Real-time measurement of free radical production using specific electrochemical sensors: new insight into the consequences of O_2^- and NO flux

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Direct real-time electrochemical measurements have offered new insight into the importance of free radical interplay in a number of cellular models. Initially the interrelationship between O_2^- and NO generation in astrocytic, neuronal and mixed astrocytic/neuronal cell populations was examined. Results indicated a novel function for astrocytic nitric oxide synthase (cNOS) in regulating extracellular O_2^- release and therefore controlling neuronal nitric oxide availability. Further, the pathological consequences of extracellular O_2^- production on rat astrocytes was assessed. Findings show that extracellular O_2^- generation initiated the production of NO by glial cells. Other findings facilitated by electrochemical measurements included the characterisation of a novel motor neuron like cell line for its ability to produce O_2^- and NO in response to extracellular glutamate and AMPA, the interaction of O_2^- with NO in an animal models of migraine headache and the modulatory effect of α-melanocyte stimulating hormone (α-MSH) on NO production by human epidermal melanocytes.

Introduction

Oxygen and nitrogen derived free radicals play a central role in normal physiological processes. As part of the host defence system and as cell/cell signalling molecules they perform an essential function. Under normal metabolic conditions, these reactive oxygen species (ROS) are produced at a rate which is matched by the capacity of tissue to catabolise them [1]. When their production exceeds the body's natural ability to deal with these potentially cytotoxic species a variety of pathological conditions may result including cancer, stroke and neurodegeneration [2]. Damage to protein, DNA and lipid is central in the pathological importance of free radicals to biological systems, especially if the natural defences of such a system to these radicals are compromised [2]

In order to gain a full understanding of the role that free radicals play in pathology it is essential to measure these species in a variety of *in vitro* and *in vivo* models. However, the high reactivity of most free radicals makes their detection difficult. Not withstanding these inherent difficulties attempts have been made to study free radicals in a variety of ways.

Free radical analysis

Superoxide measurement

The most widely used method of O_2^- measurement is the spectrophotometric measurement of cytochrome c reduction at 550 nm [3]. As the technique is so simple it is often the method of choice for estimating rates of O_2^- production [3]. The analysis of end products resulting from free radical formation utilises well established analytical techniques but does not provide unequivocal evidence of free radical production by a biological system. The spectrophotometric determination of uric acid produced by the action of xanthine oxidase on xanthine resulting in superoxide formation is an example of such a procedure [4]. The extent of free radical induced tissue injury is another indirect approach for radical determination with particular application to *in vivo* measurements. The end products of lipid peroxidation can be measured in human body fluid or tissue samples. Of such tests, the thiobarbituric acid (TBA) reaction has proven to be the most widely used [5]. This assay relies on the interaction between TBA and malondialdehyde (MDA), a volatile side product of oxidative lipid degeneration [5]. Combination of TBA with MDA results in a fluorescent red adduct which can be measured using fluorimetry. However, TBA does not react exclusively with MDA and as such this assay is open to non-specific interferents [6].

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Electron spin (paramagnetic) resonance (ESR or EPR) detects, through the use of microwave absorbance spectra, an unpaired electron present in a free radical. As such, it is the only approach so far reported that can provide direct evidence for the presence of a free radical. In addition the analysis of the ESR spectrum generally enables the identification of the free radical species [7]. However, ESR requires that the free radicals are relatively stable. "Spin trapping" of the radical may be necessary if it's decay is very rapid [7].

NO measurement

The presence of NO in biological systems is usually inferred based on one of its physiological effects [8]. These include the relaxation of blood vessels, activation of guanylate cyclase activity, increased cGMP concentrations, production of citrulline, or inhibition of platelet aggregation [8]. Also, inhibitors of nitric oxide synthase such as the L-arginine analogues or molecules such as haemoglobin, which can trap and deactivate NO, have been used to estimate NO production [8]. All of these methods have variable degrees of NO specificity and provide indirect evidence of NO production. Only cGMP or citrulline concentrations can be used to obtain quantitative information related to the amount of NO produced. Such indirect assays may provide misleading information. Spectrophotometric methods for NO analysis include chemiluminescence [9], UV-visible spectroscopy [10], electron spin resonance spectroscopy [11] and flow cytometry [12]. The UV-visible spectroscopy method for NO detection is based on the Griess reagent, which is a mixture of sulphanilic acid and N-(1-naphthyl) ethylenediamine [10]. N-(1-naphthyl) ethylenediamine reacts with NO and the product of this reaction absorbs at 548 nm. The absorbance of this peak is proportional to the concentration of NO present. Chemiluminescence is a sensitive method but requires at least two steps in sample preparation. Initially NO_2^- and/or NO_3^- must be chemically reduced back to nitric oxide. Further, NO must be transferred from the liquid phase to gas phase. Both steps may introduce significant errors. [9]. ESR can be used to measure NO [11]. In this case, a spin trap, such as haemoglobin or nitroxide, is used to stabilise the NO.

Electrochemical methods for free radical detection

Traditional methods of free radical analysis are limited by their indirect nature and the fact that analysis may only be possible several hours after the free radical event of interest has taken place. Recent attempts to measure free radicals have centred on electrochemical methods which enable direct, real-time measurement in biological systems with minimal disturbance to the sample under investigation.

Electrochemical O_2^- detection

In the late 1970's it was realised that suitably modified (or functionalised) electrode surfaces could interact in a specific and non-degradative manner with proteins, to allow stable and reversible direct electrochemistry that was not affected by artifacts [13]

Eddowes and Hill [14] showed the essentially reversible electrochemistry of horse heart cytochrome c could be demonstrated at a 4,4'-bipyridyl disulphide modified Au electrode. A further example of cytochrome c electochemistry obtained from a functionalised Au electrode was reported by Cooper *et al.* [15]. In this work the Au electrode electrode was modified with N-acetyl cysteine. This procedure was further extended to the covalent attachment of cytochrome c at the modified electrode surface through a carbodiimide initiated condensation reaction. Once covalently attached, the immobilised cytochrome c was used as an integral part of an amperometric O_2^- sensor. Superoxide generated by xanthine/XOD caused the one electron reduction of cytochrome c3+ to cytochrome c2+. The reduced protein was then reoxidised at the electrode surface (poised at + 25 mV with respect to a Ag/AgCl reference electrode). Current rates recorded were directly proportional to the rate of O_2^- production by XOD [15]. McNeil *et al.* [16] used this immobilisation procedure to detect O_2^- production by stimulated human neutrophils. The neutrophils, stimulated with phorbol-12 myristate-13 acetate (PMA), produced current changes which were cell number dependent. Using the protocol previously described by McNeil *et al.* [16], Fabian *et al.* [17] have recently used a platinised activated carbon electrode (PACE) to passively adsorb cytochrome c onto the electrode surface and have used this sensor *in vivo* to measure O_2^- production by rat brain during hypoxia, focal ischaemia, reperfusion and fluid percussion brain injury. Despite the inherently high background noise detected by the large surface area of this porous electrode material, some O_2^- generation was seen following brain injury in rats.

A novel development of the cytochrome c based electrode has recently been described [18]. Here cytochrome c was covalently attached to a gold working electrode through surface modification with 3,3'-dithiobis (sulphosuccinimidyl-propionate) (DTSSP). This linking molecule possesses a disulphide group for covalent attachment to the gold surface. Following attachment the molecule presents two carboxyl groups which readily undergo amide linkage with cytochrome c. The resulting electrode is poised at + 100 mV (*vs.* Ag/AgCl). Superoxide generated by xanthine/XOD caused the one electron reduction of cytochrome c3+ to cytochrome c2+. The reduced protein was then reoxidised at the electrode surface. This electrode has successfully been used to detect O_2^- in a wide variety of *in vitro* biological applications. Recently, the response of this electrode to enzymatically generated O_2^- has been mathematically modelled [19]. The investigation of the electrochemical kinetic behaviour of immobilised cytochrome c to enzymatically generated O_2^- has enabled estimation of O_2^- concentrations. Before detailed study of the application of this electrode to biological systems is undertaken, a brief summary of this modelling study will be presented.

Superoxide electrode based on covalently immobilised cytochrome c: modelling study [19]

Full experimental details are given in [19]. In brief, mathematical models were developed that related the rate of cytochrome c reduction by enzymatically generated O_2^- (using the xanthine/xanthine oxidase system) to the surface coverage of the protein on a modified gold electrode surface. In this way a theoretical electrode sensitivity towards O_2^- of 5.71 μM nA^{-1} was calculated. This modelling study has added the benefit of absolute O_2^- concentrations determination to the already highlighted advantages of direct, real time free radical monitoring.

Electrochemical NO detection

An electrochemical sensor based on a modified Clark oxygen electrode has been described [20]. A platinum wire formed the working electrode (anode) and silver wire formed the counter/reference electrode (cathode). Both electrodes were mounted in a capillary tube filled with sodium chloride/hydrochloric acid solution and separated from the solution containing the analyte by a gas-permeable membrane (chloroprene rubber). Application of a potential of 0.9 V resulted in a current which was measured secondarily to the oxidation of NO at the platinum electrode surface. The electrode, however, cannot be totally specific to NO as O_2^-, which can also diffuse through the membrane, will react with NO generating NO_2^-. The nitrite thus formed can also be oxidised at the electrode surface giving a current that would be additive to that already observed from NO.

An electrochemical microsensor based on NO oxidation at a polymeric metalloporphyrin (*n*-type semiconductor) electrode has been developed [21]. This reaction occurs at + 630 mV which is 270 mV lower than the potential required for NO oxidation at a metal or graphite electrode. This relatively sensitive device (detection limit of 10 nM NO) has a fast response time (10 ms). When coated with a thin layer of the anion exchanger Nafion, the porphyrinic semiconductor does not suffer from non-specific interference by NO_2^- [21].

A popular, commercially available NO electrode is supplied by World Precision Instruments (Sarasota, USA). Their ISO-NO meter is based on the direct oxidation of NO to NO^+ at a platinum electrode poised at + 850 mV. The selectivity of this device for NO comes from a perm-selective membrane which covers the working electrode.

The central role of this review is to highlight the numerous practical applications of the DTSSP/cytochrome c based superoxide electrode described by Manning *et al*. (1998) used in conjunction with a commercially available NO meter (ISO-NO, World Precision Instruments). In combination these electrodes have provided important insight into the pathological consequences of the interplay between O_2^- and NO in a number of *in vitro* and *in vivo* models.

Superoxide generation from constitutive nitric oxide synthase in astrocytes *in vitro* regulates extracellular nitric oxide availability [24]

Free radicals may play an important role in brain cell physiology and pathophysiology. The true extent of free radical involvement for reasons of analytical difficulty already outlined have not yet been clearly defined. Such analytical difficulties have been addressed by the use of novel, specific electrochemical sensors for the direct, real time detection of O_2^- and NO production. This study investigates the complex interrelationship between O_2^- and NO generation in primary rat cortical and hypothalamic brain cells. Direct, real time electrochemical measurement of free radical flux in astrocytic, neuronal and mixed astrocytic/neuronal populations may elucidate the importance of O_2^- and NO in neurophysiology.

Methods

Primary cortical and hypothalamic mixed cell cultures

Prepared from Sprague-Dawley rat fetuses as described by Clarke *et al*. [23]. Characterisation of each new set of cultures was performed using immunochemistry to determine the expression of cNOS, GFAP, N200 and OX-42.

Primary neuronal cell cultures

The glial element of these cultures was decreased through incubation with the antimitotic agent 5'-fluro-2-deoxyuridine on day four after plating (10 μg ml^{-1} for a further 3 days). Typical neuronal cultures consisted of 60-70 % N200 positive cells (neurons).

Primary astrocytic and microglial cell cultures

Created as described by Colton *et al.* [24] using 1-2 day old Sprague-Dawley rat pups.

Superoxide electrode

Prepared and characterised as previously described [18]. Sensitivity of the electrode to O_2^- was always in the region of 2 pM O_2^- pA^{-1} (as determined from calibration curves). The electrode did not react with NO, $ONOO^-$ or any electroactive component of the tissue culture medium.

Nitric oxide electrode

A commercially available NO sensor was used (ISO-NO Mark II, World Precision Instruments Inc., Stevenage, Herts., UK). This electrode did not respond to nitrite, nitrate or any other electroactive component of the tissue culture medium. NO concentrations as low as 1 nM could be detected. No loss of sensitivity was recorded after 12 h of continuous use.

Measurements

All experiments were carried out in a humidified atmosphere of 5 % CO_2/95 % air at 37 °C. Immediately prior to experimentation, the culture media in which the cells were grown (Minimal Essential Medium (MEM) supplemented with 2 mM glutamine, 10 % FCS, 10 % heat inactivated horse serum, 50 Uml^{-1} penicillin, 50 $\mu g\, ml^{-1}$ streptomycin and 2.5 $\mu g\, ml^{-1}$ amphotericin was replaced with serum free, unsupplemented MEM. Both electrodes were positioned directly over the cells and allowed to equilibrate for 20 min before experimentation. 2M NaOH (pH 10) was added to each well in order to provoke free radical release and act as a positive control. Attempts to quenched free radical production elicited by NaOH were made by the addition of SOD (100-200 $U\, ml^{-1}$) and cPTIO (2-10 µM).

Total nitrite accumulation was measured using a modification of the Griess reaction [25]. Differences between means was assessed using two way ANOVA.

Results

Results from cortical and hypothalamic cells were identical and are presented together.

Mixed brain cell cultures

Following electrode equilibration in serum free medium, a persistent and continuous O_2^- current was observed in 80 % of mixed population wells tested without chemical stimulation of the cells. This current was quenched by 100-300 Uml^{-1} SOD. No NO release was observed (confirmed by the addition of 2-20 µM cPTIO). Addition of 0.5-1 µM L-arginine caused no detectable NO production but a significant decrease in O_2^- production. The relationship between NOS and O_2^- production was investigated using specific NOS inhibitors. The non-specific NOS inhibitor L-NAME (500 µM) caused a decrease in O_2^- release as did the cNOS specific inhibitors Ng-NLA (500 µM) and 7-nitroindazol (7-NI) (5 µM). However, the iNOS specific inhibitor aminoguanidine (AD) (500 µM) had no effect on the O_2^- baseline.

Further evidence suggesting cNOS involvement included i) media lacking in Ca^{2+} and Mg^{2+} abolished basal O_2^- release and ii) the calmodulin inhibitor N-(6-aminohexyl)-5-chloro-1-napthalene sulfonamide (W-7, 50 µM) abolished O_2^- baseline (Fig. 1). Exposure of mixed cell populations to 2M NaOH caused both O_2^- and NO release that was quenched by 100 $U.ml^{-1}$ SOD and 5 µM cPTIO respectively (Fig. 2). High K^+ (40 mM, pH 7.6) caused no O_2^- release and no current was observed at the NO electrode. However, some nitrite accumulation was recorded.

Neuronal cultures

No O_2^- production was detected. Nitrite accumulation was observed which was inhibited by Ng-NLA, L-NAME, and 7NI. Nitrite accumulation was increased in the presence of 40 mM K^+. 2M NaOH caused rapid cell death but no O_2^- or NO. Less than 15 % of neurons produced NO that could be detected at the electrochemical sensor. This current was sensitive to aminoguanidine indicating iNOS.

Astrocyte cultures

O_2^- was seen which was SOD sensitive, decreased by cNOS inhibitors and responded to calcium manipulation as for mixed cultures. L-arginine or high [K^+] however did not decrease the NO baseline response. Nitrite estimation showed 60 % lower levels of basal NO production which was insensitive to L-NAME, Ng-NLA, 7-NI and low Ca^{2+}.

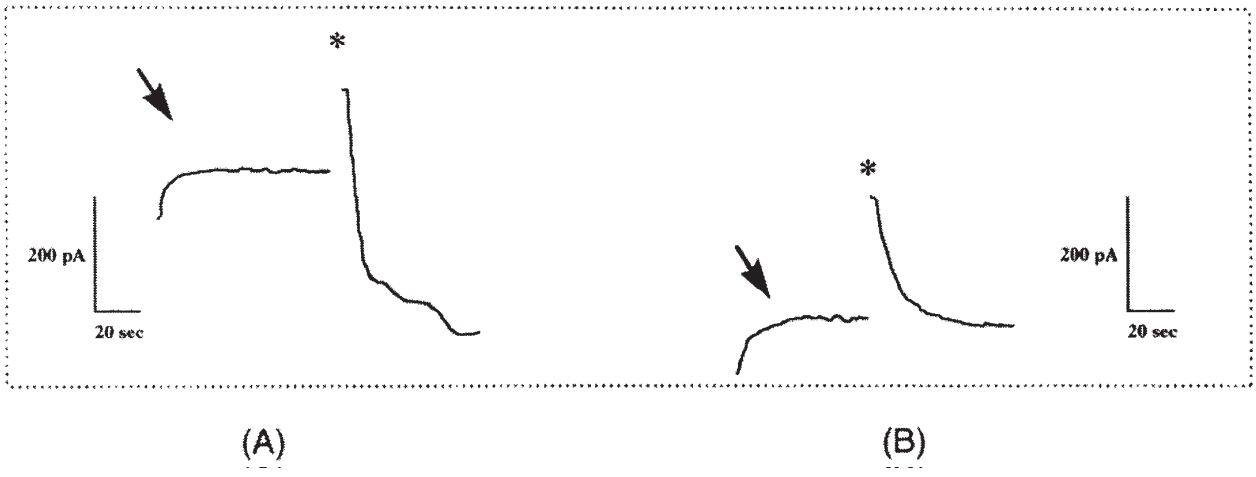


Figure 1. Typical superoxide electrochemical sensor trace demonstrating the effect of the calmodulin inhibitor W-7 on primary mixed and astrocytic cell cultures. (A) Superoxide baseline (arrow). *Addition of 100 U SOD results in a significantly depressed baseline, indicating the presence of superoxide in the well. **(B)** The same experimental well after exposure to 50 mM W-7. *Addition of 100U SOD caused no decrease in the baseline indicating superoxide production had ceased.

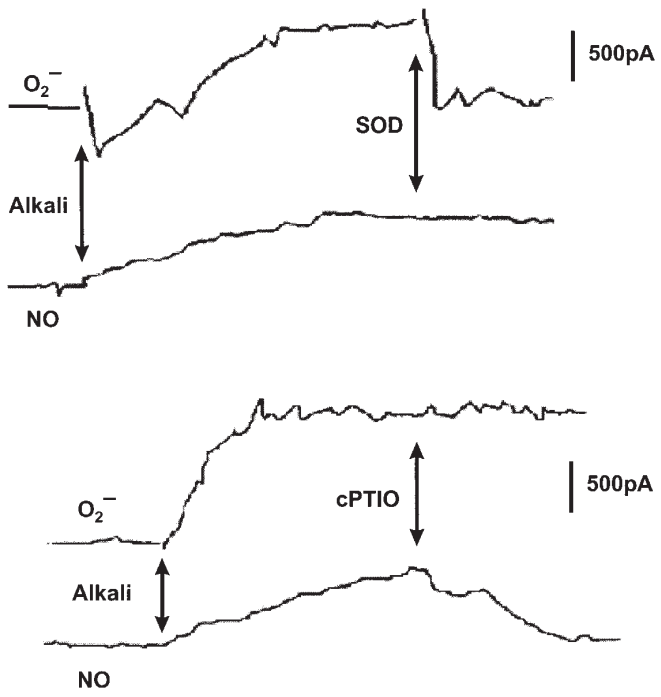


Figure 2. Representative chart recorder traces of NO and O_2^- production from mixed cell cultures following exposure to 2 M NaOH. Superoxide current was quenched by the addition of 100 U ml^{-1} SOD. Nitric oxide responses were quenched by the addition of 5 μM cPTIO.

Microglial cultures

Microglia released O_2^- transiently in response to changes of medium. They also produced an O_2^- burst in response to PMA (Data not shown).

Immunocytochemistry

Only those wells that produced a baseline response of O_2^- in response to serum withdrawal stained positive for cNOS.

Discussion

Mixed and pure astrocytic populations produced O_2^- in a Ca^{2+}/calmodulin dependent manner. This production consistently responded to NOS manipulation. It is possible that the decreased NO production resulting from NOS inhibition leaves more O_2^- to be detected at the O_2^- sensor. However, specific cNOS inhibitors decreased O_2^- detection in mixed populations with simultaneous NO detection (at levels too low to be detected at the NO electrode). A comparison of nitrite levels from astrocytic, neuronal and mixed cultures suggests NO arises predominantly from neuronal cultures via cNOS. Nitrite accumulation in astrocyte populations was minimal and independent of NOS inhibition. The observed behaviour resembles NOS uncoupling during substrate depletion which favours O_2^- production. Substrate depletion, however, is unlikely in this model. Further, addition of L-arginine had no effect on astrocytic O_2^- production. This apparently contradictory information can be explained if the coexistence of all cell population in a mixed culture is considered. L-arginine causes NO bursts from NOS in neurons. This causes a small but registerable fall in the O_2^- current as O_2^- and NO combine to form $ONOO^-$. This is additionally supported by high $[K^+]$ which i) quenched O_2^- release in mixed cultures and ii) increased nitrite accumulation in mixed and neuronal populations probably through NOS activation. High $[K^+]$ had no effect on the astrocytic cultures.

The findings presented in this work suggest a constant interplay between astrocytes and neurons that regulates NO availability. Results indicate a novel function for astrocytic cNOS in regulating extracellular O_2^- release and therefore controlling neuronal nitric oxide availability. These findings were facilitated by the use of novel electrochemical sensors that enabled the direct, real time measurement of O_2^- and NO.

Superoxide-induced nitric oxide release from cultured glial cells: possible contribution of a non-nitric oxide synthase pathway [26]

Oxidative stress has been linked to several neurological diseases including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS) [27]. The reaction of O_2^- with NO to form $ONOO^-$ may have significant implications in central nervous system (CNS) pathology as $ONOO^-$ has been shown to be toxic to neurons in culture, neuronal cell lines and glia [28]. iNOS has been shown to be expressed in astrocytes and glia from pathological samples suggesting they may be a significant source of free radicals resulting in pathology. Superoxide has been shown to be produced by neurons after stimulation of ionotropic glutamate receptors [29]. We therefore examined the effect of extracellular O_2^- generation on free radical flux in several glial cell preparations using direct, real time electrochemical measurements.

Methods

Cell culture

C6 rat glial cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 10 % FCS, penicillin and streptomycin (500 u/ml^{-1}). Primary glial cells were prepared from neonatal rat cortex as previously described [30]. From glial fibrillary acid protein (GFAP) staining it was determined that 95 % of cells in culture were astrocytes.

Generation and characterisation of SOD1 transfected C6 cell lines

Normal human SOD1 cDNA was cloned into the expression vector pCEP4 as described previously [31]. DNA was intro-

duced into C6 glia using the liposomal reagent DOSPER. Cells that successfully expressed the cloned DNA were selected for by inclusion of hygromycin in the tissue culture medium. The presence of human DNA in the glial cell line was confirmed by Western Blotting methods.

NOS activity assay and NOS isoform detection

NOS activity in cell culture samples was measured using a commercially available kit based on the conversion of [^{3}H]-arginine to [^{3}H]-citruline (Stratagene). The existence of NOS isoforms within the cell culture was assessed using RT-PCR.

Statistical analysis

Differences in free radical current observed were assessed using one way ANOVA with Dunnet's post-hoc test to assess individual groups used where appropriate.

Results

Nitric oxide is released by cultured glial cells in response to extracellular superoxide generation

Measurement of O_2^- generation in DMEM by xanthine (500 nM) and xanthine oxidase (XOD) (0.5 µM) revealed continuous radical generation over several minutes (Fig. 3A). In the absence of cells a maximal current value of 3650 ± 86.6 pA was recorded. In the presence of untransfected C6 cells this current decreased to 758 ± 76.4 pA (an 80 % decrease, n = 3) which equated to O_2^- concentrations of 14.5 ± 0.34 nM in control wells as compared to 3 ± 0.3 nM in wells containing cells. The quenching of O_2^- current in the presence of cells could be attributed to the cellular antioxidant capacity of the culture. Generation of O_2^- in the presence of C6 cells also causes a concomitant rise in NO detection (Fig. 3B). A current response of 120 pA equated to a NO concentration of 20 nM. This procedure was repeated for primary glial cells. A range of XOD concentrations from 0.1-1 µM were used. Figure 4 shows that NO was readily detected at every concentration of XOD used. Maximal NO was elicited by 0.5 µM XOD beyond which the NO response was saturated.

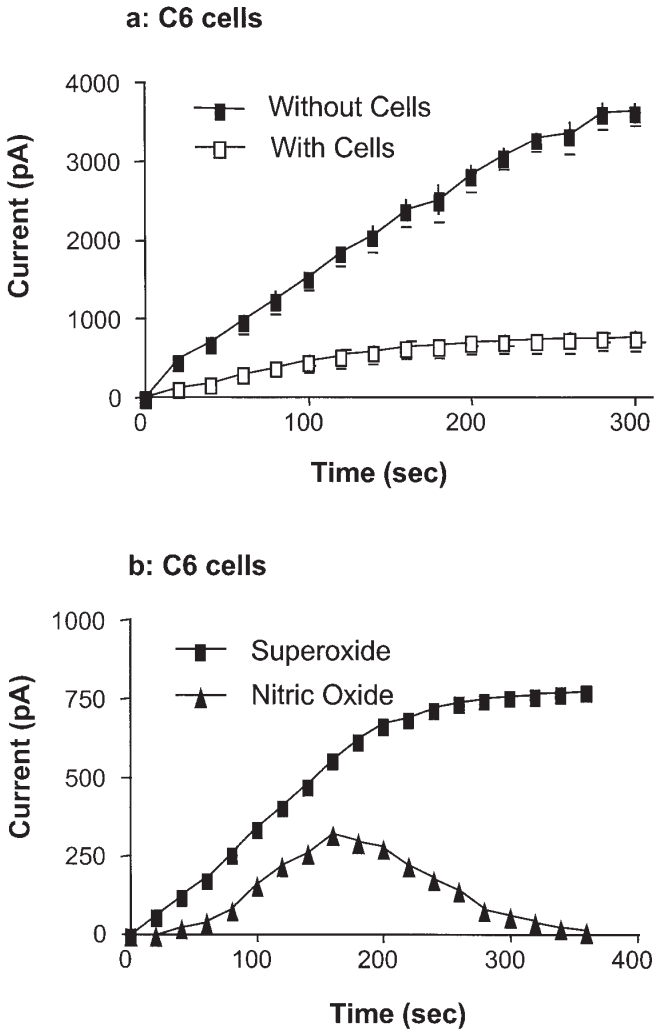


Figure 3. Superoxide induced nitric oxide release from C6 cells. A) Comparison of O_2^- calibration curves in the absence and presence of C6 cells. Superoxide generated by 500 nM XOD and 0.5 mM xanthine. **B)** Generation of O_2^- in the presence of C6 cells produced a concomitant NO release. Readings are expressed as the mean ± SEM (n = 3).

NO release is blocked by O_2^- scavengers or by over-expression of SOD1

The cell permeable SOD1 mimetic MnTBAP (50-250 µM) decreased recorded O_2^- current values in a dose dependent manner. NO currents were also simultaneously decreased. MnTBAP is selective for O_2^- and $ONOO^-$ but does not scavenge NO [32]. Confirmation that the observed NO signal was due to variations in intracellular O_2^- levels was achieved by observations made with C6 cells that overexpressed SOD1. O_2^- generated in the presence of C6 cells that overexpressed SOD1 led to a significantly decreased O_2^- current and also a smaller NO current compared to currents seen in the presence of untransfected C6 cells. Cells expressing the pCEP4 expression vector alone responded as untransfected C6 cells.

Authenticity of observed NO release and lack of detectable NOS

The NO scavenger cPTIO (100-500 µM) caused a concentration dependent decrease in the observed NO current without affecting recorded O_2^- levels. This suggests NO is generated as a consequence of extracellular O_2^- generation. However, no significant NOS activity was detected in basal, unstimulated C6 or primary glial cells. Positive control rat cerebellum showed readily detectable NOS activity. Further, no measurable amounts of mRNA for any NOS isoform could be found using RT-PCR.

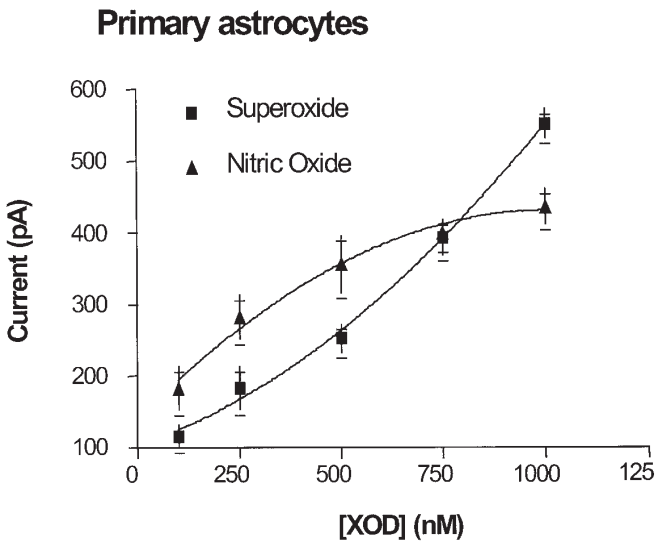


Figure 4. NO production by primary astrocytes in response to extracellular superoxide. Readings are expressed as the mean ± SEM (*n* = 3).

Discussion

Findings in this study suggest that O_2^- can stimulate the release of NO from glia. This is potentially important information as O_2^- and NO readily combine to produce cytotoxic $ONOO^-$. Superoxide can be produced by neurons in response to activation of cell surface glutamate receptors. If this O_2^- release in turn causes NO production by surrounding glia, $ONOO^-$ may play an important role in neuropatholoy. NO production by glia is critically dependent on O_2^- as demonstrated by the inclusion of SOD mimetics or the overexpression of SOD1. Although NO production does seem to be authentic (due to selective scavenging by cPTIO) its source does not appear to be NOS. Although NO production in combination with O_2^- does result in cytotoxic $ONOO^-$ formation, NO can also act as a chain terminator of lipid peroxidation. Further work is required to delineate the precise molecular mechanisms involved in this newly observed superoxide-dependent NO release from glia.

In summary, both C6 rat glioma cells and primary astrocytes have been shown to possess a mechanism in which O_2^- and NO may be simultaneously produced and combine to form highly oxidative products such as $ONOO^-$ which are thought to be involved in numerous neuropathological processes.

Development and characterisation of a glutamate sensitive motor neuron cell line [33]

Abnormal activation of glutamate receptors has been linked to neuronal damage and cell death in the CNS. "Excitotoxicity" resulting from the over-stimulation of glutamate receptors is a major focus of research into the aetiology of motor neuron disease (MND/ALS) [34]. Glutamate receptor stimulation may cause free radical release which could be central to the mechanism of excitotoxicity. Current *in vitro* studies rely on primary motor neuron cultures which are limited by several factors including the short life-span of these cultures *in vitro*, the heterogeneity of such cultures and the embryonic nature of *in vitro* neuronal cultures which may not be represent mature characteristics. The development of a permanent cell line would address many of these problems as well as providing abundant material for experimentation. The NSC-34 cell line is of motor neuron origin and may provide a useful tool for the study of motor neuron dysfunction. However, they do not naturally express glutamate receptors.

This study has two main objectives: i) determine whether NSC-34 cells can be induced to express functional glutamate receptors and toxic effects following exposure to glutamate receptor agonists; ii) examine in real-time any free radical flux following glutamatergic activation of NSC-34 cells *in vitro*.

Methods

Cell culture

NSC-34 cells were grown in DMEM containing 10 % FCS [35]. Cellular maturation was enhanced by changing this media for 1:1 DMEM + Hams F12 (containing 1 % FCS, 1 % penicillin/steptomycin and 1 % modified Eagles medium non essential amino acids) when the cells reached confluence. Significant cell death resulted after 48 h. However the remaining sub-population could be cultured in this new medium to confluence. These differentiated cells ($NSC\text{-}34_D$) were then characterised *via* immunocytochemistry.

Immunocytochemistry (ICC)

Differentiated cells were analysed for their expression of NMDAR1, NMDAR2A/B, GluR1, GluR2/3, GluR4, GluR5/6, and KA2 receptor subgroups by ICC.

Real time radical measurement

O_2^- and NO electrodes were prepared and calibrated as previously described [18]. NSC-34D cells were allowed to grow in 24 well tissue culture plates for 1 week before experimentation. If required, the cells were preincubated with the glutamate receptor agonists (+)-5-methyl-10,11-dihydro-5H-dibenzol [a,d]cyclohepten-5,10-imine maleate (MK801, 10 μM) and/or 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoaxaline-7-sulphonamide (NBQX, 1 μM) for 15 min at room temperature. Cells were then stimulated to produce free radicals by the addition of 1 mM glutamate.

Data analysis

One way ANOVA with Dunnet's post-hoc test to assess individual groups where appropriate.

Results

Expression of glutamate receptor sub-units by NSC-34D cells

NSC-34_D cells showed expression of NMDAR1, NMDAR2A/B,GluR1, GluR2/3, GluR4, GluR5/6, and KA2 glutamate receptor subgroups when analysed by ICC.

Free radical generation following glutamate receptor activation of NSC-34D

O_2^- but no NO was observed when NSC-34_D cells were stimulated with 1 mM glutamate (Fig. 5). O_2^- production was prevented by the non-competitive NMDA receptor antagonist MK801 (1 μM) (Fig. 5). The O_2^- response was abolished in the presence of 500 U/ml SOD. No O_2^- was recorded when the cells were bathed in Ca^{2+}-free medium. Repeated 1 mM glutamate additions produced repeated but sequentially diminishing O_2^- responses until no response was seen on the fifth addition. However, when cells were pretreated with 100 μM cyclothiazide to prevent desensitisation of AMPA receptors, O_2^- was continuously seen on each successive glutamate addition. Activation of non-NMDA receptors using 10 μM AMPA in the presence of 100 μM cyclothiazide resulted in NO release from NSC-34_D cells (Fig. 6).

Discussion

This study has shown that under appropriate culture conditions NSC-34 cells can differentiate to produce cells that exhibit many motor neuron-like properties and that respond to glutamate stimulation. These differentiated cells expressed glutamate receptor subunits. Glutamate exposure was able to induce O_2^- production from differentiated cells. Direct action of glutamate at cell surface receptors was implicated by the finding that O_2^- production was blocked by the presence of 1 μM MK801. This represents the first real time demonstration of O_2^- production by a motor neuron-like cell line in response to glutamate. NO could only be elicited in the presence of cyclothiazide which is known to prevent AMPA receptor desensitisation.

The motor neuron origins of this new cell line make it, in combination with direct, real time free radical measurement, a powerful tool for the further investigation of motor neuron injury.

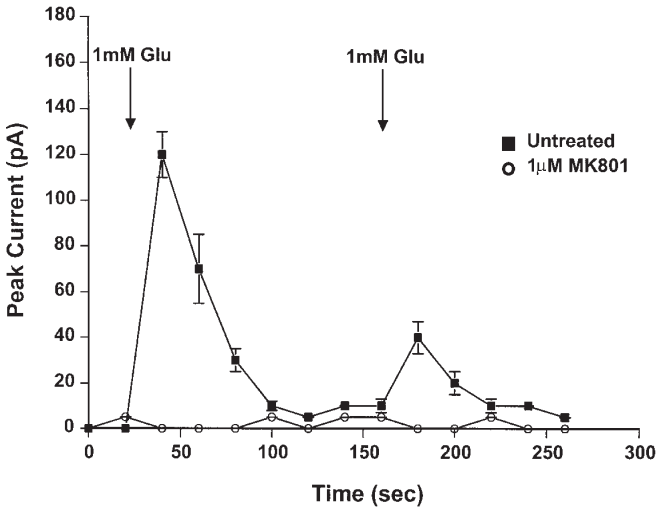


Figure 5. Superoxide production by NSC34_D cells in response to glutamate was blocked by preincubation with 1 μM MK801. Readings are expressed as the mean ± SEM (*n* = 3).

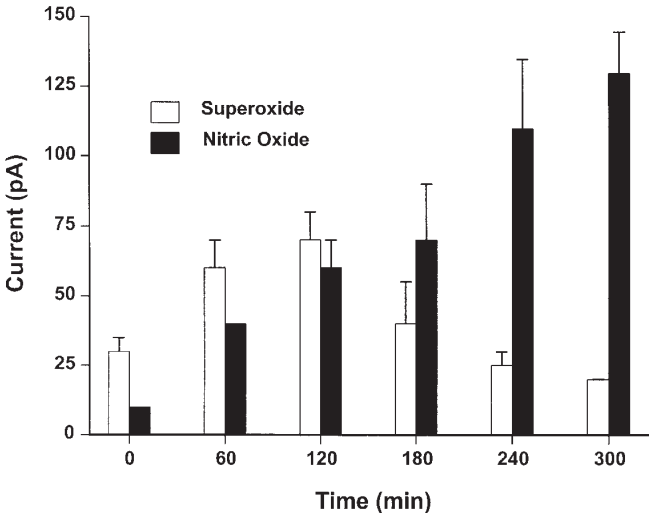


Figure 6. Superoxide and nitric oxide production by NSC34_D cells preincubated with 100mM cyclothiazide in response to 10 μM AMPA. Readings are expressed as the mean ± SEM (*n* = 3).

Effects of sumatriptan on nitric oxide and superoxide balance during glyceryl trinitrate infusion in the rat: implications for antimigraine mechanisms [36]

A hyperexcitable brain is thought to form the basis of migraine pathophysiology [37]. Propagation of cortical excitability is thought to be by spreading depression of Leao (SD) [38]. SD causes marked NO release. In turn NO release is known to mediate onset and maintenance of migraine headache. It is also known that infusion of the NO donor glyceryl trinitrate (GTN) into patients who suffer from migraine causes a delayed migraine attack several hours

after termination of NO donor infusion [39]. The mechanism for this prolonged and sustained cortical NO release is not understood. The course of O_2^- release during NO production is not known. It has been proposed that O_2^- may be released as a homeostatic mechanism for controlling NO within the cortex [40]. This work aims to examine the interrelationship between O_2^- and NO during GTN perfusion at clinically relevant doses.

Methods

Surgical procedure

Cranial wells were formed in male Sprague-Dawley rats using Home Office approved techniques.

Free radical measurement

The O_2^- electrode was prepared and calibrated as previously described. Under conditions used in this set of experiments a maximum *in vitro* O_2^- sensitivity of 5.7 μM nA^{-1} was achieved. A Model NO-501 Pt/Ir alloy NO sensor was used (200 μm tip) (Intermedical). Calibration of the electrode *in vitro* gave a NO sensitivity of 1 μM nA^{-1}. Both electrodes were placed directly above the parietal cortex surface in the aqueous layer which was between the cortex parenchyma and mineral oil which filled the crainiotomy.

Cortical O_2^- and NO release during GTN infusion: effect of sumatriptan vs. vehicle

Rats were instrumented for O_2^- and NO recording and then pre-dosed with either vehicle (saline, 1 ml/kg^{-1} i.v.) or the migraine treatment sumatriptan (300 $\mu g/kg^{-1}$ i.v.) (n = 3 per group), 15 min prior to GTN infusion in each group. Both groups were then infused with GTN at 2 $\mu g/kg^{-1}/min^{-1}$ for 30 min and then recordings were continued for a further 30 min.

Results

Corticol O_2^- and NO release during GTN infusion: effect of sumatriptan

Saline had no effect on either O_2^- or NO levels *vs.* baseline throughout the pre-treatment period. Initiation of GTN infusion caused an initial NO peak followed by a gradual rise to a maximum of 141 ± 13 % of baseline current (n = 3) 17 min after initiation of GTN infusion. NO levels remained elevated until the conclusion of the experiment (Fig. 7). O_2^- levels showed a close but inverse relationship to NO in all animals tested. Elevated NO levels suppressed O_2^- production and *vice versa*. During pre-treatment with sumatriptan the NO baseline *fell* to 57 ± 13 % of its original baseline level (Fig. 8a). Subsequent infusion with GTN produced no cortical NO release. The recorded current value remained below baseline for the duration of the experiment. Sumatriptan caused O_2^- production following GTN infusion which continued after infusion had stooped eventually reaching a current value 196 ± 4 % of baseline (Fig. 8b).

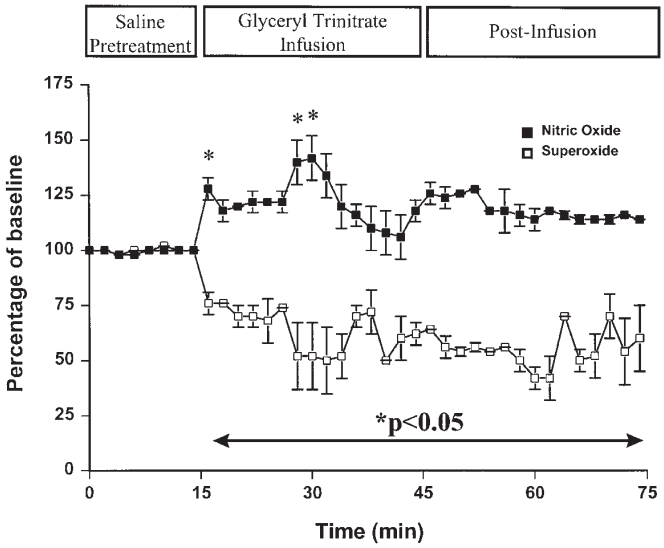
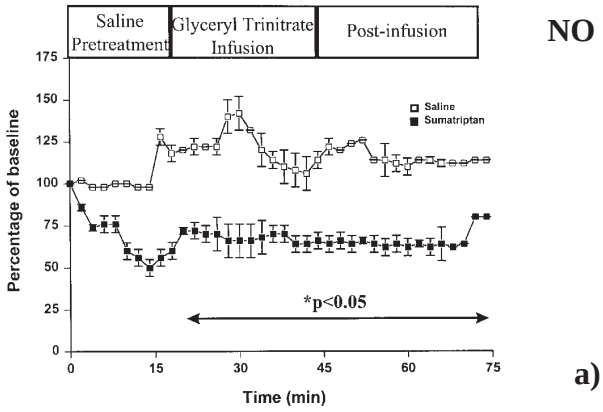


Figure 7. Cortical O_2^- and NO release during GTN infusion. Readings are expressed as the mean ± SEM (n = 3).



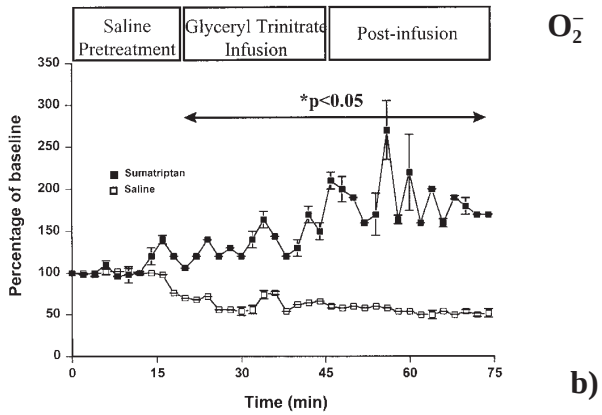


Figure 8. Cortical O_2^- (A) and NO (B) release following GTN infusion in response to sumatriptan pretreatment. Readings are expressed as the mean ± SEM (n = 3).

Sumatriptan peak effect on each free radical corresponded to an increase in O_2^- production of 105 ± 14 nM (n = 3) and a decrease in NO of 129 ± 18 nM (n = 3) from baseline.

Discussion

These findings suggest that migraine headache induced by GTN infusion will be predominantly NO mediated with little or no contribution from O_2^- or $ONOO^-$. Sumatriptan, whilst capable of limiting NO production, either by direct scavenging or through alteration in cellular redox states, also causes elevated O_2^- production. Although it is unknown whether these results would be mirrored in human patients, the consequences of raised O_2^- production in a population at risk from subsequent cerebral infarction would merit further study.

In summary, the three principle findings of this study are i) the close interrelationship between O_2^- and NO has been described *in vivo* using novel electrochemical technology; ii) GTN infusion at concentrations used to induce migraine in patients causes an increase in cortical NO production with a corresponding suppression of O_2^- levels and iii) sumatriptan pre-treatment reverses this relationship with a net increase in O_2^- and decrease in NO compared to basal levels.

Alpha-melanocyte stimulating hormone modulates nitric oxide production in melanocytes [41]

It has recently been observed that α-MSH protects melanocytes from oxidative stress [42,43]. The mechanism of this protective effect could involve tyrosinase, an enzyme involved in melanin production, but precise details are as yet unclear. Compared to other cells in the skin (*e.g.* keratinocytes and fibroblasts) melanocytes are particularly vulnerable to oxidative attack [43]. Although the reason for this is not known it could relate to their ability to generate NO. This highly reactive molecule has many physiological effects and may be linked to numerous pathophysiological actions through its ability to readily combine with O_2^- to form $ONOO^-$ which in turn results in the generation of the highly cytotoxic hydroxyl radical ($OH^\bullet$). In preliminary experiments it has been observed that melanocytes produce NO in response to ultraviolet (UV) irradiation and lipopolysaccharide (LPS). This present work examines the ability of α-MSH to modulate NO production by melanocytes in response to these stimuli.

Methods

Cell culture

Human epidermal melanocytes and keratinocytes were isolated from samples of human skin into supplimented MCDB medium (Sigma) as previously described [44]. Human fibroblasts were cultured in Dulbecco's medium (Gibco BRL) containing 10 % foetal calf serum (ICN Flow), penicillin (50 IU/ml) and streptomycin (50 μg/ml) (ICN Flow).

NO measurement

Cells were seeded into 35 mm tissue culture dishes and incubated in the presence or absence of α-MSH (Ciba-Geigy Ltd.) at concentrations ranging from 10^{-11}-10^{-7} M, LPS (15-120 ng/ml) or α-MSH and LPS together for varying times up to 72 h. The cells were then stimulated to produce NO with either 60 ng/ml LPS or UV irradiation. Subsequent NO production was recorded using the WPI ISO-NO electrochemical sensor which was positioned directly over the cellular monolayer before application of the chosen stimulus. UV irradiation was provided by a Helarium 40W lamp (Wolff B1.01) which emitted predominantly in the UVB range. Where appropriate attempts were made to quench any NO signal with 25 mM aminoguanidine.

Immunocytochemical staining for NOS

Cells incubated for 6h in the presence of 10^{-9} M α-MSH or 60 ng/ml LPS were washed three times in PBS before fixing in 1 % glutaraldehyde for 30 min at room temperature. Immunostaining was achieved using polyclonal antibodies to iNOS (Affinity Laboratories, UK) and a standard avidin-biotin horseradish peroxidase technique.

Western blotting

Further evidence of iNOS expression was sought using the procedure outline previously [41].

Melanin quantification

Cells were solubilised in 1 M NaOH and absorbance values spectrophotometrically compared to synthetic melanin.

Results

The addition of LPS to melanocytes produced immediate, dose dependent NO production. From these results a concentration of 60 ng/ml was chosen as being optimal for subsequent work. Pre-incubation with varying concentrations of α-MSH(10^{-11}-10^{-9} M) for 3, 6, 24 and 72 h prior to LPS stimulation produced concentration dependent increases in NO production (Fig. 9). However, concentrations of α-MSH greater than 10^{-9} M actively inhibited NO production (Fig. 9). Pre-incubation with 20-125 ng/ml LPS for 3, 6, 24 and 72 h prior to LPS stimulation also produced concentration dependent increases in NO production. However, NO production in the presence of both α-MSH and LPS was substantially less than that seen in the presence of either α-MSH or LPS. This data suggested that α-MSH and LPS are mutually antagonistic in their effect on NO production in melanocytes.

From Western blotting data, cells exposed to 10^{-9} M α-MSH for 6 or 24 h were found to express more iNOS than untreated control cells. This was also observed for LPS treated cells (60 ng/ml). However, cells exposed to both

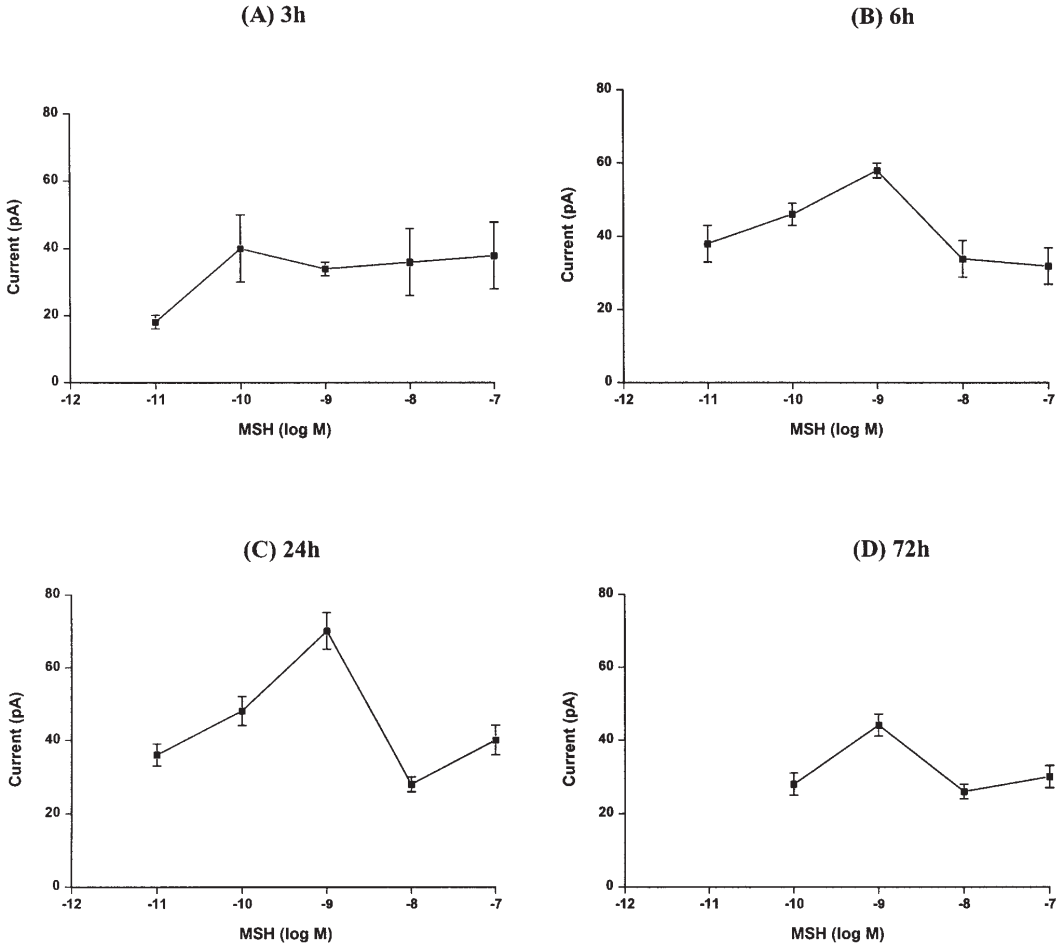


Figure 9. Effect of α-MSH incubation on NO production by LPS stimulated cells. Readings are expressed as the mean ± SEM (*n* = 3).

α-MSH and LPS showed lower levels of iNOS expression compared with those exposed to LPS alone. These findings were confirmed by immunostaining.

It has been suggested that NO increases melanin formation in melanocytes [45]. This was investigated in human melanocytes by exposing them for three days to the NO donor sodium nitroprusside (SNP). From spectrophotometric measurements it was found that those cells exposed to the NO donor did produce more melanin than untreated controls. This clearly underlines the importance of NO in melanogenesis.

Having established the importance of NO in melanogenesis, the next step was to investigate whether UV irradiation, a known stimulus of melanogenesis, caused NO production and if this production could be modulated by α-MSH. From figure 10 it can be seen that UV dosages between 20-100 mJcm^2 caused a dose dependent increase in NO production by human melanocytes. This response was enhanced by pre-incubation of the cells with α-MSH. UV irradiation also evoked NO production from human fibroblasts and keratinocytes but to a far lesser degree.

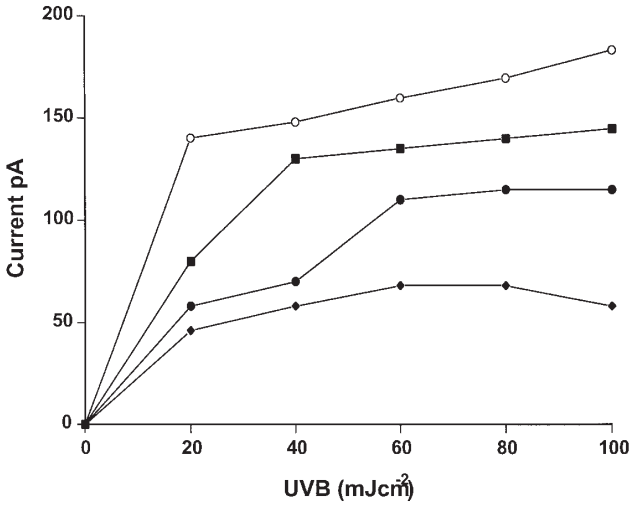


Figure 10. Effect of α-MSH on NO production by UV stimulated melanocytes. ○ Control, ■ 10^{-9} M α-MSH, ● 10^{-8} M α-MSH, ◆ 10^{-7} M α-MSH. These results are representative of three seperate experiments.

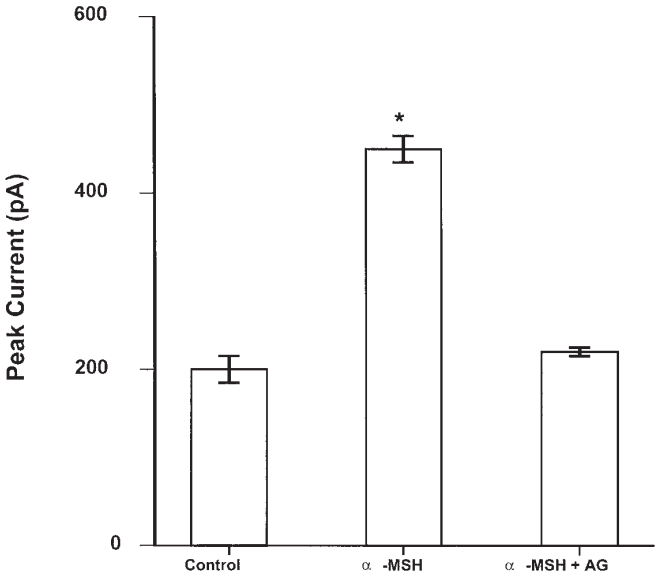


Figure 11. Effect of iNOS inhibitor on NO production by human melanocytes. The iNOS inhibitor aminoguanidine (AG) (25 mM) was added to cells along with 10^{-8} M α-MSH 24 h prior to UVB irradiation. Readings are expressed as the mean ± SEM (*n* = 3).

NO production from either of these cell types was unaffected by α-MSH.

Melanocytes required 24 h pre-incubation with α-MSH before any affect on NO generation was observed. This strongly suggested the involvement of iNOS. The presence of aminoguanidine eliminated the observed NO current (Fig. 11).

Discussion

These results clearly suggest that α-MSH can regulate NO production in human melanocytes. It has been shown that NO exposure causes these cells to produce melanin. Given that the effects on NO production by α-MSH were maximal between 6-24 h after addition it would be reasonable to suggest that iNOS is involved in this mechanism rather than a constitutive isoform. This theory is supported by the NO inhibition exhibited in the presence of aminoguanidine, a weak but selective inhibitor of iNOS. Western blotting and immunocytochemistry confirmed the presence of iNOS in α-MSH and LPS treated cells. No other isoforms could be detected when appropriate antibodies were used.

This work further suggests that α-MSH and LPS are mutually antagonistic in their effects on NO production in human melanocytes. The mechanism behind this antagonism is not yet understood, however, it does appear that the action of α-MSH on melanocytes is dependent on whether another iNOS inducing agent is present. The exact role of NO in melanogenesis is still unclear. However, the ability of melanocytes to produce NO in considerably greater quantities than keratinocytes or fibroblasts suggests NO may be an autocrine factor to regulate melanogenesis and further may mediate the melanogenic action of α-MSH on the cell.

Overall summary and future developments

In conclusion it can be seen that through the application of novel technology that facilitates the direct, real-time measurement of free radicals new insight into the importance of these species in a variety of cellular models has been gained. Such technology not only gives better understanding of pathological processes but may also lead to the development of novel pharmaceutical compounds. The ability of new compounds to modulate free radical release from cellular models may form the basis of new high throughput drug screening programs.

Further development of these electrode systems will, it is envisaged, lead to the production of miniaturised electrodes suitable for implantation and direct *in vivo* measurement in animal models of reperfusion injury. Recently reported work details the use of the O_2^- electrode in ischaemic/reperfusion studies in the femoral artery of rat [46]. There are also multidisciplinary collaborative programmes in place to develop biocompatible implantable sensors for the simultaneous, real-time measurement of superoxide, nitric oxide and glutamate in studies of focal cerebral ischaemia. With the aid of these new technologies it is envisaged that further understanding of free radical related pathologies can be gained.

Acknowledgements

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