 in some neurons (Fig. 5E). In other cases, FCCP failed to produce a large rise in slope in some cells, especially type 1 astrocytes (data not shown). When applied first, H_2O_2 always increased O_2^- production. CN blocked the H_2O_2 stimulation partially, the rate reverting to one resembling the initial basal level (Fig. 6A). One of the possible mechanisms of the H_2O_2 increase was by its block of Cu/Zn-SOD. In support of this hypothesis, Cu^{2+} -preferring metal chelators that inactivated SOD also increased HET oxidation. Application of ammonium 1-pyrrolidinedithiocarbamate (APDC; 10 mM) significantly increased the slope 4.4-fold above basal (basal = 0.45 ± 0.04 vs 1.98 ± 0.15 FI.U/min in APDC; $n = 69$; $p < 0.05$), and was not significantly increased further by H_2O_2 (H_2O_2 rate = 2.63 ± 0.22 FI.U/min; $p > 0.05$, Friedman repeated-measures ANOVA/Student's t test).

Figure 6. *A*, H₂O₂ produced an apparent increase in O₂^{•−} production by a CN-sensitive mechanism. The base rate (0.67 ± 0.08 FI.U/min) increased to 2.29 ± 0.22 FI.U/min in the presence of H₂O₂ (H₂O₂; 175 mM), and CN (3 mM) decreased the slope to 0.69 ± 0.26 FI.U/min. Data are the mean of nine neurons. Similar results were observed in four experiments. *B*, O₂^{•−} levels can be influenced by NOS activity. The mean response of $n = 13$ neurons is depicted by filled squares. Basal rate of HET oxidation was depressed by 50 K application in most cells (11/15) in this example. Neurons were washed immediately with Mg²⁺-free saline (0 Mg) containing L-Arg (L-Arg; 3 mM) and remained in Mg²⁺-free saline for the remainder of the experiment. NMDA (100 μM) + L-Arg increased the rate of HET oxidation from 1.9 ± 0.05 to 5.8 ± 0.09 FI.U/min in 13 neurons, but the rate remained constant in 2 neurons (open squares). Replacement of L-Arg with the NOS inhibitor LNA (100 μM) resulted in an increase in slope in both of these neurons. This action was not attributable to direct radical scavenging by L-Arg or LNA (data not shown). Finally, CN was able to block HET oxidation in all cases. *C*, Basal rate of O₂^{•−} production was not decreased by DPI (10 μM). DPI did not prevent the increase in O₂^{•−} production induced by FCCP (1 μM). *D*, The FCCP-mediated increase in HET oxidation was blocked by the NO-generator SNOC (300 μM). The inhibition was long-lived after washout of SNOC despite the continued presence of FCCP. This action may be caused by consumption of O₂^{•−} by NO and peroxynitrite formation during SNOC presence, but may indicate an inhibition of O₂^{•−} generation after SNOC washout. Data are the mean of 23 neurons. *E*, The SNOC-mediated block of HET oxidation is not caused by vehicle or cysteine. Recovery after SNOC (100 μM) washout was quicker than for the case shown in *D*. Nitrate-free L-Cys (L-Cys) was ineffective, whereas a second application of SNOC also blocked HET oxidation. Data are the mean of 32 neurons.

dent–Newman–Keuls contrasts). Similar results were obtained with the chelator *o*-phenanthroline (10 mM; data not shown).

A subpopulation of hippocampal pyramidal neurons is known to possess NOS (Valtschanoff et al., 1993; Chiang et al., 1994; Dinerman et al., 1994). NOS also can be activated by the increase in [Ca²⁺]_i produced by glutamate receptor agonists (Lipton and Stamler, 1994) and previously has been shown to increase O₂^{•−} production in rat neurons (Lafon-Cazal et al., 1993). NO might decrease the O₂^{•−} concentration via peroxynitrite formation similar to that seen in cell-free assays (Fig. 2). Alternatively, NOS is able to generate O₂^{•−} directly if the substrate L-Arg is limiting (Pou et al., 1992; Culcasi et al., 1994). In the example shown in Figure 6*B*, most neurons increased O₂^{•−} production on treatment with NMDA + L-Arg, suggesting that weak, or no, NO generation took place. However, the two neurons depicted by the open symbols did not show an increase in HET oxidation during NMDA stimulation until the NOS antagonist LNA was added. Inhibition of NOS by LNA would block peroxynitrite production and associated O₂^{•−} consumption. Overall, 12 of 48 neurons showed NMDA responses consistent with NOS activity and sensitivity to arginine

derivatives (range 0–63% of neurons per experiment; $n = 6$ experiments). The low numbers of neurons with active NOS are consistent with the low prevalence of diaphorase staining in CA1 and CA3 regions (range 2–32% of pyramidal cells) (Valtschanoff et al., 1993) (but see also Dinerman et al., 1994).

Because diaphorase activity can produce O₂^{•−} directly in the absence of L-Arg, we also tested whether NOS contributed to basal and FCCP-stimulated O₂^{•−} generation. NOS and NADPH oxidase are blocked selectively by diphenylene iodonium (DPI) (Rossi, 1986; Stuehr et al., 1991). Application of DPI (10 μM) failed to inhibit the basal rate of O₂^{•−} production and also failed to block the FCCP-mediated rise in slope (Fig. 6*C*; overall, $n = 33$ neurons, 3 experiments). This was not unexpected, because NOS may not be active under these conditions and also perhaps as a consequence of the low percentage of neurons expressing NOS activity (Fig. 6*B*). The NO generator SNOC potentially inhibited the FCCP-mediated oxidation of HET (Fig. 6*D,E*). A 5 min exposure to 300 μM SNOC suppressed the slope for >15 min in the continued presence of FCCP. As discussed, this inhibition may be attributable to peroxynitrite formation during SNOC application

Figure 7. HET produced region-specific and time-dependent staining in rat hippocampal slices. Two examples are shown. The *top left panel* shows a phase-contrast image of the CA3/CA2 region with the prominent, translucent cell somas of the pyramidal neurons. The *middle and right panels* show Et staining at time = 0 ($t=0$) and after 28 min under resting conditions ($t= +28 \text{ min}$). Initial staining was slight and increased over time. The increase in Et fluorescence was largely localized to the pyramidal cell somas, proximal dendrites, and scattered cells. This also is evident in the example (CA3 region) in the *bottom row*. The width of each image originally was 1.3 mm.

(see above), but also may be attributable to inhibition of oxidative phosphorylation after SNOC washout. Figure 6E shows that the duration of inhibition was shorter with lower SNOC concentration ($100 \mu\text{M}$) and was not observed for nitrate-free L-CYS/HCl. The slope again was suppressed by the final application of SNOC.

O_2^- production in brain slices

Treatment of hippocampal slices with HET (3.2 nM) resulted in a time-dependent increase in fluorescence. The increases were not

uniform spatially but, typically, hippocampal pyramidal neurons (especially CA1 and CA3) and the dentate gyrus stained heavily (Figs. 7, 8). Figure 7 shows two examples of the HET staining. Most of the Et was localized to the pyramidal cell soma layer and dendrites. Similar to cultured cells, O_2^- production was increased by NMDA, KA, and FCCP (data not shown). As seen in cell-free assays and in cultured neurons, CN and NO generation decreased HET oxidation by O_2^- (Fig. 8). Staining data are summarized in Figure 8B. The exogenous NO generators SNP and SNOC

Figure 8. *A*, Oxidation of HET to Et in rat hippocampal slices is reduced by CN and NO generators. Representative examples of the staining pattern after 20 min incubations in saline containing $3.2 \mu\text{M}$ HET are shown for Control, $300 \mu\text{M}$ SNP, 3 mM CN, 3 mM L-Arg, and $100 \mu\text{M}$ LNA. SNP and CN blocked Et accumulation in the pyramidal neuron soma layer; soma staining was reduced by L-Arg. The width of each image originally was 3.3 mm. *B*, Summary of mean fluorescence intensity in hippocampal slices treated with CN, SNP, SNOC ($300 \mu\text{M}$), L-Arg, and LNA. All treatments except LNA produced a significant reduction in HET oxidation ($p < 0.05$).

blocked basal rates of HET oxidation effectively in brain slices. The basal oxidation rate was equally sensitive to block by CN^- . Addition of L-Arg (3 mM), the substrate for endogenous NOS, inhibited HET oxidation significantly and suggests that NOS was active in slices. The NOS inhibitor LNA consistently decreased O_2^- production slightly, albeit nonsignificantly, versus unstimulated controls, and typically reduced the effectiveness of L-Arg (data not shown). Similar results were obtained with DPI (10 μM ; data not shown). The data show that O_2^- assay by HET is applicable to tissue slice preparations and that O_2^- concentration in rat hippocampal slices is sensitive to CN and NO.

The charged Et oxidation product and its ability to stain polynucleotides in cells that convert HET provided a permanent record of cells that oxidized HET. Slices could be fixed and retained Et record similar to tumor cells (Bucana et al., 1986). However, we found that the fluorescence intensity could decrease slightly during overnight fixation at 4°C (data not shown). The binding to DNA and RNA also may protect the Et from additional actions of ROS that may promote either the re-reduction of Et to HET or its decomposition to nonfluorescent forms.

DISCUSSION

Among the numerous methods available for detecting ROS production in cells, there are none that are selective for O_2^- and that also are able to indicate the site of its production. We have demonstrated that HET is oxidized only by O_2^- under the present assay conditions. The dye was not oxidized by $\cdot\text{OH}$, NO, ONOO^- , H_2O_2 , hypochlorite, or singlet O_2 . Compared with other fluorescent dyes, HET displays good photostability, especially under the longer excitation wavelength used to observe the oxidation product, Et. The higher quantum efficiency of Et bound to DNA enhanced detection. The method was amenable to kinetic measurements of O_2^- production, and the stable oxidized product allowed tissues to be fixed and used for end-point analyses. Thus far, HET has been used primarily in flow-cytometric studies (Olive, 1989; Rothe and Valet, 1990; Perticarari et al., 1991; Shi et al., 1991) and can be used in some plate readers for automated analyses (Bucana et al., 1986; Saiki et al., 1986). We have demonstrated now that the HET method has distinct advantages for analyzing O_2^- production in a variety of neuronal preparations by digital imaging microfluorimetry.

One of the possible limitations of HET as an indicator for the quantitation of O_2^- production is the ~20-fold increase in quantum efficiency that occurs with the binding of Et to DNA or RNA (LePecq and Paoletti, 1967). Absolute quantitation of O_2^- , therefore, must consider the presence of DNA and RNA. This complication is weighed by the possibility that the intercalation of Et into polynucleotides also serves to protect the Et from additional oxidation/reduction and also provides a permanent record of cells that oxidized HET. Et generated outside of cells (e.g., by phagocytes) will be excluded from the nucleus, provided that cell membranes remain intact, in accord with the use of Et and similar dyes in cell viability assays. Another possible disadvantage is the non-ratiometric measurement of Et fluorescence. Despite the fact that HET is fluorescent, it may be ill suited for ratios because of its possible sequestration into vesicles (Bucana et al., 1986; Saiki et al., 1986), although not observed in neurons in present studies, and the short excitation wavelength would promote photo-oxidation. Although high concentrations (>100 μM) of Et can interfere with mitochondrial respiration (Miko and Chance, 1975), our standard imaging conditions permitted detection of Et

estimated to range between 10 nM and 30 μM (Fig. 1D) and, therefore, avoided potential acute toxicity of Et.

Cell-free assays demonstrated that HET oxidation was mediated by O_2^- (Fig. 2) generated with KO_2 (Fig. 1E), as reported previously (Rothe and Valet, 1990), and by X + XO (Fig. 2), and was blocked by Cu/Zn-SOD (Fig. 2A). A 1:1 molar ratio for the interaction of HET with O_2^- has been reported recently (Biziukin and Korkina, 1994). The observed insensitivity to $\cdot\text{OH}$ probably is the result of the relatively low concentration of HET compared with the ~4300- and 3100-fold excesses of the potent $\cdot\text{OH}$ scavengers DMSO and HEPES, and perhaps the trapping of O_2^- before its conversion to $\cdot\text{OH}$. Because DMSO is freely permeable, it seems unlikely that any cellular domain would escape the $\cdot\text{OH}$ scavenging and react with HET to generate a significant Et signal. Insensitivity to ONOO^- was shown by the low conversion produced by the ONOO^- generator morpholinonydronimine (*SIN-1*; Fig. 2B) and by the inhibition of HET oxidation in XO + X + NO generators (Fig. 2B,C). The assay thus may underestimate total O_2^- production if there are high concentrations of dismutases or competing scavengers. By the same reasoning, changes in HET oxidation rate can be used as an indirect measure of these competing processes.

The rate of HET oxidation varied greatly in different types of cells but always exceeded the auto-oxidation rate seen in cell-free assays. The basal rate of O_2^- generation increased as cultured neurons aged (Fig. 4B). The highest rates typically were observed in senescing cultures, in accord with previous reports (Nohl and Hegner, 1978; Shigenaga et al., 1994).

Depolarization (50K) did not increase O_2^- generation measured by HET (Fig. 4C), but this may be an aberration attributable to swelling of the soma. Increased O_2^- production was observed for depolarization in Na^+ -free saline, in which swelling was minimal. An alternate explanation is related to differences in $[\text{Ca}^{2+}]_i$ in Na^+ -free saline (see below).

Our data extend previous reports (Bondy and Lee, 1993; Lafon-Cazal et al., 1993; Marin et al., 1993; Culcasi et al., 1994) that glutamate receptor agonists increase O_2^- production by demonstrating changes in O_2^- production within individual hippocampal neurons. This increase did not occur in glia. Neuronal O_2^- production increased after treatment with AMPA (data not shown), KA (Figs. 4D, 5A,F), and NMDA (Figs. 5B,D,E, 6B). The NMDA-mediated increase was small or absent in neurons younger than 8 DIV, presumably because of lack of development of NMDA receptors at this stage (data not shown). In mature neurons, O_2^- generation increased roughly twofold after treatment with NMDA (Fig. 5). The lag between NMDA application and the rise in O_2^- production also varied in different neurons (Fig. 3). The nonadditivity of treatments that increased O_2^- production, for example, the lack of additional increase in O_2^- production by FCCP in neurons with high rates of O_2^- generation after NMDA treatment (Fig. 5), suggested that either cellular O_2^- production or its reporting by HET could be saturated. Because this ceiling was observed in cells with widely varying rates of HET oxidation, we favor the hypothesis that O_2^- production was saturable.

Role of $[\text{Ca}^{2+}]_i$ in O_2^- generation

Enhanced O_2^- production appeared to require Ca^{2+} entry, because little effect was observed when KA or NMDA was applied in Ca^{2+} -free saline (Fig. 5). On the other hand, short application of a Ca^{2+} ionophore producing $[\text{Ca}^{2+}]_i$ that equaled or exceeded that produced by glutamate agonists (Prehn et al., 1994) did not increase O_2^- production greatly (Fig. 5D,E). Similar results were

observed in a rat brain microsome preparation (Bondy and Lee, 1993). A high $[Ca^{2+}]_i$ requirement for ROS generation by isolated mitochondria was demonstrated by Dykens (1994). However, maximal ROS generation in that study required the presence of high intracellular Na^+ and Ca^{2+} . Nevertheless, it appears that high $[Ca^{2+}]_i$ may be sufficient for enhanced O_2^- production in intact neurons, because rate increases were observed for KA (Fig. 6A) and NMDA (data not shown) applied in Na^+ -free saline.

The route and intracellular localization of the $[Ca^{2+}]_i$ signal may be important parameters for enhancement of O_2^- generation, because O_2^- production did not increase greatly after Ca^{2+} ionophore or 50K depolarization. The importance of entry locus or intracellular compartment also may explain why only certain mitochondria appeared to oxidize HET (Fig. 3C). Perhaps these mitochondria reside near synaptic areas containing high concentrations of receptors and thus are exposed to large $[Ca^{2+}]_i$ gradients. Indeed, we have demonstrated recently that initial sites of neurodegeneration produced by KA stimulation are associated with such domains of high $[Ca^{2+}]_i$ at synapses (Bindokas and Miller, 1995). Much of this Ca^{2+} can be seen to enter the mitochondria. High microdomain concentrations of $[Ca^{2+}]_i$ thus may be critical for increasing O_2^- generation. Hepatocyte mitochondrial state similarly was found to be influenced more by inositol triphosphate-mediated $[Ca^{2+}]_i$ elevations than by sustained elevations of $[Ca^{2+}]_i$ (Hajnóczky et al., 1995). It is interesting to note that 50K was able to increase O_2^- generation in the absence of extracellular Na^+ . With Ca^{2+} removal compromised by lack of Na–Ca exchange, Ca^{2+} influx through voltage-sensitive Ca^{2+} channels now may be more influential. It seems likely that increased $[Ca^{2+}]_i$ provides a link between glutamate receptor stimulation, mitochondrial function, and O_2^- generation.

Role of mitochondria in O_2^- generation

It is estimated that 1–2% (Turrens and Boveris, 1980) of the electron flow in mitochondria normally leaks from the electron transport chain to form O_2^- and that 20% of the O_2^- escapes mitochondrial SOD (Nohl and Hegner, 1978). O_2^- may arise via ubisemiquinone oxidation or NADH dehydrogenase activity (cf. Turrens et al., 1985; Nohl, 1990). Our data are consistent with mitochondrial respiration as the main source of the basal O_2^- production and as the major source of the FCCP-, H_2O_2 -, and glutamate-receptor agonist-activated O_2^- production. In support of this conclusion, the initial Et staining pattern was colocalized with a population of R123-stained mitochondria (Fig. 3C) similar to that recently reported by Reynolds and Hastings (1995). Block of the respiratory chain with CN could eliminate basal O_2^- generation completely (Fig. 5C). We are not aware of any other effects of FCCP or CN other than on mitochondrial state that would explain the observed O_2^- rate data. Because NOS did not contribute to basal O_2^- generation in cultured neurons (Fig. 6C), the inhibition of O_2^- production was not attributable to possible inhibition of NOS by CN (Hobbs et al., 1994). Of interest, the protonophore FCCP was able to restore HET oxidation in CN-blocked neurons (Fig. 5C). FCCP acts to stimulate mitochondrial electron transport maximally, suggesting that the increase in O_2^- generation was caused by increased mitochondrial respiration. This also implicates ubisemiquinone as the major leak site, because O_2^- generation by ubisemiquinone in isolated preparations has been observed under similar conditions (Nohl, 1990). The production of O_2^- in the presence of KA and NMDA was inhibited by CN (see Fig. 5), but neurons with especially high rates of O_2^- production continued to generate O_2^- at a slower rate. This

suggests that O_2^- can be generated by other mechanisms as well (cf. Siesjö et al., 1989).

It is noteworthy that some neurons (e.g., superior cervical ganglion cells) and glia present in some hippocampal cultures appeared to have especially low rates of O_2^- production and often were unaffected by FCCP (data not shown). Because it is unlikely that FCCP failed to uncouple respiration, either these cells did not produce large concentrations of O_2^- via electron transport or, more likely, they possessed more efficient ROS-scavenging mechanisms. For example, glial cells have been shown to have significantly higher glutathione content (Sagara et al., 1993).

H_2O_2 is toxic to cells and recently has been shown to secondarily generate O_2^- in endothelial cells (Carter et al., 1994). Application of H_2O_2 increased or promoted HET oxidation in all cells tested. This may be attributable to H_2O_2 overwhelming cellular defense mechanisms (for example, by blocking Cu/Zn-SOD) and unmasking O_2^- production normally undetected, and/or an actual increase in production of O_2^- . This is consistent with the increase in HET oxidation observed after treatment with the Cu^{2+} chelators APDC and *o*-phenanthroline. The effect of H_2O_2 was not additive in cells that already had a high rate of O_2^- production because of treatment with FCCP, KA, or NMDA (see Fig. 5E), and was sensitive to block by CN (Fig. 6A). Because O_2^- dismutates spontaneously or by enzymatic action to H_2O_2 and inhibits catalase (Kono and Fridovich, 1982), and its generation is enhanced by H_2O_2 , this could create a regenerative cycle that magnifies oxidative damage to cellular components.

It has been reported that NOS and other NADPH oxidases can generate O_2^- under certain conditions (Pou et al., 1992; Culcasi et al., 1994). The selective inhibitor diphenylene iodonium (Stuehr et al., 1991) failed to block the basal rate of O_2^- production as well as the FCCP-stimulated increase in cultured cells (Fig. 6C), suggesting that these oxidases are not active under these conditions. Exogenous generation of NO did inhibit the oxidation of Et by X/XO (Fig. 2) and within neurons (Fig. 6). This action probably was caused by O_2^- consumption via peroxynitrite formation (Lipson et al., 1993). The prolonged suppression of FCCP-stimulated O_2^- generation after NO may be an indication that nitrogen radicals disrupt mitochondrial electron transport. Various cytochromes and enzymes, as well as mitochondrial potential, are known to be sensitive to NO or $^{\cdot}ONOO$ (Bolanos et al., 1994; Cleeter et al., 1994; Schweizer and Richter, 1994).

Slices of rat hippocampi showed time-dependent staining intensity primarily in neuronal somas from pyramidal layers and dentate gyrus (Figs. 7, 8). Staining was sensitive to CN and NO similar to that observed in cultured neurons (Fig. 8). Increasing the L-Arg concentration reduced staining significantly, presumably by stimulating NOS activity. NOS inhibitors reduced this effect. The slight but consistent reduction of baseline O_2^- production by NOS inhibitors suggests further that NOS activity is an important regulator of O_2^- levels in brain slices.

Given the success of the assay of O_2^- production within slice preparations, it also may be possible to monitor O_2^- production in whole tissue by using HET in perfusion solutions. This would allow end-point monitoring of O_2^- production during situations such as ischemia/reperfusion.

REFERENCES

- Abele AE, Scholz K, Miller RJ, Scholz WK (1990) Excitotoxicity induced by enhanced excitatory neurotransmission in cultured hippocampal neurons. *Neuron* 4:413–419.
- Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M (1983) Flow cytometric studies of oxidative product formation by neu-

- trophils: a graded response to membrane stimulation. *J Immunol* 130:1910–1917.
- Bensasson RV, Land EJ, Truscott TG (1993) Excited states and free radicals in medicine: contributions from flash photolysis and pulse radiolysis. New York: Oxford UP.
- Bindokas VP, Miller RJ (1995) Excitotoxic degeneration is initiated at non-random sites in cultured rat cerebellar neurons. *J Neurosci* 15:6999–7011.
- Biziukin AV, Korkina LG (1994) Use of the fluorescent indicator hydroethidine to study the oxidative metabolism of phagocytes [in Russian]. *Klin Lab Diagn* 1:41–42.
- Bolaños JP, Peuchen S, Heales SJR, Land JM, Clark JB (1994) Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. *J Neurochem* 63:910–916.
- Bondy SC, Lee DK (1993) Oxidative stress induced by glutamate receptor agonists. *Brain Res* 610:229–233.
- Bucana C, Saiki I, Nayar R (1986) Uptake and accumulation of the vital dye hydroethidine in neoplastic cells. *J Histochem Cytochem* 34:1109–1115.
- Cadenas E (1989) Biochemistry of oxygen toxicity. *Annu Rev Biochem* 58:79–110.
- Carter WO, Narayanan PK, Robinson JP (1994) Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J Leukocyte Biol* 55:253–258.
- Cathcart R, Schwieters E, Ames BN (1983) Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal Chem* 134:111–116.
- Chiang LW, Schweizer FE, Tsien RW, Schulman H (1994) Nitric oxide synthase expression in single hippocampal neurons. *Mol Brain Res* 27:183–188.
- Cleeter MWJ, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AHV (1994) Reversible inhibition of cytochrome C oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. *FEBS Lett* 345:50–54.
- Coyle JT, Puttfarcken P (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262:689–695.
- Culcasi M, Lafon-Cazal M, Pietri S, Bockaert J (1994) Glutamate receptors induce a burst of superoxide via activation of nitric oxide synthase in arginine-depleted neurons. *J Biol Chem* 269:12589–12593.
- Dawson TM, Snyder SH (1994) Gases as biological messengers: nitric oxide and carbon monoxide in the brain. *J Neurosci* 14:5147–5159.
- Dinerman JL, Dawson TM, Schell MJ, Snowman A, Snyder SH (1994) Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. *Proc Natl Acad Sci USA* 91:4214–4218.
- Dugan LL, Sensi SL, Canzoniero LMT, Goldberg MP, Handran SM, Rothman SM, Choi DW (1994) Imaging of mitochondrial oxygen radical production in cortical neurons exposed to NMDA. *Soc Neurosci Abstr* 20:1532.
- Dykens JA (1994) Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca^{2+} and Na^{+} : implications for neurodegeneration. *J Neurochem* 63:584–591.
- Elstner EF, Heupel A (1976) Inhibition of nitrite formation from hydroxylammonium-chloride: a simple assay for superoxide dismutase. *Anal Biochem* 70:616–620.
- Emaus RK, Grunwald R, Lemasters JJ (1986) Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim Biophys Acta* 850:436–448.
- Fabian RH, DeWitt DS, Kent TA (1995) In vivo detection of superoxide anion production by the brain using a cytochrome C electrode. *J Cereb Blood Flow Metab* 15:242–247.
- Feelisch M (1991) The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J Cardiovasc Pharmacol* 17[Suppl 3]:S25–S33.
- Feelisch M, Ostrowski J, Noack E (1989) On the mechanism of NO release from sydnonimines. *J Cardiovasc Pharmacol* 14[Suppl 1]:S13–S22.
- Forman HJ, Kennedy J (1976) Dihydroorotate-dependent superoxide production in rat brain and liver. *Arch Biochem Biophys* 173:219–224.
- Gallo PM, Paz MA, Henson E, Latt SA (1984) Dynamic approaches to the delivery of reporter reagents into living cells. *Biotechniques* 3:32–36.
- Hajnóczky G, Robb-Gaspers LD, Seitz MB, Thomas AP (1995) Decoding of cytosolic calcium oscillations in mitochondria. *Cell* 82:415–424.
- Halliwell B (1989) Oxidants and the central nervous system: some fundamental questions. Is oxidant damage relevant to Parkinson's disease, Alzheimer's disease, traumatic injury or stroke? *Acta Neurol Scand* 126:23–33.
- Halliwell B, Gutteridge JMC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 219:1–14.
- Hobbs AJ, Fukuto JM, Ignarro LJ (1994) Formation of free nitric oxide from L-arginine by nitric oxide synthase: direct enhancement of generation by superoxide dismutase. *Proc Natl Acad Sci USA* 91:10992–10996.
- Hogg N, Darley-Usmar M, Wilson MT, Moncada S (1992) Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem J* 281:419–424.
- Huie RE, Padmaja S (1993) The reaction rate of nitric oxide with superoxide. *Free Radic Res Commun* 18:195–199.
- Ignarro LJ (1990) Nitric oxide: a novel signal transduction mechanism for transcellular communication. *Hypertension* 16:477–483.
- Ikedo Y, Long DM (1990) The molecular basis of brain injury and brain edema: the role of oxygen free radicals. *Neurosurgery* 27:1–11.
- Iqbal J, Whitney P (1991) Use of cyanide and diethyldithiocarbamate in the assay of superoxide dismutases. *Free Radic Biol Med* 10:69–77.
- Key Jr LL, Ries WL, Taylor RG, Hays BD, Pitzer BL (1990) Oxygen derived free radicals in osteoclasts: the specificity and localization of the nitroblue tetrazolium reaction. *Bone* 11:115–119.
- Kono Y, Fridovich I (1982) Superoxide radical inhibits catalase. *J Biol Chem* 257:5751–5754.
- Koody NW, Royall JA, Ischiropoulos H, Beckman JS (1994) Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radic Biol Med* 16:149–156.
- Kuppusamy P, Zweier JL (1989) Characterization of free radical generation by xanthine oxidase. *J Biol Chem* 264:9880–9884.
- Lafon-Cazal M, Pietri S, Culcasi M, Bockaert J (1993) NMDA-dependent superoxide production and neurotoxicity. *Nature* 364:535–537.
- Lei SZ, Pan Z-P, Aggarwal SK, Vincent Chen H-S, Hartman J, Sucher NJ, Lipton SA (1992) Effect of nitric oxide on the redox modulatory site of the NMDA receptor-channel complex. *Neuron* 8:1087–1099.
- LePecq J-B, Paoletti C (1967) A fluorescent complex between ethidium bromide and nucleic acids: physical-chemical characterization. *J Med Biol* 27:87–106.
- Lipton SA, Stamler JS (1994) Actions of redox-related congeners of nitric oxide at the NMDA receptor. *Neuropharmacology* 33:1229–1233.
- Lipton SA, Choi Y-B, Pan Z-H, Lei SZ, Vincent Chen H-S, Sucher NJ, Loscalzo J, Singel DJ, Stamler JS (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364:626–632.
- Lissi E, Pascual C, Del Castillo MD (1994) On the use of the quenching of luminol luminescence to evaluate SOD activity. *Free Radic Biol Med* 16:833–837.
- Lokesh BR, Cunningham ML (1986) Further studies on the formation of oxygen radicals by potassium superoxide in aqueous medium for biochemical investigations. *Toxicol Lett* 34:75–84.
- Lynch RE, Fridovich I (1978) Permeation of the erythrocyte stroma by superoxide radical. *J Biol Chem* 253:4697–4699.
- Maines MD (1993) Carbon monoxide: an emerging regulator of cGMP in the brain. *Mol Cell Neurosci* 4:389–397.
- Marin P, Quignard J-F, Lafon-Cazal M, Bockaert J (1993) Non-classical glutamate receptors, blocked by both NMDA and non-NMDA antagonists, stimulate nitric oxide production in neurons. *Neuropharmacology* 32:29–36.
- Miko M, Chance B (1975) Ethidium bromide as an uncoupler of oxidative phosphorylation. *FEBS Lett* 54:347–352.
- Nakano M (1990) Determination of superoxide radical and singlet oxygen based on chemiluminescence of luciferin analogs. *Methods Enzymol* 186:585–591.
- Nohl H (1990) Is redox-cycling ubiquinone involved in mitochondrial oxygen activation? *Free Radic Res Commun* 8:4–6.
- Nohl H, Hegner D (1978) Do mitochondria produce oxygen radicals *in vivo*? *Eur J Biochem* 82:563–567.
- Olive PL (1989) Hydroethidine: a fluorescent redox probe for locating hypoxic cells in spheroid tumours. *Br J Cancer* 60:332–338.
- Perticarari S, Presani G, Mangiarotti M, Banfi E (1991) Simultaneous flow cytometric method to measure phagocytosis and oxidative products by neutrophils. *Cytometry* 12:687–693.

- Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM (1992) Generation of superoxide by purified brain nitric oxide. *J Biol Chem* 267:24173–24176.
- Prehn JHM, Bindokas VP, Marcuccilli CJ, Krajewski S, Reed JC, Miller RJ (1994) Regulation of neuronal Bcl2 protein expression and calcium homeostasis by transforming growth factor type β confers wide-ranging protection on rat hippocampal neurons. *Proc Natl Acad Sci USA* 91:12599–12603.
- Reynolds IJ, Hastings TG (1995) Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. *J Neurosci* 15:3316–3327.
- Rossi F (1986) The O_2^- -forming NADPH oxidase of phagocytes: nature, mechanisms of activation and function. *Biochim Biophys Acta* 853:65–89.
- Rothe G, Valet G (1990) Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *J Leukocyte Biol* 47:440–448.
- Sagara J-I, Miura K, Bannai S (1993) Maintenance of neuronal glutathione by glial cells. *J Neurochem* 61:1672–1676.
- Saiki I, Bucana C, Tsao JY, Fidler IJ (1986) Quantitative fluorescent microassay for identification of antiproliferative compounds. *J Natl Cancer Inst* 77:1235–1240.
- Scaduto Jr RC (1995) Oxidation of DMSO and methanesulfinic acid by the hydroxyl radical. *Free Radic Biol Med* 18:271–277.
- Schweizer M, Richter C (1994) Nitric oxide potently and reversibly deenergizes mitochondria at low oxygen tension. *Biochem Biophys Res Commun* 204:169–175.
- Shi T, Eaton AM, Ring DB (1991) Selection of hybrid hybridomas by flow cytometry using a new combination of fluorescent vital stains. *J Immunol Methods* 141:165–175.
- Shigenaga MK, Hagen TM, Ames BA (1994) Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci USA* 91:10711–10718.
- Siesjö BK, Agardh C-D, Bengtsson F (1989) Free radicals and brain damage. *Cerebrovasc Brain Metab Rev* 1:165–211.
- Smith RP, Wilcox DE (1994) Toxicology of selected nitric oxide-donating xenobiotics, with particular reference to azide. *Crit Rev Toxicol* 24:355–377.
- Steinbeck MJ, Khan AU, Appel Jr WH, Karnovsky MJ (1993) The DAB-Mn⁺⁺ cytochemical method revisited: validation of specificity for superoxide. *J Histochem Cytochem* 41:1659–1667.
- Stuehr DJ, Fasehun OA, Kwon NS, Gross SS, Gonzalez JA, Levi R, Nathan CF (1991) Inhibition of macrophage and endothelial cells nitric oxide synthase by diphenyliodonium and its analogs. *FASEB J* 5:98–103.
- Swannell RPJ, Caplin R, Nedwell DB, Williamson FA (1992) An investigation of hydroethidine as a fluorescent vital stain for prokaryotes. *FEMS Microbiol Ecol* 101:173–182.
- Tanaka M, Sotomatsu A, Yoshida T, Hirai S, Nishida A (1994) Detection of superoxide production by activated microglia using a sensitive and specific chemiluminescence assay and microglia-mediated PC12h cell death. *J Neurochem* 63:266–270.
- Traystman RJ, Kirsch JR, Koehler RC (1991) Oxygen radical mechanisms of brain injury following ischemia and reperfusion. *J Appl Physiol* 71:1185–1195.
- Tsien RY (1989) Fluorescent indicators of ion concentrations. *Methods Cell Biol* 30:127–156.
- Turrens JF, Boveris A (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* 191:421–427.
- Turrens JF, Alexander A, Lehninger AL (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 237:408–414.
- Valtschanoff JG, Weinberg RJ, Kharazia VN, Nakane M, Schmidt HHHW (1993) Neurons in the rat hippocampus that synthesize nitric oxide. *J Comp Neurol* 331:111–121.
- Zhu H, Bannenberg GL, Moldéus P, Shertzer HG (1994) Oxidation pathways for the intracellular probe 2',7'-dichlorofluorescein. *Arch Toxicol* 68:582–587.