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Indomethacin Reduces Lipid Peroxidation in Rat Brain Homogenate by Binding Fe^{2+}

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One of the hallmarks of Alzheimer's disease (AD) is the progressive degeneration of cholinergic neurons in the cerebral cortex and hippocampus. It is generally accepted that this neuronal degeneration is due to free-radical-induced damage. These free radicals attack vital structural components of the neurons. This implies that agents that reduce free radical generation could potentially delay the progression of AD. Free radical generation in the brain is assisted by the presence of iron, required by the Fenton reaction. Thus, agents that reduce iron availability for this reaction could potentially reduce free radical formation. Since non steroidal anti-inflammatory drugs (NSAIDS) have been shown to reduce the severity of AD, we investigated the possible mechanism by which indomethacin could afford neuroprotection. Our results show that indomethacin (1 mM) is able to reduce the iron-induced rise in lipid peroxidation in rat brain homogenates. In addition, our NMR data indicate that indomethacin binds the Fe^{2+}/Fe^{3+} ion. This was confirmed by a study using UV/Vis spectrophotometry. The results imply that indomethacin provides a neuroprotective effect by binding to iron and thus making it unavailable for free radical production.

Key words: Indomethacin; free radical; iron; lipid peroxidation; neurodegeneration; Alzheimer's disease (AD).

INTRODUCTION

Alzheimer's disease (AD) is characterised by a progressive degeneration of cholinergic neurons in the cerebral cortex and hippocampus leading to loss of cognitive function (Flynn and Ranno, 1999). The prevalence of AD increases with age and doubles every 5 years after the age of 65 (Daya, 1999; Flynn and Ranno, 1999). Several attempts have been made to explain the underlying basis of this disease. Of all the hypotheses put forward, it is generally accepted that the neuronal degeneration observed in AD is due to free-radical-induced damage (Frolich and Rierder, 1995).Thus, a number of studies have been done to assess the role of free radicals in AD. There is a large body of evidence to suggest that free radicals play a role in the development of AD. For example, the major constituent of senile plaques, β-amyloid protein, is theorised to possess a direct neurotoxic action that is related to calcium overload and subsequent free radical generation (Lockhart *et al*., 1994). We now know that oxidative stress leads to formation of oxygen radicals, which in turn attack vital structural components of the neurons resulting in neurodegeneration (Southgate and Daya,

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1999). It, therefore, appears that agents that reduce free radical generation could potentially delay the onset of AD. For example, the use of α-tocopherol has been shown to be effective in delaying functional deterioration in moderately impaired AD patients (Sano *et al*., 1997).

The brain is particularly vulnerable to free-radical-induced damage, especially if one considers the high oxygen utilisation by the brain (Daya, 1999). Free radical generation in the brain is further assisted by the presence of large amounts of iron (Braughler and Hall, 1989; Halliwell, 1992), required by the Fenton reaction (Fahn and Cohen, 1992). The role of iron in free radical and lipid peroxidation reactions has been studied extensively (Fahn and Cohen, 1992) and its role in this regard is widely accepted (Fahn and Cohen, 1992). Free iron or chelates of iron are involved in radical reactions at a number of different levels (Halliwell, 1992). The autoxidation of Fe^{2+} results in the formation of O_2^-. Conversely, the reduction of Fe^{3+} to Fe^{2+} by O_2^- also occurs and competes with the dismutation of O_2^- for available O_2^-. Thus in theory, Fe^{2+} autoxidation could result in the redox cycling of iron due to the reaction of O_2^- produced with Fe^{3+}. Furthermore, Fe^{2+} is also oxidised in the presence of H_2O_2 (Fenton's reagent) to form hydroxyl radicals ($^{\cdot}OH$) or a ferryl ion (Fe^{3+}-OH). Both these are very potent oxidants and will react with a wide range of biological substrates such as lipids, DNA, and protein (Halliwell, 1992; Ottino and Duncan, 1997).

Recent studies (Mcgeer and Rogers, 1992; Rich *et al*., 1995; Rogers *et al*., 1993) have shown that indomethacin and other nonsteroidal anti-inflammatory drugs (NSAIDS) appear to protect mild to moderately impaired AD patients from cognitive decline. While these studies imply that NSAIDS are neuroprotective in AD, besides the prototypic NSAIDS, namely the salyicylates serving as a trap for hydroxyl radicals, there are currently no known reports explaining the underlying mechanism(s) involved in the neuroprotective effects of the NSAIDS. In the present study we examined the possible underlying neuroprotective effects of indomethacin.

MATERIALS AND METHODS

Chemicals and Reagents

All reagents used were of analytical grade. Indomethacin, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (98%), and butylated hydroxytoluene (BHT) were purchased from the Sigma Chemical, St Louis, MO, USA. Methanol (HPLC grade), anhydrous ferric chloride and ferric sulphate were purchased from BDH Laboratory Supplies, Poole, England. Trichloroacetic acid (TCA), resorcinol, ferrous sulphate, and glacial acetic acid were purchased from Saarchem (PTY) Ltd., Krugersdorp, South Africa. Isolute J C_{18} solid phase extraction (SPE) columns were obtained from International Sorbent Technology, Mid Glamorgan, UK.

Animals

Adult male rats of the Wistar strain, weighing between 250–300 g were used. The animals were housed in a controlled environment with a 12 h light:dark cycle, and were given access to standard laboratory chow and water *ad libitum*. Protocols for the experiments were approved by the Rhodes University Ethics Committee.

Instrumentation

Samples were analyzed on a modular, isocratic high performance liquid chromatographic (HPLC) system. The chromatographic system used consisted of a Spectraphysics Iso Chrom LC Pump, a Linear UVIS 200 Detector, and a Rikadenki Recorder. Samples were introduced into the system using a Rheodyne fixed loop injector, fitted with a 20 μL loop. An N-EVAP analytical evaporator was used to evaporate the methanol.

Chromatographic Conditions

Separation was achieved using a C_{18} (Waters Spherisorb, 5 μm, 250 H 4.6 mm i.d.) column, fitted with an in-line precolumn filter. The mobile phase composition for the analysis was 14% methanol in Milli-Q water and was degassed using a 0.45 μm membrane filter prior to use. The mobile phase flow rate was 1.2 mL/min. The detector sensitivity was set at 0.1 AUFS (absorbance units full scale) and the thiobarbituric acid-malondialdehyde (TBA-MDA) complex was detected at 532 nm. Resorcinol (0.1 mg/mL in water) was used as an external standard.

Homogenate Preparation

Rats were killed by cervical dislocation and the brains were rapidly removed and homogenised (10% w/v) with 0.1 M phosphate buffered saline (PBS), pH 7.4. The homogenate was frozen in liquid nitrogen and stored at -70°C until needed. All samples were used within 2 days of homogenate preparation.

Lipid Peroxidation Determination

The method used in this experiment was a modification of the method used by Anoopkumar-Dukie *et al.* (2001). Homogenate (1 mL) containing 1 mM Ascorbate, 100 μM EDTA, 5 mM Iron Sulphate, and H_2O_2 in the absence and presence of indomethacin (0, 0.5, 1 mM) was incubated in an oscillating water bath for 1 h at 37°C. At the end of the incubation period, 0.5 mL BHT (0.5 mg/mL in methanol) and 1 mL TCA (15% in water) were added to the mixture. The tubes were sealed and incubated for 15 min in a boiling water bath to release protein-bound MDA. To avoid adsorption of MDA onto insoluble protein, the samples were cooled and centrifuged at 2000 g for 15 min. Following centrifugation, 1 mL of the protein free supernatant was removed from each tube and a 1 mL aliquot of TBA (0.33% in water) was added to this fraction. The tubes were sealed and incubated in a boiling water bath at acidic pH for 30 min.

After cooling, TBA-MDA was separated from other possible interfering thiobarbituric acid-reactive substances (TBA-RS) using a Isolute J C_{18} solid phase extraction (SPE) column that was prewashed with 2 mL of methanol followed by 2 mL distilled water. The sample (1 mL) was loaded onto the column that was subsequently washed with 2 mL distilled water. The TBA-MDA complex was eluted with 1 mL methanol. The methanol

was then evaporated using an N-EVAP analytical evaporator at 65°C under a gentle stream of nitrogen. The residue was dissolved in distilled water (0.5 mL) containing 0.1 mg/mL resorcinol. These samples were analysed by HPLC as described above. The MDA levels were obtained from a calibration curve generated using 1,1,3,3-tetramethoxypropane in the same way described above. The ratio of the peak height of TBA-MDA to the peak height of resorcinol (external standard) was plotted against the concentration of MDA in the complex injected. Final results are expressed as nmoles/mg tissue.

UV/Vis Analysis

Distilled deionised water was utilized to make up the standard 10 mM solution of iron, whilst a 50:50 ratio of ethanol to water was used to dissolve the indomethacin, of an equimolar concentration to the iron. The UV/Vis spectra were monitored with a Cary 500 UV/Vis/NIR spectrophotometer. Spectra were recorded immediately after addition of indomethacin solution to the iron solutions, thereafter periodically for 48 h.

NMR Analysis

The ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded using standard pulse sequences on a Bruker Avance 400 spectrometer. Chemical shifts are reported in ppm and referenced to residual undeuterated MeOH resonances (δ 3.30 and 49.04 for ^{1}H and ^{13}C data, respectively).

Pure indomethacin: ^{1}H NMR (400 MHz) δ 2.26 (3H, s, H_3-17), 3.64 (2H, s, H_2-18), 3.77 (3H, s, H_3-20), 4.79 (1H, br s, O*H*), 6.62 (1H, dd, $J = 2.5$, 9.1 Hz, H-7), 6.87 (1H, d, $J = 8.8$ Hz, H-8), 6.97 (1H, d, $J = 2.5$ Hz, H-5), 7.49 (2H, dt, $J = 2.3$, 8.6 Hz, H-13, H-15), 7.62 (2H, dt, $J = 2.3$, 8.8 Hz, H-12, H-16).

Statistical Analysis

The results were analysed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p < 0.05$.

RESULTS

The Effect of Indomethacin on Lipid Peroxidation

Exposure of whole rat brain homogenate to 5 mM of ferric sulphate increased lipid peroxidation (Fig. 1). As shown in Fig. 1, iron (1 mM) significantly increased lipid peroxidation compared to the control (a). Furthermore, indomethacin significantly reduced this increase but only at a concentration of 1 mM.

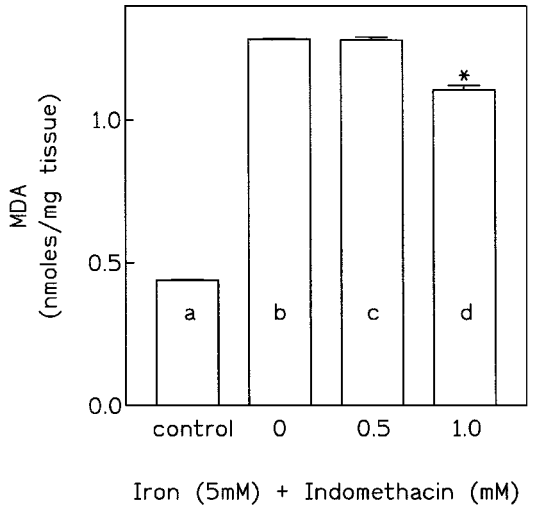


Figure 1. Effect of indomethacin on iron-induced increase in lipid peroxidation in whole rat brain homogenate. Each bar represents the mean ± SEM of triplicate determinations. $^{*}(p < 0.05)$ b vs. d; Student-Newman-Keuls test.

NMR Studies

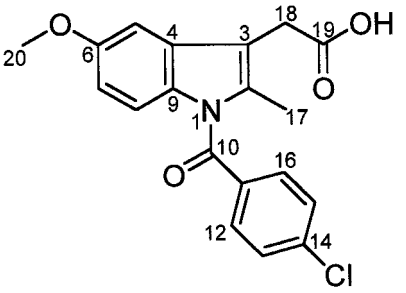
The ^{13}C NMR data for pure indomethacin and mixtures of indomethacin and Fe^{2+} or Fe^{3+} are given in Table 1. ^{1}H and ^{13}C NMR data for pure indomethacin were assigned with the aid of standard 2D NMR experiments, namely an HMQC and an HMBC experiment. Prominent two- and three-bond HMBC correlations from the methylene singlet at δ 3.64 (H_2-18) to the carbonyl carbon resonances of C-19, C-3, C-4, and C-2 delineated the acetic acid moiety at C-3, while a three-bond HMBC correlation from a 2H aromatic proton doublet at δ 7.62 (H-12 and H-16) to C-10 positioned the latter carbonyl carbon in the *p*-chlorobenzoyl substituent of indomethacin.

The ^{13}C NMR spectra for indomethacin with Fe^{2+} and for indomethacin with Fe^{3+} showed significant broadening, splitting, and slight shielding of the carbon resonances due to the paramagnetic nature of the Fe ions and also the coupling of carbon and Fe atoms. However, a comparison of the ^{13}C NMR data (Table 1) for indomethacin, indomethacin/Fe^{2+}, and indomethacin/Fe^{3+} revealed noticeable differences in the chemical shifts for C-19 (δ 174.9, 172.7, and 171.8, respectively) and C-3 (δ 114.6, 113.3, and 112.4, respectively), and excessive broadening of the C-18 and C-3 resonances, which were also of extremely low intensity. The data are compatible with the binding of the Fe^{2+}/Fe^{3+} ion to the acetic acid grouping at C-3. With the exception of C-19, the carbon resonances for indomethacin/Fe^{3+} are shifted by no more than 0.6 ppm with respect to those for pure indomethacin. Greater differences are noticeable between the carbon chemical shifts for the proposed indomethacin/Fe^{2+} versus the indomethacin/Fe^{3+} complex (referenced against pure indomethacin). The greater chemical shifts for the indomethacin/Fe^{2+} complex indicates indomethacin's binding preference for Fe^{2+} over Fe^{3+}.

Table 1. ^{13}C NMR (CD_3OD, 100 MHz) Data for Indomethacin and Indomethacin Complexes with Iron(II) and Iron(III)

	Compound [δ_C (multiplicities)[a]]		
Carbon	Indomethacin	Indo/Fe^{3+}	Indo/Fe^{2+}
2	136.9 (s)	136.4 (s)	135.2 (s)
3	114.6 (s)	113.3 (s)	112.4 (s)
4	132.2 (s)	131.7 (s)	130.4 (s)
5	102.5 (d)	102.2 (d)	100.9 (d)
6	157.6 (s)	157.0 (s)	155.8 (s)
7	112.7 (d)	112.3 (d)	111.0 (d)
8	115.9 (d)	115.5 (d)	114.2 (d)
9	132.2 (s)	131.7 (s)	130.4 (s)
10	170.0 (s)	170.0 (s)	168.2 (s)
11	140.1 (s)	139.6 (s)	138.4 (s)
12	132.3 (d)	132.1 (d)	130.8 (d)
13	130.2 (d)	130.1 (d)	128.7 (d)
14	135.7 (s)	135.2 (s)	134.0 (s)
15	130.2 (d)	130.1 (d)	128.7 (d)
16	132.3 (d)	132.1 (d)	130.8 (d)
17	13.5 (q)	13.5 (q)	12.0 (q)
18	30.6 (t)	30.5 (t)	29.3 (t)
19	174.9 (s)	172.7 (s)	171.8 (s)
20	56.2 (q)	56.5 (q)	54.9 (q)

[a] Multiplicities given are with respect to decoupled protons.



UV/Vis Studies

The spectra obtained for the interaction between ferrous sulphate and indomethacin are seen in Fig. 2 (a)–(d); where plot (a) is that of indomethacin alone and plots (b)–(d) are in the presence of the Fe^{2+}. The peak for indomethacin alone is observed at 318 nm. Immediately following the addition of Fe^{2+} to the indomethacin, a small shift in wavelength of 2 nm is observed along with an increase in indomethacin peak height, Fig. 2(a); indicating a possible coordination. The indomethacin peak also sharpens on addition of Fe^{2+} confirming the interaction between the two. A second peak observed at 266 nm in the presence of Fe^{2+} is noted in Fig. 2(b)–(d). Figure 2(c) is the spectrum obtained for indomethacin and Fe^{2+} after an hour and shows that indomethacin degrades with time in the presence of ferrous sulphate. A 2 nm shift is consistently observed between the peak in Fig. 2(a) and 2(c), whilst the second

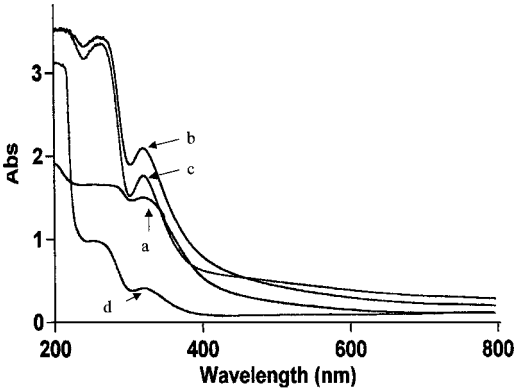


Figure 2. Absorption spectral changes observed for indomethacin on the addition of Fe^{2+}. Spectra for (a) indomethacin (10 mM) alone, (b) immediately after addition of Fe^{2+} (10 mM), (c) after 1 h of interaction between Fe^{2+} and indomethacin, and (d) after 48 h of interaction between Fe^{2+} and indomethacin.

peak remains constant in the 266 nm region. This confirms that the interaction between Fe^{2+} and indomethacin still exists even though the complex is degrading. After 48 h, Fig. 2(d) was obtained and it shows the indomethacin–Fe^{2+} complex had degraded considerably. The spectrum shown in Fig. 2(d) exhibit features similar to those of indomethacin, displaying that Fe^{2+} is no longer interacting with indomethacin at this stage.

Fe^{3+} in the form of ferric chloride or ferric sulphate did not exhibit spectral data indicative of an interaction between the Fe^{3+} oxidation state and indomethacin. Only one large undefined peak was obtained in the 260 nm region, but it overlapped with the intense solvent peak and thus no electronic interactions could be measured between indomethacin and Fe^{3+}.

DISCUSSION

The brain is rich in phospholipid content (Southgate and Daya, 1999), consumes a large percentage of oxygen (Daya, 1999) and has little antioxidant defense mechanisms. This makes the cell membranes of the neurons very vulnerable to oxidative attack, particularly by free radicals. Many neurodegenerative diseases, such as AD and Parkinsons disease, may have as their basis, free radical damage of neurons. The loss of neurons in turn results in the loss of receptors and subsequent loss of neuronal function. Thus, an important aspect of the prevention or inhibition of the progression of such neurodegenerative processes involves the use of free radical scavengers (Evans and Morris, 1996; Potocnik and van Rensburg, 1999). Compounds that show neuroprotection in AD include the NSAIDS. Recent studies have shown that indomethacin, a NSAID, reduces the incidence or severity of AD (Heidrich *et al*., 1997; Mcgeer and Rogers, 1992; Rich *et al*., 1995; Rogers *et al*., 1993). However, the authors do not describe a probable mechanism for the neuroprotection. The results of the present study suggest that indomethacin is able to partly blunt the iron-induced rise in lipid peroxidation at a concentration of 1 mM. Although this concentration

is suprapharmacological, it is necessary to use such high concentrations as the concentration of iron used to generate the free radicals and lipid peroxidation, is high. This protective effect of indomethacin may explain why this agent has been shown to delay the progression of AD. One of the important reactions resulting in free radical, and in particular $^{\cdot}OH$ generation in the brain, is the Fenton reaction, which requires Fe^{2+} (Halliwell, 1992). Thus, without Fe^{2+}, the reaction will not proceed (Braughler and Hall, 1989). In addition, the autoxidation of Fe^{2+} results in the formation of O_2^- (Halliwell, 1992). As shown in the results above, the NMR data indicate that indomethacin binds the Fe^{2+}/Fe^{3+} ion and that this binding takes place on the acetic acid grouping at C-3. Such binding will prevent iron from taking part in the Fenton reaction and will thus reduce free radical generation. Binding by indomethacin is further supported by the UV/Vis study that shows sharpening of the indomethacin peak on addition of Fe^{2+} confirming the interaction between the two. This interaction appears to be a stable one. This study represents the first report to shed some light on the possible reasons for the neuroprotective effects of indomethacin. Similar studies need to be done on the other NSAIDS to elucidate the possible neuroprotective effects of these agents.

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