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Effect of a novel NSAID derivative with antioxidant moiety on oxidative damage caused by liver and cerebral ischaemia-reperfusion in rats

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Abstract

Tissue ischaemia-reperfusion evokes toxic and harmful biochemical processes such as oxidative stress and inflammation. The aim of this study is to investigate the indices of tissue damage in rat liver and brain after ischaemia-reperfusion injury of these organs, and to study prospective cytoprotection of molecules such as the novel anti-inflammatory *N*-(2-thiolethyl)-2-{2-[*N*′-(2,6-dichlorophenyl)amino] phenyl}acetamide (compound **1**) and α-tocopherol. Two experimental models were studied: firstly, 30 min liver ischaemia via hepatoduodenal ligament clamping followed by 60 min reperfusion; and secondly, 45 min cerebral ischaemia via bilateral common carotid artery occlusion followed by 90 min reperfusion. Compound **1** and α-tocopherol were administered intraperitoneally before induction of ischaemia. We hereby report that compound **1**, a molecule that combines potent in-vitro antioxidant and in-vivo anti-inflammatory activity with low gastrointestinal toxicity, offered protection in-vivo against liver or brain ischaemia-reperfusion-induced damage. Both compound **1** and α-tocopherol prevented changes in lipid peroxidation in the rat liver and brain tissue and in tumour necrosis factor (TNF-α) levels in brain. Also compound **1** attenuated glutathione depletion, evoked by ischaemia-reperfusion, in the rat brain but not in the liver. These results could be explained on the basis of the antioxidant/anti-inflammatory properties of compound **1** and suggest its beneficial effect and potential therapeutic use in post-ischaemic injury.

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Introduction

Ischaemic-reperfusion injury, which occurs in conditions such as cerebral stroke or myocardial infarction, is attributed primarily to oxidative injury resulting from oxygen toxicity and free-radical-mediated destruction of sarcoplasmic and other cell membranes (Yamamoto et al 1983; Fridovich 1988; Stohrer et al 1998). Two of the major consequences of such damage are lipid peroxidation and depletion of the cellular antioxidant glutathione that leads to the oxidation of protein thiols (Mizui et al 1992; Shivakumar et al 1992). Therefore, the inhibition of excessive lipid peroxidation and the maintenance of thiol homoeostasis are important factors in cell survival. An obvious means of preventing or repairing oxidative damage is the use of tissue protective agents such as antioxidants (Kourounakis et al 1992; Halliwell 2000). The use of antioxidants in ischaemic conditions has been studied to some extent (Horakova et al 1991; Block et al 1995; Cowley et al 1996) and several reviews on their prospective therapeutic potential are available (Maxwell & Lip 1997; Nagel et al 1997). In addition to antioxidants, other agents such as nitrates, lipid-lowering agents or beta blockers, and also cyclooxygenase–lipoxygenase inhibitors have been considered for the prevention of ischaemia-reperfusion injury or post myocardial infarction risks (Chen et al 1995; Rapaport & Gheorghiade 1996). It is believed that inflammation, arachidonic acid metabolites and pro-inflammatory cytokines are important mediators of oedema and tissue injury after cerebral (or hepatic) ischaemia-reperfusion (Watanabe & Egawa 1994; Chen et al 1995; Coimbra et al 1996; Colleti & Green 2001).

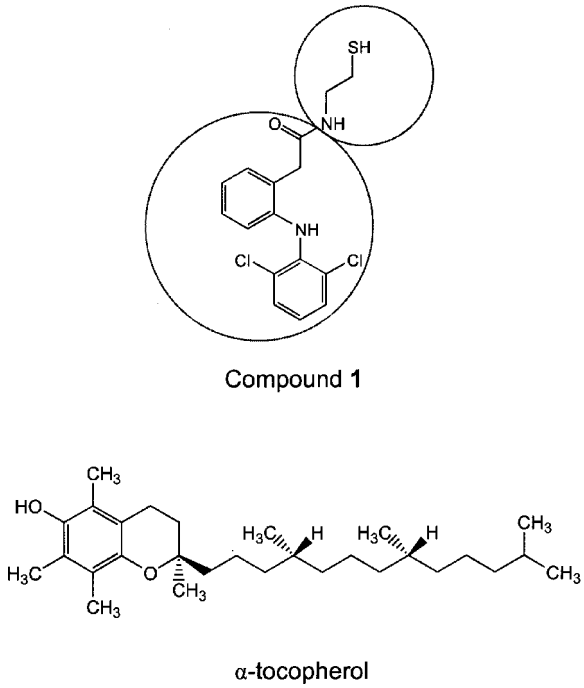

Figure 1 Structure of compound **1**, indicating the anti-inflammatory (diclofenac) and antioxidant (cysteamine) moieties, and of α-tocopherol.

In this study we investigated the protective effect of a novel anti-inflammatory molecule *N*-(2-thiolethyl)-2-{2-[*N*′-(2,6-dichlorophenyl)amino]phenyl}acetamide (**1**; Figure 1), which had been designed to incorporate an anti-inflammatory component (diclofenac) and an antioxidant moiety (cysteamine) in its structure (Kourounakis et al 1999), as well as the effect of α-tocopherol, on some indices of cellular damage after ischaemia-reperfusion of the rat liver and brain. α-Tocopherol, a known antioxidant molecule, has already been studied in tissue ischaemia-reperfusion injury (e.g. Yamamoto et al 1983; Rhoden et al 2001) and was used here for reasons of comparison.

While α-tocopherol is a classical potent antioxidant, compound **1** is a hybrid molecule we have previously synthesized, which has significant in-vitro antioxidant activity, suppressing lipid peroxidation of rat hepatic microsomal membranes and interacting with the stable free radical, 1,1-diphenyl-2-picryl hydrazyl (Kourounakis et al 1999). We have also shown that it possesses potent anti-inflammatory activity with very much reduced gastrointestinal toxicity compared with diclofenac (Kourounakis et al 1999, 2000). Therefore, we investigated its effect in in-vivo ischaemia-reperfusion injury of brain and liver, an oxidative stress model, in an attempt to evaluate this compound as a potential cyto- and neuroprotective agent.

The levels of lipid peroxidation, reduced glutathione and the pro-inflammatory cytokine tumour necrosis factor (TNF-α), were determined in liver or brain tissue to evaluate the effectiveness of compound **1** in inhibiting or reverting the oxidative and cellular damage produced by ischaemia-reperfusion. We could also compare the effects of compound **1** and α-tocopherol on lipid peroxidation, glutathione and TNF-α.

Materials and Methods

All chemicals, solvents and reagents were obtained from standard sources.

Synthesis

Compound **1** (*N*-(2-thiolethyl)-2-{2-[*N*′-(2,6-dichlorophenyl)amino]phenyl}acetamide) was synthesized by the classical method for amide formation with N, N-dicyclohexylcarbodiimide using diclofenac acid and cysteamine according to Kourounakis et al (1999).

Administration of compound 1 and α-tocopherol

In all cases compounds were suspended in water with a few drops of Tween 80.

To evaluate the effect of compounds on rat liver ischaemia, compound **1** (0.3 mmol or 106.6 mg kg^{-1}) or α-tocopherol (0.3 mmol or 129.2 mg kg^{-1}) was administered intraperitoneally once daily for 4 days and 1 h before the induction of ischaemia.

For evaluating the effect on rat cerebral ischaemia, two dosage schemes were applied: short-term administration wherein compound **1** or α-tocopherol (0.3 mmol kg^{-1}) were administered intraperitoneally once 1 h before ischaemia and long-term administration wherein compound **1** (0.2 mmol kg^{-1}) or α-tocopherol (0.3 mmol kg^{-1}) were administered intraperitoneally once daily for 7 days and 1 h before ischaemia.

Ischaemia-reperfusion

Experiments were carried out on male Fischer rats, weighing 110–130 g, which were kept in an environmentally controlled room (22–28°C, 50–60% humidity, 12-h day illumination) with food and water available freely. Rats were anaesthetized with intramuscular injection of fentanyl (0.0625 mmol kg^{-1}) and midazolam (6.25 mmol kg^{-1}). Anesthesia was supplemented when necessary.

For cerebral ischaemia, tracheotomy and intubation was applied after a midline incision of the front surface of the neck and the common carotid arteries were exposed and clamped with aneurysm clips, to induce incomplete cerebral ischaemia (Yamamoto et al 1983). Rats were subjected to 45 min of carotid occlusion, after which the clips were removed and the brain was reperfused for 90 min. Rats were subsequently killed and their brains were quickly removed with a surgical spatula, homogenized for 60 s in ice-cold isotonic KCl solution (1.15%) and stored at −80°C for further evaluation.

For liver ischaemia, a laparotomy was performed via a midline incision and the liver bilus was exposed. The portal vein, hepatic artery and bile duct of the left lateral and median lobes were occluded by a small vascular clamp (Eleftheriadis et al 1997). The blood supply to the omental

(caudate) and right lobes was uninterrupted and portal blood flow was maintained through them without evidence of vascular congestion of the alimentary tract. This procedure is considered to yield approx. 70% partial ischaemia of the liver by weight. After 30 min of normothermic ischaemia, recirculation of blood through the ischaemic liver was achieved by removing the clamp. After 60 min of reperfusion, the rats were sacrificed and the ischaemic hepatic lobes excised and rapidly homogenized in ice-cold Tris buffer (1:4 w/v). The liver microsomal fraction was prepared as described previously (Rekka et al 1989) while the cytosolic fraction was also isolated and both stored at -80°C for further evaluation.

Sham-operated rats, submitted to anaesthesia, were used as controls. During all ischaemia-reperfusion experiments precautions were taken for the rats to maintain normal body temperature. All groups consisted of 7–9 rats.

In-vitro assays

Measurement of lipid peroxidation

Lipid peroxidation of the rat brain (homogenate) and liver (hepatic microsomal fraction) was determined fluorometrically (excitation wavelength at 513 nm, emission at 553 nm) as thiobarbituric acid reactive material (TBARs) by a modified method of Kourounakis et al (1999), Ohkawa et al (1979) and Vajragupta et al (2000). The level of lipid peroxides is expressed in terms of nmol malonic di-aldehyde (MDA) per mg of tissue or microsomal protein, which was calculated by the fluorometric intensity using MBDA (malonaldehyde bis(dimethyl acetal)) as an external standard and expressed as mean$\pm$s.e.m. (n = 7–8).

Measurement of glutathione levels

This assay was performed in the cytosolic fraction of the liver and in brain homogenate supernatant (9000 *g*, 20 min). Reduced glutathione was determined by a spectrophotometric method based on the reaction of glutathione with DTNB (5,5-dithio-bis(2-nitro-benzoic) acid), according to literature (Akerboom et al 1982). Test samples (or different concentrations of glutathione as standards) were incubated at 37°C in a phosphate buffer (pH 7.4) containing a solution of NADPH (4 mg mL^{-1}) and glutathione reductase (6 U mL^{-1}). The reaction was initiated with the addition of DTNB and after 3 min absorbance was measured at 412 nm. The level of glutathione is expressed in terms of μmol (mg protein)$^{-1}$.

Cytokine assay

The levels of TNF-α in liver and brain homogenate were measured in duplicate by ELISA according to the manufacturer's instructions. The ELISA kit for TNF-α was purchased from Endogen, Woburn, MA. Test samples and standards were added to the anti-rat TNF-α precoated stripwell plate followed by a series of incubations (room temperature, 20–25°C), washings, addition of the biotinylated antibody and the appropriate substrate. The absorbance was measured on an ELISA reader set at 450 and 550 nm, and TNF-α levels are expressed in terms of ng (mg protein)$^{-1}$.

Protein determination

The microsomal protein content was estimated by the method of Lowry et al (1951) using bovine serum albumin as a standard.

Statistical analysis

Statistical analysis between groups was performed by one-way analysis of variance followed by Bonferroni's multiple comparison post test to evaluate differences between the means of individual groups (treatments). Differences were accepted as statistically significant at $P < 0.05$.

Results

Effects of compound 1 and α-tocopherol on liver and brain lipid peroxidation levels

Basal levels of lipid peroxidation in the liver expressed as MDA (33.8$\pm$2.6 nmol (mg microsomal protein)$^{-1}$) increased approx. 3-fold (81.5$\pm$8.7 nmol (mg microsomal

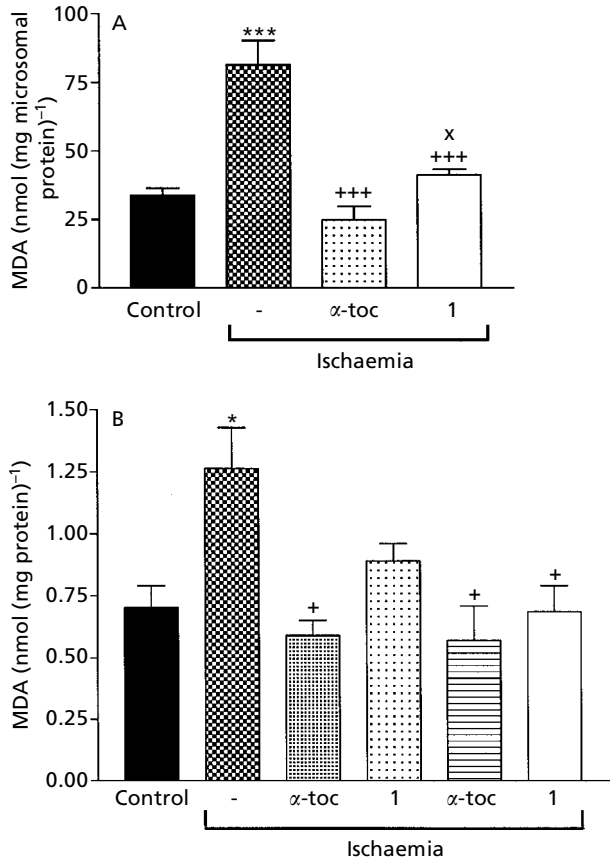

Figure 2 Lipid peroxidation levels expressed as nmol MDA per mg protein (mean$\pm$s.e.m.) in liver microsomes (A) or brain homogenate (B) after short-term administration of compound **1** or α-tocopherol (**1** or α-toc) or after long-term administration of compound **1** or α-tocopherol (**1**′ or α-toc′). $^{*}P < 0.05$, $^{***}P < 0.001$, vs control group; $^{+}P < 0.05$, $^{+++}P < 0.001$, vs ischaemia group; $^{\times}P < 0.05$, vs α-tocopherol group.

protein)$^{-1}$) after 30 min ischaemia and 60 min reperfusion. Pretreatment with 0.3 mmol kg^{-1} α-tocopherol or compound **1** significantly suppressed this increase to 24.9 ± 4.9 nmol and 41.3 ± 1.9 nmol MDA respectively (Figure 2A).

Lipid peroxidation in the rat brain increased 1.4- to 2-fold after 45 min ischaemia and 90 min reperfusion. Short-term pretreatment (1 h before induction of ischaemia-reperfusion) with α-tocopherol or compound **1** offered significant protection against this increase, and the lipid peroxidation remained close to control levels (Figure 2B). The same effect of pretreatment by α-tocopherol was observed using the long-term administration protocol. Long-term administration of compound **1** before ischaemia-reperfusion also provided full protection (Figure 2B).

Effects of compound 1 and α-tocopherol on liver and brain glutathione levels

Liver glutathione levels were significantly reduced in the ischaemic-reperfused liver, while α-tocopherol, but not compound **1**, inhibited this reduction to some extent (Figure 3A). In the brain, glutathione levels also dropped significantly after ischaemia-reperfusion. However, both short- and long-term administration of α-tocopherol and **1** had a significant effect: both compounds maintained glutathione concentration after ischaemia and reperfusion at control levels (Figure 3B).

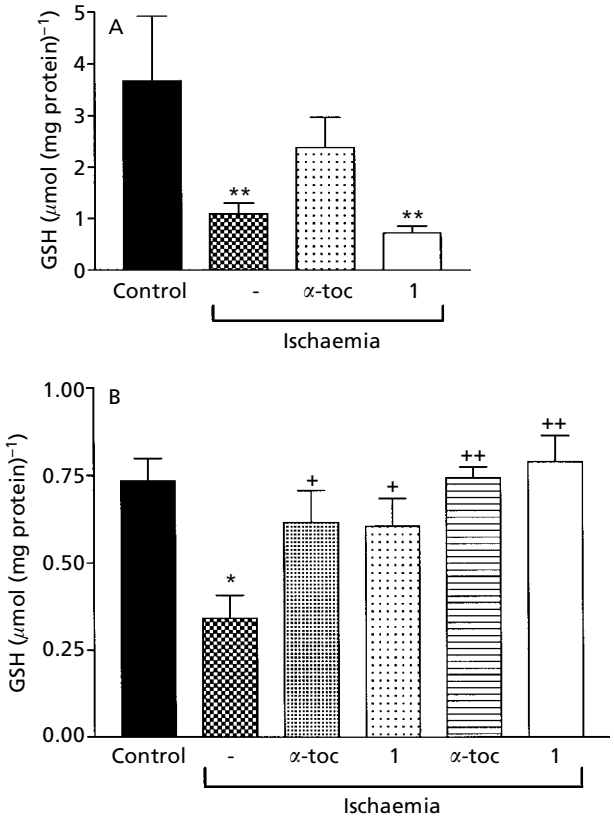

Figure 3 Glutathione (GSH) levels expressed as μmol (mg protein)$^{-1}$ (mean ± s.e.m.) in liver (A) or brain (B) after short-term administration of compound **1** or α-tocopherol (**1** or α-toc) or after long-term administration of compound **1** or α-tocopherol (**1**′ or α-toc′). $^{*}P < 0.05$, $^{**}P < 0.01$, vs control group; $^{+}P < 0.05$, $^{++}P < 0.001$, vs ischaemia group.

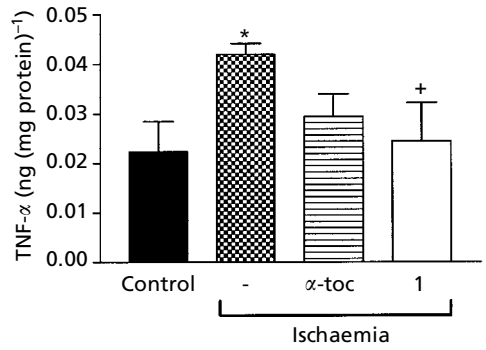

Figure 4 TNF-α levels expressed as ng (mg protein)$^{-1}$ (mean ± s.e.m.) after long-term administration of compound **1** or α-tocopherol (**1**′ or α-toc′). $^{*}P < 0.05$, vs control group; $^{+}P < 0.05$, vs ischaemia group.

Effects of compound 1 and α-tocopherol on liver and brain TNF-α levels

No significant difference in liver TNF-α levels was found between groups (levels were in the range 4–5 ng (g liver)$^{-1}$). In contrast, we found a significant elevation of TNF-α levels in the rat brain of the ischaemia/reperfused group. Short-term administration of either α-tocopherol or compound **1** did not seem to affect the increase in TNF-α (results not shown). However, long-term administration significantly suppressed TNF-α elevation such that control levels were maintained after ischaemia-reperfusion (Figure 4).

Discussion

Since ischaemia-reperfusion injury is caused by oxygen toxicity (active oxygen species – oxidative stress), as well as the ensuing inflammation (Emerit et al 1991; Gute et al 1998), we investigated the influence of compound **1** and α-tocopherol on the ischaemia-reperfusion injury of liver and brain tissue, using the increase of lipid peroxidation (measured as TBARs and expressed as MDA) as well as glutathione depletion as indices of oxidative damage, and TNF-α production as an index of a preliminary test for inflammation (Zhang & Rivest 2001).

Hepatic ischaemia, in the rat, for 30 min followed by 1 h of reperfusion resulted, as expected, in a significant rise of lipid peroxidation and a decrease of cytosolic glutathione levels, demonstrating the oxidative damage inflicted upon this tissue. These results are in agreement with previous studies (Sewerynek et al 1996; Portakal & Inal-Erden 1999). In our experiment, both α-tocopherol and compound **1**

reduced the consequences of oxidative damage, α-tocopherol by maintaining both lipid peroxidation and glutathione at control/baseline levels and compound **1** by protecting only against lipid peroxidation. From our results it appears that the oxidative damage is greater in the liver than in the brain (compare control group with the ischaemia-only group), and this may perhaps explain why compound **1** is not able to counteract this damage in the liver compared with that in the brain. The demonstrated protective effect of α-tocopherol is explained by its potent antioxidant properties and constitutes a confirmation of previously reported findings (Vardareli et al 1998). Compound **1** is also quite potent in this model concerning protection against lipid peroxidation. Interestingly, the effect of diclofenac sodium on lipid peroxidation and liver injury in the ischaemia-reperfusion model in rats has been investigated to some extent. Takayama et al (1993) showed that both diclofenac sodium and α-tocopherol (administered orally for 5 days) protected against liver injury expressed as increases in plasma phosphatidylcholine hydroperoxide and elevation of serum glutamic oxaloacetic transaminase and lactate dehydrogenase (Takayama et al 1993, 1994).

There were no significant alterations in the TNF-α levels of the liver after ischaemia-reperfusion injury and the treatment with α-tocopherol or compound **1** under our experimental conditions. A longer ischaemic challenge is probably needed for expression of this cytokine.

We further investigated the oxidative damage after ischaemia-reperfusion in the rat brain and the effect on this of α-tocopherol and compound **1**. Both lipid peroxidation and the proinflammatory cytokine TNF-α increased significantly while glutathione levels were reduced by the free radical assault. It was previously shown that the glutathione depletion observed during ischaemia is accompanied by a further depletion during reperfusion (Durmaz et al 1999) while restoration of glutathione levels in the brain follows only after 24 h of reperfusion (Shivakumar et al 1995). Compound **1** and α-tocopherol demonstrated a clear protective effect against this ischaemia-reperfusion (oxidative) injury by lowering levels of brain tissue lipid peroxidation and inhibiting reduction of glutathione concentration. α-Tocopherol has previously been shown to protect against such cellular damages (Yamamoto et al 1983). We have also demonstrated the action of other free radical scavengers (e.g. guaiazulene) in the paracetamol hepatotoxicity model where both lipid peroxidation and glutathione depletion were reduced (Kourounakis et al 1997a, b). Since disturbance of thiol homoeostasis is closely related to lipid peroxidation levels and generation of oxidative stress (Shivakumar et al 1992) in the brain, rapid restoration of glutathione levels may help the brain to recover from reperfusion injury.

Furthermore, in this work we have shown that TNF-α increases significantly with brain ischaemia-reperfusion injury, indicating that in this damage, inflammation also participates as expected. We also found that both compound **1** and α-tocopherol attenuated the elevated levels of TNF-α in the brain during ischaemia-reperfusion. α-Tocopherol acts by its free radical scavenging properties (i.e., indirectly), while compound **1**, being both a potent anti-inflammatory agent and an efficient antioxidant, may act protectively by a dual mechanism.

Conclusion

We found that compound **1** is effective in protecting tissue from oxidative damage induced by ischaemia-reperfusion. Both compound **1** and α-tocopherol prevented changes, that were evoked by ischaemia and reperfusion, in lipid peroxidation in the rat liver and brain tissue and in TNF-α levels in brain. Also compound **1** attenuated the depletion of glutathione, evoked by ischaemia-reperfusion, in the rat brain but not in the liver. Thus, compound **1**, structurally designed to acquire a combination of potent anti-inflammatory and antioxidant properties with very low gastrointestinal toxicity, could prove to be a potential candidate for the prevention or treatment of ischaemia-reperfusion injury considering the underlying pathobiochemistry of this condition.

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