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Changes in oxidative stress in the rat brain during post-cardiac arrest reperfusion, and the effect of treatment with the free radical scavenger idebenone

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Abstract

The study was designed to determine the effect of idebenone, an electron-trapping agent and free radical scavenger capable of crossing the blood–brain barrier, on cardiac arrest-induced oxidative brain stress. Stress indices used were the brain contents of thiobarbituric acid-reactive material (TBAR), conjugated dienes and protein and non-protein thiols. Twenty-four hours after receiving one oral dose of placebo or 100 mg kg^{-1} idebenone, the rats were anaesthetized with diethyl ether and either decapitated immediately, or subjected to 7.5 min cardiac arrest induced by compression of the heart vessel bundle. The next groups of rats were sacrificed at the end of the cardiac arrest session, or resuscitated by external chest compression and artificial ventilation with air and sacrificed 15 min, 60 min, 24 h, and 72 h later while re-anesthetized with diethyl ether. Subsequent placebo or idebenone (100 mg kg^{-1}) doses were given to the appropriate surviving rats once daily, beginning 8–10 min after the end of cardiac arrest session. Compared to pre-arrest values, TBAR and conjugated dienes' contents increased, respectively, by 339 and 286%, and protein and non-protein thiol contents decreased, respectively, by 69 and 85% within 60 min after the resuscitation in placebo-treated rats. Normalization of all oxidative stress indices in these rats was slow and incomplete even at 72 h. Idebenone treated rats showed no increase in TBAR contents, and a marked attenuation of changes in the other indices. These results show that oral idebenone greatly reduces oxidative brain stress following transient circulatory arrest in the rat. This effect could not be explained by simple stoichiometric scavenging of free radicals. Possible mechanisms of idebenone action are discussed.

Keywords: Global cerebral ischaemia; Cardiopulmonary resuscitation; Drug therapy; Free radical; Post-resuscitation period

1. Introduction

Oxidative stress, which is usually defined as a preponderance of production of free radicals over their elimination (this phenomenon may also involve certain non-radical reactive oxygen species, ROS), is commonly implicated in the development of postischaemic brain damage [1,2]. While some of those chemical entities may already be generated during ischaemia through autooxidation of components of the 'electron-saturated' mitochondrial respiratory chain [3], a major part of their flux forms during reperfusion. Free radicals-generating mechanisms contributing to postischaemic brain injury may involve numerous factors, such as activation of arachidonic acid cascade [4], conversion of xanthine dehydrogenase to xanthine oxidase in brain capillaries, excessive release of neurotransmitters, mitochondrial

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perturbations, acidosis, mobilization of tansition metal ions, activation and/or induction of nitric oxide synthases, and activation of neutrophils and microglial cell [1,2].

The efficacy of many compounds with different mechanisms of reducing free radical-mediated ischaemic damage was tested in a variety of brain ischaemia models. For instance, the xanthine oxidase inhibitor allopurinol [5], the free radical spin trap *N-tert*-butyl-α-phenylnitrone [6], and the free radical scavengers/antioxidants α-lipoic acid [7], dimethylthiourea [5] and tirilazad mesylate [8] were found to provide protection in different degrees. This latter drug appeared promising enough to enter phase III clinical trials in a number of oxidative stress-involving settings including ischaemic stroke [8]. However, its major site of action—due to poor penetration of the blood–brain barrier—appears to be the brain perivascular compartment, and free radical scavengers capable of crossing the barrier more easily were postulated to provide better protection [8,9].

Free radical scavengers/antioxidants are usually considered candidate drugs to be given shortly after the occurrence of a brain ischaemic insult, that is, during the period of most intense postischaemic free radical formation. Nevertheless, a need to explore their preventive use in patients at risk of cardiovascular failure or stroke and in a number of clinical settings involving a 'man-made' risk of cerebral ischaemia (e.g. in coronary artery bypass graft or carotid endarterectomy) has also been acknowledged [10]. The goal of the present study was to investigate the effects of periischaemic treatment with idebenone, an electron-trapping agent and free radical scavenger known to inhibit lipid peroxidation in vitro [11,12], on oxidative brain stress produced by reversible cardiac arrest in the rat. Idebenone penetrates the brain rapidly after oral administration [13] and ameliorates neurological deficit in rats with experimental cerebral ischaemia [14]. Oxidative stress indices used included the brain contents of thiobarbituric acid-reactive material (TBAR), conjugated dienes (conjugated double bonds, CDB) and non-protein and protein SH group-containing compounds (thiols).

2. Materials and methods

2.1. Animals

One hundred and ninety-one Wistar rats of either sex, 160–210 g body weight, were used for the study. All procedures were in compliance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the local animal care committee.

The rats had free access to standard food and purified tap water throughout the experiment. Idebenone (2,3 - dimethoxy - 6 - (10 - hydroxydecyl) - 5-methyl-1,4-benzoquinone, donated by Takeda Chemical Industries, Tokyo, Japan) was mixed with 5% gum arabic solution to form an 80 mg ml^{-1} suspension. The rats were randomly assigned to drug or placebo treatment, and were given one oral dose of the placebo (1.25 ml kg^{-1}, 93 rats) or idebenone (100 mg kg^{-1}, 98 rats) 24 h prior to further manipulations. On the next day, all rats were anaesthetized with diethyl ether. Six idebenone treated and five placebo-treated rats serving as controls were killed by decapitation 3–4 min after induction of anaesthesia. In the remaining rats cardiac arrest was induced according to the method of Korpachev et al. [15,16]. Briefly, after chest skin incision, a blunt-end hook-like metal device was inserted transmurally into the chest cavity using right parasternal approach and positioned to compress the heart vessel bundle against the sternum. This caused cessation of cardiac blood flow, and subsequent cardiac arrest. The device was removed 3–4 min later, and the skin cut was sutured. Seven placebo-treated and eight idebenone treated rats were decapitated 7.5 min after the cessation of blood flow. In the remaining rats resuscitation was begun by external chest compressions 7.5 min after the induction of cardiac arrest and continued until return of a spontaneous heartbeat (for 1.5 to 2.5 min in successful attempts). During resuscitation, 63 μg kg^{-1} adrenaline and 1 mEq kg^{-1} sodium bicarbonate were given intravenously to all rats. Resuscitated rats were ventilated with room air using a rodent respirator (Ugo Basile, Italy) set to produce a tidal volume of 2.5 ml at a rate of 25–30 breaths min^{-1}. The rats started to breathe spontaneously within 7 to 15 min, and reaction to pain, corneal reflex and spontaneous locomotor activity reappeared, respectively, 15–25 min, 30–45 min and about 1 h after the restoration of heartbeat. Subsequent idebenone (100 mg kg^{-1}) or placebo doses were given orally to appropriate surviving rats (except for the first post-cardiac arrest dose which was given intragastrically) every 24 h beginning 8–10 min after the restoration of spontaneous heartbeat. The next groups of rats (5–8 rats per each time point and treatment modality) were re-anaesthetized with diethyl ether and decapitated 15 min, 60 min, 24 h and 72 h after restoration of heartbeat. The brains were removed within 1.5 min, snap–frozen in liquid nitrogen and kept at −70°C until processed for oxidative stress assays. The remaining 27 rats were used for another study and were not included in the present report except for resuscitation and survival rate data.

2.2. Assessment of oxidative brain stress

Analyses were carried blindly with respect to the treatment received by the rats. TBAR was determined by the method of Slater and Sawyer [17] and quantified

as nanomoles of malondialdehyde. CDB was measured in total lipid extracts as described by Recknagel and Goshal [18]. Total and non-protein thiols were assessed according to Sedlak and Lindsay [19], and protein thiols were calculated as the difference between total and non-protein thiols. All results were expressed relative to the brain protein (Lowry) content.

2.3. Statistics

Resuscitation and survival rates were compared between the idebenone treated and placebo-treated rats by χ^2 test. Oxidative stress data were analyzed by the two-way ANOVA with treatment (idebenone or placebo) and time point relative to cardiac arrest session (pre-arrest, end of cardiac arrest session, and 15 min, 1 h, 24 h, and 72 h postresuscitation time) as independent factors, followed by Scheffe's F test for comparisons between individual groups. Occasionally, Student's t-test for independent samples was used as indicated in the text. In all cases, $P < 0.05$ was considered significant.

3. Results

Fifteen min after the onset of resuscitation, the rate of resuscitation success in placebo treated rats was 56% and did not differ from that in idebenone-treated rats (51%, $P = 0.57$). In eight placebo- and 13 ibedenone-treated rats, the causes of resuscitation failure verified at autopsy were iatrogenic complications (haemothorax or cardiac tamponade) or respiratory insufficiency due to a pre-existing condition (e.g. lung fibrosis). After correction for these factors, the early resuscitation success rate in the placebo treated and idebenone treated rats was, respectively, 62 and 61% ($P = 0.89$). The cause of resuscitation failure in the remaining 28 idebenone treated and 28 placebo treated rats remained unknown. Seven placebo- and four idebenone-treated rats that died within 3 days after resuscitation were not autopsied. In agreement with earlier reports [15,16,20], daily visual examination showed no neurological deficit in the surviving rats during this period. Three days after resuscitation the uncorrected overall survival rate in the placebo- and ibedenone-treated rats was, respectively, 33.3 and 33.9%. Using the above correction, the 3 day survival rate was, respectively, 39.6 and 43.5% ($P = 0.70$).

The two-way ANOVA showed significant effects of treatment and time, and significant interaction between the two main factors on all of the oxidative brain stress indices studied ($P < 10^{-6}$ for all).

In the placebo treated rats, the brain contents of TBAR and CDB showed significant increases at 15 min postresuscitation time and the end of cardiac arrest session, respectively, and reached their corresponding maxima (439% of control for TBAR, 386% of control for CDB) 1 h after resuscitation (Fig. 1A-B). Protein and non-protein thiol contents were already reduced at the end of the cardiac arrest session and reached a minimum at 1 h postresuscitation time (67 and 10% of the appropriate pre-arrest value, respectively); the apparent further drop in non-protein thiols at 24 h postresuscitation time was not significant (Fig. 1C–D). In the placebo treated rats, the TBAR content began a return to normal between 1 and 24 h postresuscitation time ($P < 0.001$), whereas protein and non-protein thiol contents showed a recovery between 24 and 72 h postresuscitation time ($P < 0.001$), and a similar tendency ($P = 0.07$) was apparent in CDB content. None of these indices had reached the respective pre-arrest value on postresuscitation day 3 in these rats.

In the idebenone treated rats, there was no change in brain TBAR content (Fig. 1A), whereas the brain CDB content increased at a markedly attenuated rate, reached a maximum (177% of control) at 24 h, and become normal at 72 h (Fig. 1B). Brain non-protein and protein thiols were maximally depleted (by 29 and 26%, respectively) at 60 min in these rats (Fig. 1C–D). The content of non-protein thiols, but not that of protein thiols, showed a tendency to return to normal ($P = 0.06$) at 72 h postresuscitation time.

Prior to the cardiac arrest session, the brain contents of CDB, TBAR and protein and the non-protein thiols were virtually identical in the placebo- and idebenone-treated rats ($P > 0.99$). Immediately after the session, the only significant difference between the effects of the two treatment modalities was a lower non-protein thiol content in the placebo treated rats. During the postresuscitation period all indices employed showed less oxidative stress in the idebenone treated rats compared to their placebo treated counterparts except that the protein thiol content did not differ at 72 h postresuscitation time (Fig. 1A–D).

4. Discussion

Non-protein thiols are important constituents of the cellular defence against free radicals [2], whereas protein thiols are critical for structure and function of many proteins (e.g. see [21]). Ischaemia-induced depletion of these brain thiol pools is considered a sign of oxidative stress [2,22,23]. TBAR and CDB, the products of polyunsaturated lipids' peroxidation, are other indices of oxidative free radical-mediated injury in biological systems which are often used [2,18,24]. The changes in these indices found in placebo treated rats in the present study are in general agreement with those reported in other models of global cerebral ischaemia. However, the declines in brain thiols reported here (see

Fig. 1C–D), in particular that in the non-protein pool, were relatively large and long-lasting compared with those reported in earlier studies [7,22,23,25,26].

Because the non-protein brain thiol pool consists mostly (> 95%) of reduced glutathione (GSH) under normal circumstances [22,26,27], the above inconsistency should not be related to a difference in oxidative stress indices employed (most researchers utilized GSH measurements). The pattern and magnitude of postischaemic changes in cerebral blood flow also do not notably differ between rat models of 'isolated' global brain ischemia and the Korpachev model (cf. [28–32]), and the brain intracellular pH normalizes within 5 min after transient cardiac arrest in the rat [33]. The differences in oxidative stress should therefore be related to the other characteristics of the experimental model employed in the present study. First, in contrast to 'isolated' global brain ischaemia models (for review see [34]), there is no residual blood flow [16] and ischaemia-induced pathologies affect all of the major brain regions in the Korpachev model [16,20,35–37]. Second, cardiac arrest causes severe and prolonged systemic acidosis. Arterial acid-base balance was not monitored in the present study. However, in a previous study an average blood pH 6.9–7.1 ($n = 5$) was found in unmedicated rats 7–60 min after 10 min of cardiac arrest using this model (Majkowska J. Changes in the rat brain after clinical death. Pathophysiological and morphological characteristics. Ph.D. Thesis, Polish Academy of Sciences Medical Research Centre, Warsaw, 1991; see also [29]). This acidosis could not be nullified with bicarbonate dose employed in the present study [29], and would promote oxyhemoglobin dissociation (Bohr effect) and ROS formation [24,38,39]. No-

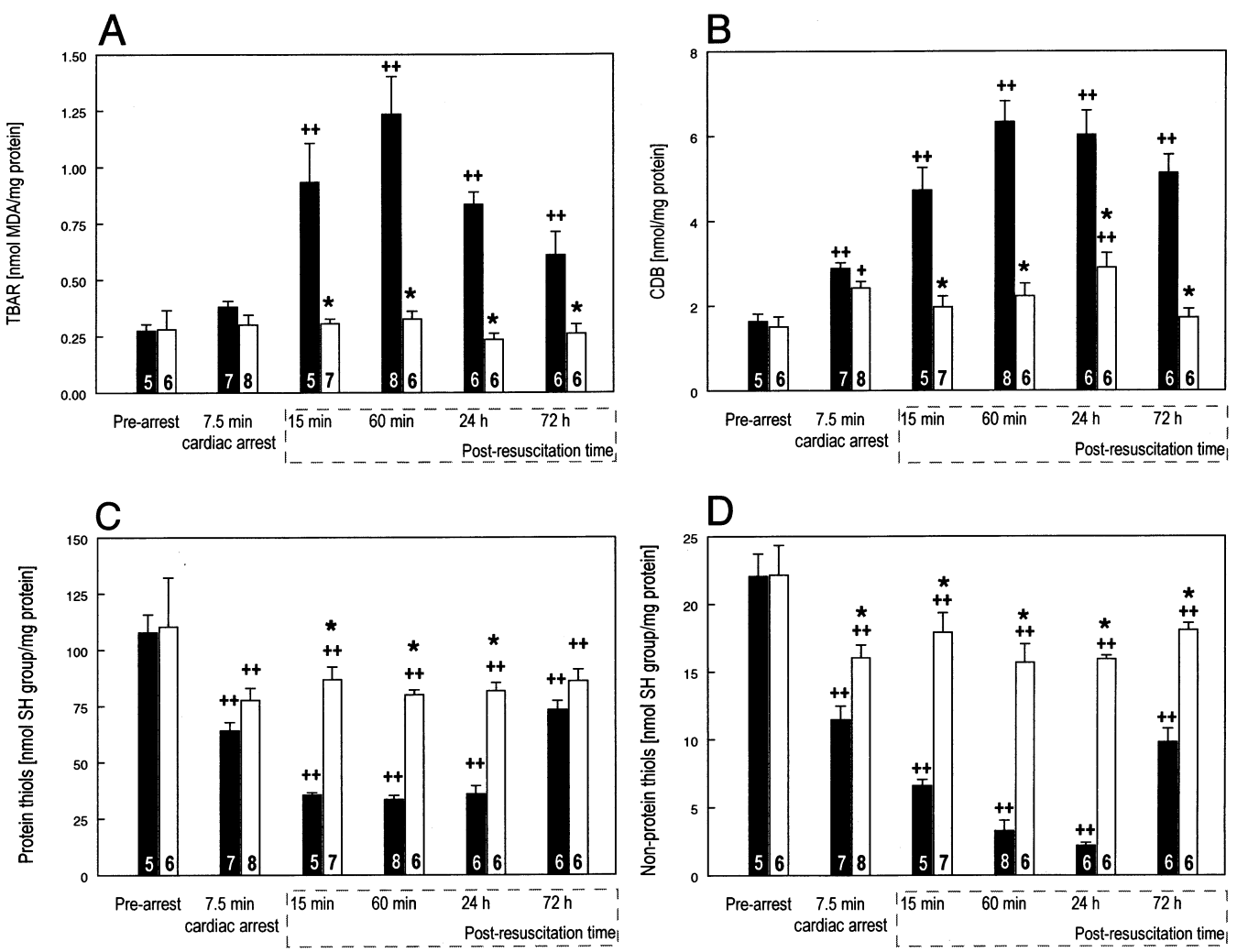

Fig. 1. Changes in oxidative brain stress in placebo-treated (closed bars) and idebenone treated (open bars) rats during cardiac arrest and reperfusion. (A) Thiobarbituric acid-reactive material (TBAR, quantified as nmoles of malondialdehyde (MDA)). (B) Conjugated dienes (CDB). (C) Protein thiols. (D) Non-protein thiols. Results are shown as mean ± S.D.; number of rats per group is given inside the respective bar. + − $P < 0.03$, + + − $P \leq 0.003$ versus the respective pre-arrest value,* − $P < 0.001$ versus the corresponding placebo-treated group, post-hoc Scheffe's F test.

tably, the period of fastest increase in oxidative stress in the present study (15–60 min after resuscitation) coincided with the time of systemic acidosis and 'reactive' brain hyperaemia as reported in the literature. The cardiac arrest-induced prolonged systemic acidosis may cause flooding of the entire body with ROS and generalized depletion of low-molecular weight antioxidants. This would preclude the mitigation of brain oxidative stress by influx of peripheral antioxidants' which might otherwise be facilitated by the multiphase opening of the blood–brain barrier observed both in the Korpachev [34] and other brain ischaemia models [31,40].

The question may arise as to how the brain could survive and function after such a profound depletion of non-protein thiols. However, a majority of this pool probably represents sacrificial thiols. This possibility is indirectly supported by the fact that non-protein thiols continued to decrease after their protein counterparts reach a minimum. Furthermore, a majority of non-protein thiols are contained in non-neuronal cells [27], most probably in astrocytes [41]. These, because of their high capacity of antioxidant defences and association with the blood–brain barrier [42] are in a strategic position to provide the first-line defence against reperfusion-related, ROS-mediated brain damage [2]. Moreover, the major constituent of non-protein thiol pool, GSH, is engaged in a wide variety of cellular processes [43] and the large postischaemic decrease in this pool may reflect its enhanced postischaemic turnover. Finally, it is lipid peroxidation-derived secondary free radical production, and not thiol depletion, that apparently directly parallels the severity of postischaemic dysfunction and tissue injury [44].

The aforementioned scarcity of GSH in neurones could make neuronal proteins particularly susceptible to oxidative stress. This susceptibility may not, however, be entirely detrimental, because oxidation of the SH groups of *N*-methyl-D-aspartate receptors results in their inactivation and may reduce glutamate toxicity [45,46]. Interestingly, the content of protein brain thiols showed no significant fluctuations after resuscitation in the idebenone treated rats and remained slightly, but significantly, decreased at the end of experiment compared to the respective pre-arrest value. This suggests that there is a protein thiol pool which cannot be rescued by idebenone treatment and becomes long-term depleted as a result of ischaemic brain insult. On the other hand, incomplete recovery of brain thiols may also be related to insufficient activity of antioxidant enzymes during this period, similarly to that found in focal brain ischaemia [47].

The results of the present study indicate that post-cardiac arrest oxidative brain stress is most intense (in terms of the indices utilized) during the first hour in the rat, and can be largely reduced by idebenone. In this species, about 90% of oral idebenone dose is taken up and mostly converted to shorter-side chain derivatives [12] some of which retain free radical scavenger properties [48]. Both the parental compound and its catabolites enter the brain within 5 min and reach maximum levels within 15 min following oral or intravenous idebenone administration [12]. These data suggest that idebenone may be highly useful in preventing ischaemia related brain damage in a post-insult treatment paradigm. This study was not designed to estimate the actual width of the therapeutic time window. However, certain free radicals-involving mechanisms of ischaemic brain injury evolve over a period of several hours in rodents [49] which suggests that the therapeutic time window for idebenone administration may extend beyond the first hour of reperfusion. Highly intriguing is, however, the efficacy of the first (pre-arrest) dose of this drug in attenuating changes in non-protein brain thiols found at the end of cardiac arrest session (the other oxidative stress indices showed a similar tendency at that time point, $P < 0.001$ by t-test). Only minute amounts of idebenone and its catabolites can be found in the rat brain and plasma 24 h after oral idebenone administration and (assuming the disposition of idebenone in the present study followed that after 10 mg kg^{-1} dose [12]) the expected molar levels of these compounds 24 h later shall be many times lower than that of spared thiols in the brain. Therefore a simple stoichiometric effect of the first idebenone dose via scavenging free radicals in a non-recyclable mechanism (reduced idebenone + free radical → oxidized idebenone) seems unlikely.

We can only speculate about the mechanisms which may result in the above non-stoichiometric effect. First, idebenone may reinforce recyclable cellular free radical-scavenging systems. Oral idebenone has been shown to reduce non-respiratory oxygen consumption associated with lipid peroxidation [50]. Second, idebenone may attenuate free radicals' formation by scavenging the primary rise in free radicals which may serve as signalling molecules [51], and by preventing ROS-mediated activation of certain transcription factors, e.g. nuclear TF kappa B [52] which mediate expression of defence and signalling proteins including some that can increase postischaemic free radicals' production, e.g. nitric oxide synthases (NOS) [53,54]. In fact, antioxidants can prevent inducible NOS expression in at least some in vivo systems [55]. Importantly, NOS may contribute to ROS production at high extracellular glutamate levels [56] which is the case in an ischaemic brain. Third, idebenone may indirectly reduce oxidative brain stress by elevating nerve growth factor [57,58] which acutely blocks ROS formation in the brain [59,60].

Data on short-term survival showed no clear benefit from idebenone treatment in the present study. Whether the marked attenuation of oxidative brain

stress would result in improvement of long-term outcome in cardiac arrest survivors remains to be elucidated.

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