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Disulfiram Augments Oxidative Stress in Rat Brain following Bilateral Carotid Artery Occlusion

Key Words
Disulfiram
Ischemia
Reperfusion
GSH
Oxidative stress

Abstract
We examined the brain oxidative stress which accompanies 30 min of bilateral carotid artery ligation (BCAL) in terms of changes in brain levels of glutathione; reduced (GSH) and oxidized (GSSG) forms and the exacerbation of oxidative stress by disulfiram (DSF). These results indicate that BCAL alone decreases GSH content and limits glutathione reductase (GR) activity, and these changes were enhanced by DSF pretreatment. Similar observations were recorded with DSF alone. GR activity (74.3 ± 4.0 μmol min^{-1} mg^{-1} tissue; $p < 0.001$) and GSH content (1.23 ± 0.06 μmol min^{-1} g^{-1} tissue; $p < 0.001$) was attenuated in rats subjected to synergistic effect of BCAL and DSF with a concomitant increase of GSSG (0.006 ± 0.006 μmol min^{-1} g^{-1} tissue; $p < 0.001$). Recovery of GSH/GSSG level and GR activity during reperfusion following 30 min BCAL was considerably delayed (96 h) in the BCAL and DSF group as compared to the recovery time of 24 h in the group subjected to BCAL-reperfusion alone. Perturbation of GSH/GSSG homeostasis as a result of BCAL was augmented by DSF. These findings clearly demonstrate central nervous system oxidative stress due to a BCAL-DSF synergistic effect. Based on the results obtained with this model, we conclude that DSF increases brain oxidative stress and this may be detrimental to alcoholics who might drink and develop an acetaldehyde-induced hypotension while taking DSF.

Introduction

Cerebral ischemia is attributed to a reduced blood flow and a lack of oxygen supply to the brain. In such situations, neurons subjected to oxidative stress are deprived of an energy supply, by the inhibition of Na^+-K^+ pump which results in glutamate release [28]. Oxidative stress is also associated with tissues exposed to certain xenobiotics and a reperfusion after hypoxia or ischemia. Reductive stress or energy has been proposed to play a key role in defending against oxidative stress [11]. Studies on ischemic and reperfused tissue can provide valuable insights into the biochemical events that occur after tissue is subjected to oxidative stress. The pathogenesis of ischemic neuronal injury is generally linked to reduced glutathione (GSH) synthesis and to a diminished capacity to scavenge free radicals [26]. Indication that free-radical-induced damage including peroxidation is involved in neuronal damage is provided by the beneficial effect of 21-aminosteroids [16], which are novel free-radical scavengers and lipid peroxidation inhibitors [10]. Free-radical-induced injury is potentiated by a reduced free-radical scavenging capacity and the deprivation of glucose [25]. Damage to a tissue during reperfusion following ischemia is reported to be due to oxidative stress or to the disruption of the blood brain barrier [7]. An antioxidant enzyme, superoxide dismutase (SOD) has shown neuroprotective effects in animal models of ischemia [32].

GSH, an endogenous peptide, plays an important role in protecting cells from oxidative stress by scavenging free radicals and by generating other antioxidants like vitamin E [22]. Detoxification of hydroperoxides is carried out by glutathione peroxidase (GP), which utilizes the reducing equivalents of GSH to form oxidized glutathione (GSSG).

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In turn GSSG is rapidly reduced to GSH by glutathione reductase (GR) using NADPH whose generation is limited during oxidative stress. Thus, any increase in the level of GSSG could alter GSH/GSSG ratio, which is indicative of oxidative stress [27].

Disulfiram (DSF) is commonly used in the treatment of alcoholism. DSF, an oxidative drug, may induce oxidative stress by shifting the prooxidant-antioxidant balance represented by the GSH/GSSG ratio. Earlier, we have shown that a chronic DSF treatment perturbs GSH/GSSG homeostasis in rat brain [18]. Ischemia-reperfusion in rat models is also shown to cause perturbation of GSH/GSSG homeostasis where decreased GSH level was linked to energy production [5, 23]. The lipophilicity of DSF and its metabolites facilitates easy entry to the brain and this liphophilicity was shown to be enhanced in an ischemic brain following carotid artery occlusion [8]. Further, DSF is reduced to diethyldithiocarbamate (DDTC) nonenzymatically by GSH and enzymatically by GR [17]. DDTC has been shown to inhibit Cu-Zn superoxide dismutase and to deplete the free-radical scavenging capacity of the brain in ischemic rats [30].

To protect against oxidative stress, neurons have an antioxidant system which includes SOD, GSH, glutathione peroxidase and GR. The aim of the present study was to investigate the effect of ischemia and reperfusion on rat brain glutathione content. Because DSF interferes with brain glutathione metabolism and inhibits SOD, its effect per se, as well the synergistic effect of recirculation/reperfusion after bilateral carotid artery occlusion/ligation (BCAL) on glutathione metabolism was investigated. The cerebral ischemia-recirculation was affected in rats by BCAL which allowed partial blood flow. Such a BCAL model does not produce severe damage to the animal brain tissue [6].

Materials and Methods

Chemicals

DSF, GR (yeast), reperfusion, GSSG, NADPH, N-ethylmaleimide and 5,5′-dithio-bis-(2-nitro benzoic acid), dithioerythreitol, CM-cellulose and 5′-ADP Sepharose were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). All other chemicals used were of analytical grade obtained from either Glaxo (India) or E. Merck (India) Ltd.

Animals and Treatment

Male Sprague-Dawley rats (150–180 g) were obtained from the Animal Care Facility of NIMHANS. Animals had free access to chow pellets and water. The Sprague-Dawley strain was selected as the model for inducing ischemia because it can sustain moderate ischemia [24].

Carotid Artery Ligation

The surgical procedure was carried out as described by Eklof and Seisjo [6]. After premedication with atropine sulfate (25–30 μg kg^{-1}, i.m.), anesthesia was induced and maintained throughout surgery with pentobarbital (35 mg kg^{-1} i.p.). In sham-operated rats (n = 4), the bilateral carotid arteries were clamped using aneurysm clips. A terminal vein was cannulated for drug administration. Following surgery and 30 min of occlusion, the rats were allowed to recover fully. The recirculation/reperfusion was achieved by pulling the thread out of the internal carotid artery under the same anesthetic condition as used earlier. Once again, the rats were allowed to recover and had free access to food and water until the next procedure. Control animals (n = 4) were subjected to a similar procedure without clamping the carotid arteries. Reperfusion was allowed for different periods ranging from 30 min to 144 h.

Drug Administration

Following surgery and recovery, DSF (200 mg kg^{-1}) dissolved in PEG 400 was administered (i.v.) to the rats. Control animals were given vehicle (PEG 400, 3 ml kg^{-1}) under the same experimental conditions. The rats were killed at various intervals (30 min, 6, 12, 18, 24, 48, 72, 96, 120 and 144 h) of reperfusion. The effect of DSF alone on brain glutathione content was studied by administering DSF (200 mg kg^{-1}, i.v.) to another group of rats (n = 4) via the cannulated vein, while placebos received only PEG 400. The rats were killed after 30 min of drug administration. After specified periods of recirculation, the animals were sacrificed by decapitation in a cold room. The brain was removed after extensive perfusion with 0.9% cold saline through the heart and frozen immediately over dry ice until weighed and processed. A similar procedure was carried out with rats treated with DSF alone. The brain was cut into two halves, the first half was homogenized (1:5 w/v) with cold 0.3 *M* perchloric acid and centrifuged at 10,000 *g* for 5 min. The supernatant was used for the GSH/GSSG assay. The other half of the brain was homogenized (1:5 w/v) in 20 *mM* Tris-HCl, pH 7.4 containing 1 *mM* EDTA. An aliquot of the homogenate was used for the assay of GP. GR activity in the brain homogenate was measured as described by us earlier [18].

Assays

The GSH, GSSG content and GR activity were measured by the method described by us earlier [18]. GP activity was measured by the method of Wendel [31]. Protein concentration was determined by the method of Lowry et al. [13].

Statistical Analysis

Data were analyzed by an unpaired two-tailed t test, ANOVA followed by a post hoc Bonferroni test; results were considered significant at $p < 0.05$.

Results

Thirty minutes of carotid artery ligation followed by 30 min of recirculation significantly reduced brain GSH (1.53 ± 0.06 μmol g^{-1} tissue; $p < 0.001$) with a concomitant increase of GSH (0.0042 ± 0.0002 μmol g^{-1} tissue; $p < 0.01$) content (fig. 1A,B). GSH/GSSG ratio decreased to 364 in the experimental group as compared to the con-

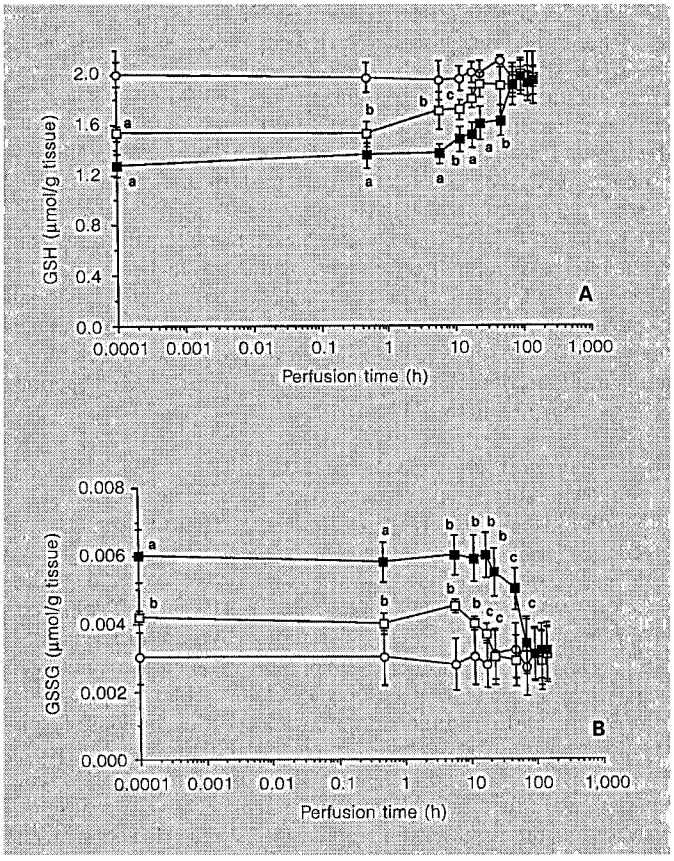

Fig. 1. GSH (A) and GSSG (B) content of rat brain following 30 min of BCAL and reperfusion (□) and BCAL-DSF administration followed by reperfusion (■); ○ = control. Values (µmol g^{-1} tissue; mean ± SD of 4 experiments) were analyzed by ANOVA followed by the post hoc Bonferroni test. [a] $p < 0.001$; [b] $p < 0.01$; [c] $p < 0.05$.

Table 1. Rat brain glutathione and GR activity after 30 min of acute DSF administration (200 mg kg^{-1})

Parameter	Control (n = 4)	DSF treated (n = 4)
Total (GSH + GSSG) µmol g^{-1} tissue	1.970 ± 0.04	1.95 ± 0.04
GSH, µmol g^{-1} tissue	1.950 ± 0.02	1.60 ± 0.2***
GSSG, µmol g^{-1} tissue	0.0026 ± 0.0004	0.005 ± 0.0013**
GR activity[a]	120.0 ± 12.0	100.0 ± 6.0*
GSH/GSSG ratio	750	320

Values are mean ± SD.

p values for unpaired two-tailed t test were * $p < 0.02$, ** $p < 0.01$, *** $p < 0.001$.

[a] µmol NADPH oxidized min^{-1} mg^{-1} protein.

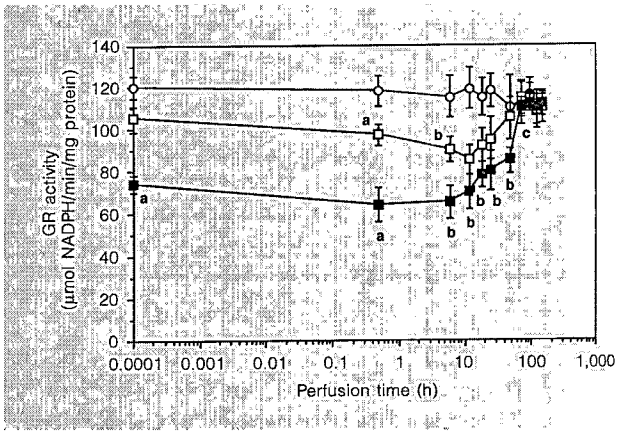

Fig. 2. GR activity (µmol NADPH oxidized min^{-1} mg^{-1} protein) of rat brain following 30 min of BCAL and reperfusion (□) and BCAL-DSF administration followed by reperfusion (■); ○ = control. Values (mean ± SD of 4 experiments) were analyzed by ANOVA followed by the post hoc Bonferroni test. [a] $p < 0.001$; [b] $p < 0.01$; [c] $p < 0.05$.

trol group's ratio of 667. This effect was consistent through 24 h of reperfusion. The group subjected to BCAL and DSF administration showed a significant decrease of GSH (1.27 ± 0.09 µmol g^{-1} tissue; $p < 0.001$) and an increase of GSSG (0.006 ± 0.0006 µmol g^{-1} tissue; $p < 0.001$) (fig. 1A, B).

GSH/GSSG ratio was reduced to 212 in the BCAL-DSF group as compared to control group's ratio of 667. DSF-induced modulation of GSH/GSSG homeostasis was observed through 96 h of reperfusion. DSF alone decreased GSH (1.60 ± 0.07 µmol g^{-1} tissue; $p < 0.001$) with a concomitant increase of GSSG (0.005 ± 0.0013 µmol g^{-1} tissue; $p < 0.01$) and GSH/GSSG ratio decreased to 320 in the DSF-treated group as compared to the control group's ratio of 750 (table 1). These values are comparable with the data obtained after BCAL-reperfusion. After 6 h of reperfusion, the recovery of GSH was not appreciable in both groups. In the group subjected to BCAL alone, GSH/GSSG returned to normal levels after 24 h of reperfusion and GSH/GSSG ratios were not significantly different from those of the controls. However, in rats subjected to both BCAL and DSF treatment, GSH/GSSG returned to normal levels only after 96 h of reperfusion. This clearly shows augmentation of oxidative stress by DSF (fig. 1A, B). Thirty minutes of BCAL alone did not affect GR, but GR activity was significantly decreased in animals subjected to BCAL and DSF treatment (74.3 ± 4.0 µmol min^{-1} mg^{-1} protein; $p < 0.001$) as

compared to the levels of the control rats (120 ± 5 μmol min^{-1} mg^{-1} protein) (fig. 2). GR acitivity was also decreased (100 ± 6 μmol min^{-1} mg^{-1} protein; $p < 0.02$) significantly in animals treated with DSF alone (table 1). GR activity in rats subjected to the BCAL alone returned to normal level after 72 h of reperfusion. However, in rats subjected to the synergistic effect of BCAL and DSF, it took 96 h of reperfusion before GR activity was restored. GP activity was not significantly altered in any of the groups studied (data not shown).

Discussion

A partial blood flow maintained through vertebral arteries in rats subjected to BCAL-reperfusion [6] was shown to cause oxidative stress and eventually ischemic neuronal injury [4]. The reduced efficiency of neurons in the ischemic brain to scavenge free radicals as a consequence of cerebral glutathione modulation and depletion of Cu-Zn SOD are possible key aspects leading to neurotoxic effects [30]. In fact, the neuronal injury caused by ischemia can be reduced by quenching the free radicals by enzymatic or nonenzymatic agents [10], pretreatment with vitamin E [33] and the administration of free-radical scavenging enzymes such as SOD and catalase [32]. The treatment with 21-aminosteroids (U74006F) was also found to have a beneficial effect on ischemic brain in gerbils subjected to transient carotid occlusion [9]. Thus, if free radicals are involved in initiating the process of ischemic brain damage, then GP activity should have been stimulated during the ischemic and postischemic periods, causing increased lipid peroxidation. Strangely enough, the GP activity did not change appreciably in this study. However, the role of free radicals in causing neuronal injury is not being ruled out. DSF is a lipophilic, oxidative drug which crosses the blood-brain barrier easily, more so in a brain subjected to BCAL [8] and may potentiate the oxidative stress if the brain GSH/GSSG homeostasis is perturbed [18].

The concentration of brain GSH/GSSG ratio in rats subjected to 30 min of BCAL and reperfusion was significantly lower than in controls. Cooper et al. [5] also found a decline of GSH but no change of GSSG under similar circumstances. The present study is unique because, we simultaneously determined postischemic GSH, GSSG, GR and GP activities which are all part of an antioxidant defense system. The central nervous system oxidative stress in our animals is reflected by an altered glutathione metabolic system. In earlier studies, DSF was shown to decrease GSH and increase GSSG content in rat brain [18], and precipitate oxidative stress in the brains subjected to ischemia [8].

Patients taking DSF develop severe aversive reactions, known as the DSF-ethanol reaction (DER) when challenged with ethanol [1]. One of the adverse reactions of DER is hypotension. In the event of a DER, there is a likelihood that a patient may experience hypotension accompanied by a partial blood flow to the brain. Therefore a prolonged DER may induce oxidative stress which may lead to neuronal damage. In the present study, the rats were administered DSF approximately 20 times higher than the human clinical dose of 8–10 mg kg^{-1} [1] in order to examine its acute effect. DSF at a clinically relevant dose may not induce oxidative stress in rats. However, a chronic administration of DSF (200 mg kg^{-1}) may induce an oxidative stress by depleting GSH [18]. Ohno et al. [21] also reported DSF-mediated loss of cell viability. The present study shows a delayed recovery of glutathione metabolism in rats subjected to the synergistic effect of DSF and BCAL. It appears from our study that DSF potentiates BCAL-reperfusion-induced oxidative stress.

DSF reacts readily with essential SH groups of proteins to form mixed disulfides [2]. It has been shown that glutathione reductase has 10 cysteine residues per monomer [3, 29], and that some of the SH groups may form mixed disulfides with DSF resulting in enzyme inactivation. Therefore, a decrease in brain GSH and increase in GSSG level as observed in the present study may be due to DSF-GSH-GR interactions [17, 18] which resulted in decreased GR activity to replenish GSH. Furthermore, in a recent study S-methyl N,N-diethylthiolcarbamate sulfoxide, a putative metabolite of DSF was shown to rapidly carbamoylate GSH [20]. Therefore, DSF may significantly reduce the tissue's ability to offset the oxidative insult by reducing GSH.

The neurotoxicity induced by ischemia is also emphasized either by an excessive activation of postsynaptic glutamate receptors or by an increased glutamate release due to reversed operation of the glutamate uptake carrier [28]. In a related study, we have reported DSF-induced reversal of carrier-mediated glutamate uptake in rat synaptosomes [14]. Ischemia has also been shown to be caused by increased levels of neurotransmitters, such as dopamine and serotonin, in rat models [24]. DSF was shown to interfere with neurotransmitter metabolism in rats [19]. Therefore, DSF may induce a neurotoxic effect by multifaceted physiological responses, such as excessive glutamate release, altered neurotransmitter metabolism and/or the inhibition of the antioxidant defensive system (GSH,

GR and SOD). Although the brain is susceptible to an oxidative insult caused by reduced blood flow, it appears to possess the capability to recover from an oxidative stress. This of course depends on the extent of damage. However, the present study has clearly demonstrated a BCAL-reperfusion-induced brain oxidative stress that is potentiated by DSF. Furthermore, a delayed recovery period of the defective glutathione metabolic system in rats subjected to BCAL-reperfusion and DSF treatment is a definite indicator of oxidative stress. To conclude, our study highlights the fact that DSF therapy can be associated with some neurotoxic effects as a consequence of drug-induced central nervous system oxidative stress.

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