Oxidative stress induced by administration of the neuroleptic drug haloperidol is attenuated by higher doses of haloperidol

Bangalore R. Shivakumar and Vijayalakshmi Ravindranath

Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences, Bangalore (India)

The effect of haloperidol administration on lipid peroxidation and glutathione/protein thiol homeostasis in the brain was examined 4 h following subcutaneous administration of a single dose of haloperidol; 1.0, 1.5, 2.0 or 2.5 mg/kg b.wt. Glutathione (GSH) levels decreased significantly in cortex, striatum and midbrain after haloperidol administration. Maximal decrease of GSH was observed in the striatum. The depleted GSH was recoverable as protein glutathione mixed disulfide (Pr-SSG) with concomitant loss of protein thiols (Pr-SH) in all the regions of the brain examined. Administration of 1.5 mg/kg b.wt. of haloperidol resulted in significant depletion of GSH in striatum and midbrain as compared to that after administration of the lower dose of 1.0 mg/kg b.wt. of haloperidol. However, administration of higher doses of haloperidol (2.0 and 2.5 mg/kg b.wt.) did not result in greater depletion of GSH; the GSH levels were not significantly different from that observed following the administration of 1.5 mg/kg b.wt. of haloperidol. However, Pr-SSG levels increased dose-dependently following haloperidol administration. The total GSH recovered as sum of GSH and Pr-SSG was significantly higher than controls in striatum and midbrain following administration of higher doses of haloperidol, namely, 2.0 and 2.5 mg/kg b.wt. The depleted GSH was not recoverable as glutathione disulfide (GSSG). GSSG levels were not significantly different from that after administration of 1.5 mg/kg b.wt. of haloperidol. The levels of malondialdehyde (indicative of lipid peroxidation) increased significantly as compared to control levels (280–220%) following administration of 1.0 and 1.5 mg/kg b.wt. of haloperidol. Thereafter, the malondialdehyde levels in brain regions decreased and were only (186–150%) of control levels after administration of 2.0 and 2.5 mg/kg b.wt. of haloperidol, respectively. The present study demonstrates that administration of low doses of haloperidol results in depletion of GSH and increased levels of malondialdehyde. However, administration of higher doses of haloperidol results in attenuation of peroxidative damage with concomitant increase in the total GSH recovered as sum of free GSH and GSH bound to protein thiols (Pr-SSG).

INTRODUCTION

Haloperidol, an antipsychotic drug, is commonly used for the treatment of schizophrenia[2]. Haloperidol has high affinity for the dopamine receptor (D_2 receptor) and administration of haloperidol results in blockade of D_2 receptor[29,30]. Administration of neuroleptics like haloperidol also results in the increased turnover of dopamine leading to increased production of hydrogen peroxide (H_2O_2) following metabolism of dopamine by monoamine oxidase[3,4,12]. One of the major side effects of neuroleptic therapy is the development of extrapyramidal symptoms following administration of the drug[17]. These extrapyramidal symptoms are alleviated following the administration of higher doses of haloperidol[10,11,18]. The biochemical mechanisms underlying the pathophysiology of these side

effects has been a subject of intense study and various hypotheses have been put forth[7,9,24]. Recent studies have focussed on the possible role of reactive oxygen species in the pathogenesis of extrapyramidal effects[8]. The generation of reactive oxygen species due to increased turnover of dopamine is a source of oxidative stress[5].

Glutathione (GSH), the major non-protein thiol in the cell, acts as an antioxidant and helps to maintain the protein-thiol homeostasis in the cell[19]. Glutathione peroxidase plays an important role in the detoxification of hydrogen peroxide with the concomitant oxidation of GSH to its oxidized form GSSG. Oxidized glutathione which is normally present in the cells in very low amounts is reduced back to GSH by glutathione reductase utilizing reducing equivalents of NADPH[16]. If the production of GSSG overwhelms the NADPH

Correspondence: V. Ravindranath, Department of Neurochemistry, NIMHANS, Hosur Road, Bangalore 560 029, India. Fax: (91) (812) 643130.

supply in the cell, the GSSG/GSH homeostasis is disturbed. Thus, increase in GSSG is often an indication of oxidative stress.

Administration of haloperidol (1.0 mg/kg b.wt.) to mice has been shown to result in increased levels of GSSG in striatum, 1 h after dosage. This increase was alleviated by the administration of monoamine oxidase inhibitor deprenyl, indicating that the oxidative stress was probably generated following haloperidol administration, which could be prevented by inhibition of dopamine turnover[5].

The present study was carried out to determine the GSH-protein thiol homeostasis following administration of varying doses of haloperidol. The formation of reactive oxygen species was also measured using the fluorescent probe (2',7'-dichlorofluorescin diacetate)[14] and the levels of malondialdehyde (MDA) were determined as an estimate of lipid peroxidation products.

MATERIALS AND METHODS

Materials

Reduced glutathione (GSH), oxidized glutathione (GSSG), NADPH, glutathione reductase, o-phthaldialdehyde, dithiothreitol, iodoacetic acid and 5,5, dithio bis-(2-nitrobenzoic acid) were obtained from Sigma Chemical Co., St. Louis, MO, USA. 2',7'-Dichlorofluorescin diacetate (DCFH-DA) and 2',7'-dichlorofluorescein (DCF) were obtained from Molecular Probes Inc. (Eugene, OR, USA). Haloperidol (injectable ampoules; 5 mg/ml) was obtained from Searle India Ltd., Bombay, India. All other reagents were of analytical grade.

Animals

The experiments were performed on Sprague–Dawley rats weighing 200–250 g and aged 3–4 months, obtained from the Central Animal Research Facility of the Institute. Animals had free access to pelletted diet (Lipton India Ltd., Calcutta, India) and water ad libitum. Haloperidol was diluted in saline and administered subcutaneously to groups of rats at doses of 1.0, 1.5, 2.0 and 2.5 mg/kg b.wt., respectively. Control animals, which received vehicle alone were incorporated in each experiment. After 4 h, the animals were anesthetized with ether and perfused transcardially with ice-cold normal saline (20 ml) to remove blood from the brain tissue. The rats were decapitated, brains were rapidly removed, and the following regions were dissected out: cortex (CT), striatum (ST) and midbrain (MB). The tissues were frozen immediately in liquid nitrogen.

Assay of GSH and GSSG

Brain regions (CT, ST and MB) from one half of the cerebral hemisphere were weighed in frozen condition and homogenized in 2 vols. of perchloric acid (10% v/v containing 1.0 mM EDTA). The homogenate was centrifuged at 8,000×g for 2 min. One aliquot of the supernatant was used for the assay of total GSH by enzymatic recycling method[31]. An aliquot of the acid supernatant (0.1 ml) was transferred to an Eppendorf tube containing solid sodium bicarbonate (~ 50 mg). Immediately 10 μl of iodoacetic acid (0.2 M in water) was added. The tube was kept in dark at room temperature for 1 h. Later the contents of the tubes were centrifuged and an aliquot was used for analysis of GSSG by enzymatic recycling method[25]. GSSG levels were also measured following derivatization of GSH with N-ethylmaleimide as described[26].

Assay of protein glutathione mixed disulfides

The levels of protein glutathione mixed disulfide (Pr-SSG) in the acid precipitated pellet was measured as described[1,21]. The pellet was washed twice in ethanol (1.0 ml) and sonicated for 4 min. The pellets were resuspended in 4-morpholinepropanesulfonic acid buffer (MOPS, 50 mM, pH 8.0) containing dithiothreitol (25 mM). The mixture was sonicated for 5 min and incubated for 1 h at 37°C. Later the samples were deproteinized with perchloric acid (70% v/v), mixed and frozen in liquid nitrogen, thawed and centrifuged. An aliquot of the supernatant was treated with o-phthaldialdehyde and the reaction mixture was kept in dark at room temperature for 10 min. The fluorescence was recorded following excitation at 350 nm and emission at wavelength 420 nm in an Aminco Bowman spectrophotofluorometer. The amount of GSH equivalents present as Pr-SSG was quantified from a standard curve of GSH.

Assay of protein thiols

The brain regions from the other half of the cerebral hemisphere were homogenized in 9 vols. of potassium phosphate buffer (0.1 M, pH 7.4). An aliquot of the homogenate was treated with an equal volume of perchloric acid (5% v/v) centrifuged and the concentration of protein thiol was estimated in the acid precipitated pellet as described earlier[6].

Aliquots of the remaining homogenate were used for the assay of malondialdehyde, measured as thiobarbituric acid reacive products[20].

An aliquot of the homogenate was incubated with 2',7'-dichlorofluorescin diacetate (5 μM in methanol) for 15 min at 37°C for the estimation of reactive oxygen species. The reaction was terminated by chilling the reaction mixture in ice. The formation of fluorescent oxidized derivative, namely, 2',7'-dichlorofluorescein was monitored using an excitation wavelength of 488 nm and emission wavelength of 525 nm in an Aminco Bowman spectrophotofluorometer. The equivalent levels of oxygen radicals was quantified from a DCF standard curve[14].

Data were analysed using one-way analysis of variance (ANOVA) and Duncan's test. Values are represented as mean ± S.D.

RESULTS

Following administration of a single dose of haloperidol (1.0 and 1.5 mg/kg b.wt.), GSH levels in cortex were 85 and 83% of control levels (Fig. 1A). Administration of a higher dose (2.0 mg/kg b.wt.) did not result in further GSH loss and the GSH levels were 84% of controls. Substantial amount of the depleted GSH was recoverable as Pr-SSG. Pr-SSG levels increased dose-dependently in the cortex as shown in Fig. 1B. The amount of GSH gained as Pr-SSG following lower doses of haloperidol (1.0 and 1.5 mg/kg b.wt.) was 0.33 and 0.37 μmol of GSH equivalent/g tissue over the control levels. An increase in the dose (2.0 mg/kg b.wt.) resulted in an increase in the amount of Pr-SSG formed and 0.45 μmol of GSH equivalents/g tissue was recovered as Pr-SSG. The formation of Pr-SSG was accompanied by the loss of protein thiols (Pr-SH). The Pr-SH lost in cortex (Fig. 1C) following 1.0 and 1.5 mg/kg b.wt. of haloperidol was 1.17 and 1.37 μmol of thiol equivalent/g tissue. An increase in the dose (2.0 mg/kg b.wt.) resulted in lesser loss of protein thiols (1.13 μmol of thiol equivalent/g tissue). Further increase in the dose (2.5 mg/kg b.wt.) resulted in lower loss of protein thiols (0.78 μmol of thiol

equivalent/g tissue). The total GSH recovered (GSH + Pr-SSG) was not significantly different from untreated controls following administration of 1.0 and 1.5 mg/kg b.wt. of haloperidol in the cortex (Fig. 1D). The total GSH levels recovered in the cortex were 108 and 106% of control levels after administration of 2.0 and 2.5 mg/kg b.wt. of haloperidol, respectively.

Following subcutaneous administration of a single dose of haloperidol (1.0 and 1.5 mg/kg b.wt.), GSH levels in the striatum were 76 and 74% of controls (Fig. 2A). On increasing the dose (2.0 and 2.5 mg/kg b.wt.), the GSH levels were 74 and 78% of control levels. The depleted GSH was recoverable as Pr-SSG as shown in Fig. 2B. The Pr-SSG levels increased in a dose-dependent manner following administration of increasing
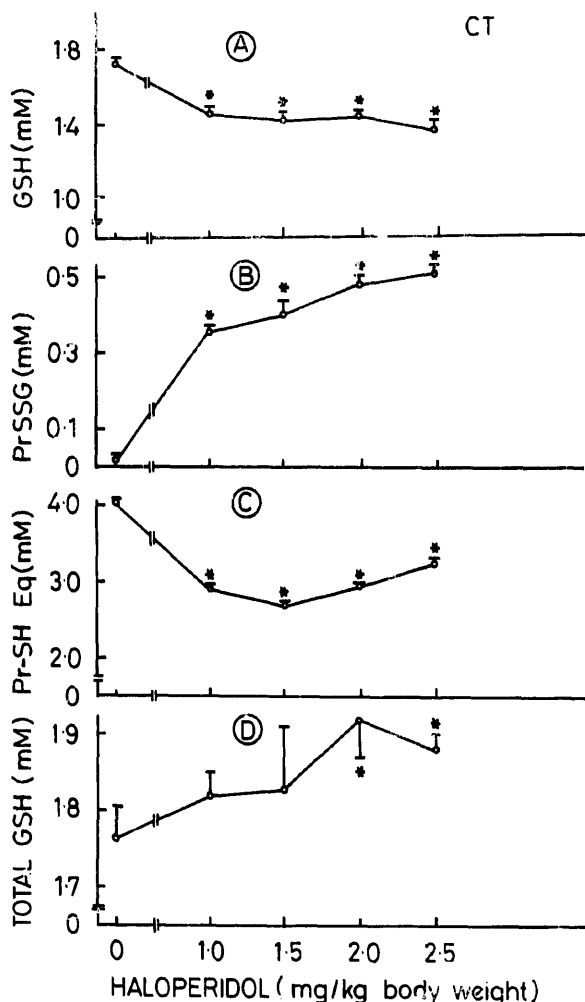

Fig. 1. The effect of haloperidol administration on cortical concentrations of (A) GSH, (B) Pr-SSG, (C) Pr-SH and (D) total GSH (GSH + Pr-SSG) following administration of increasing doses of haloperidol. Rats were administered varying doses of haloperidol (1.0, 1.5, 2.0 and 2.5 mg/kg b.wt.) subcutaneously. Animals were sacrificed 4 h following the dosage. Values are expressed as μmol of GSH equivalent/g tissue (mM) and are mean ± S.D. (n = 4–11, except for the dose of 1.0 mg/kg b.wt. of haloperidol, wherein 3 animals were used). Asterisks indicate values significantly different from corresponding controls (P < 0.05).
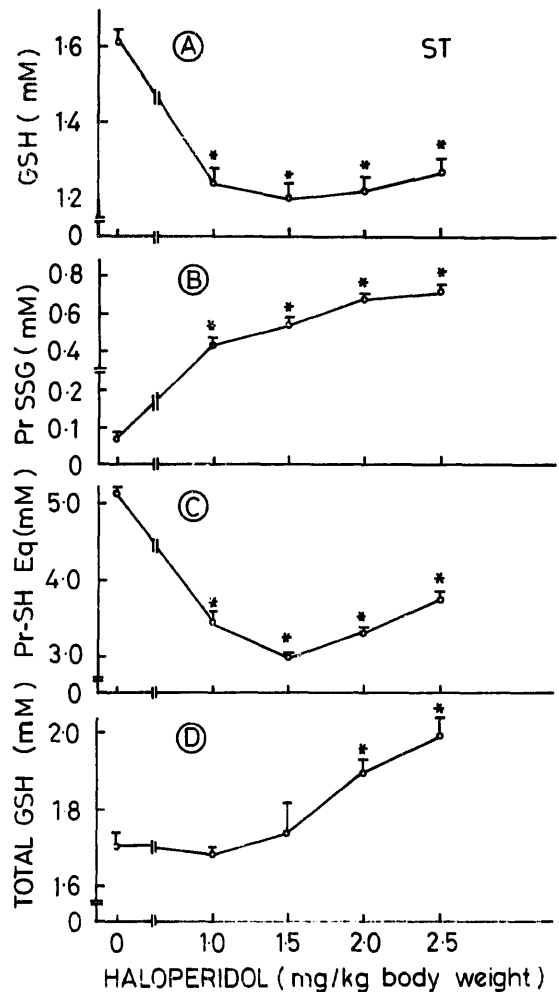

Fig. 2. The effect of haloperidol administration on striatal concentrations of (A) GSH, (B) Pr-SSG, (C) Pr-SH and (D) total GSH (GSH + PrSSG) following administration of increasing doses of haloperidol. Rats were administered varying doses of haloperidol (1.0, 1.5, 2.0 and 2.5 mg/kg b.wt.) subcutaneously. Animals were sacrificed 4 h later following the dosage. Values are expressed as μmol of GSH equivalent/g tissue (mM) and are mean ± S.D. (n = 4–11, except for the dose of 1.0 mg/kg b.wt. of haloperidol wherein 3 animals were used). Asterisks indicate values significantly different from controls (P < 0.05).

doses of haloperidol (1.0, 1.5, 2.0 and 2.5 mg/kg b.wt.). The levels were 0.37 and 0.65 μmol of GSH equivalent/g tissue as compared to control levels of 0.07 μmol of GSH equivalent/g tissue. The amount of protein thiols lost in the striatum was also dose-dependent following administration of 1.0 and 1.5 mg/kg b.wt. of haloperidol as shown in Fig. 2C. The concentration of protein thiols were 68 and 57% of control levels. In contrast, after administration of higher doses of haloperidol (2.0 and 2.5 mg/kg b.wt.) the levels of protein thiols were 64 and 73% of corresponding controls indicating a recovery of protein thiol status. Further, the total GSH recovered as sum of GSH and Pr-SSG was not significantly different from untreated controls following administration of 1.0 and 1.5 mg/kg

b.wt. of haloperidol in the striatum (Fig. 2D). However, the concentrations of total GSH recovered was significantly higher (112 and 117% of control) in the striatum following administration of higher doses of haloperidol (2.0 and 2.5 mg/kg b.wt.).

The levels of MDA in the cortex (indicative of lipid peroxidation) increased significantly as compared to untreated controls (228–220%) following administration of 1.0 and 1.5 mg/kg b.wt. of haloperidol (Fig. 3A). After administration of 2.0 and 2.5 mg/kg b.wt. of haloperidol they were 186–170% of controls. The reactive oxygen species were measured using the fluorescent probe 2′,7′-dichlorofluorescin diacetate. The levels of oxy-free radicals measured as 2′,7′-dichlorofluorescein were 153 and 202% after 1.0 and 1.5 mg/kg b.wt. of haloperidol. However, the levels of 2′,7′-dichlorofluorescein decreased significantly and were 126 and 109% of controls after administration of 2.0 and

Fig. 4. The effect of haloperidol administration on the concentration of (A) GSH, (B) Pr-SSG and (C) total GSH (GSH+PrSSG) in midbrain following adminstration of increasing doses of haloperidol. Rats were administered varying doses of haloperidol (1.0, 1.5, 2.0 and 2.5 mg/kg b.wt.) subcutaneously. Animals were sacrificed 4 h later. Values are expressed as mean ± S.D. ($n = 4-11$, except for the dose of 1.0 mg/kg b.wt. of haloperidol wherein 3 animals were used). Asterisks indicate values significantly different from corresponding controls ($P < 0.05$).
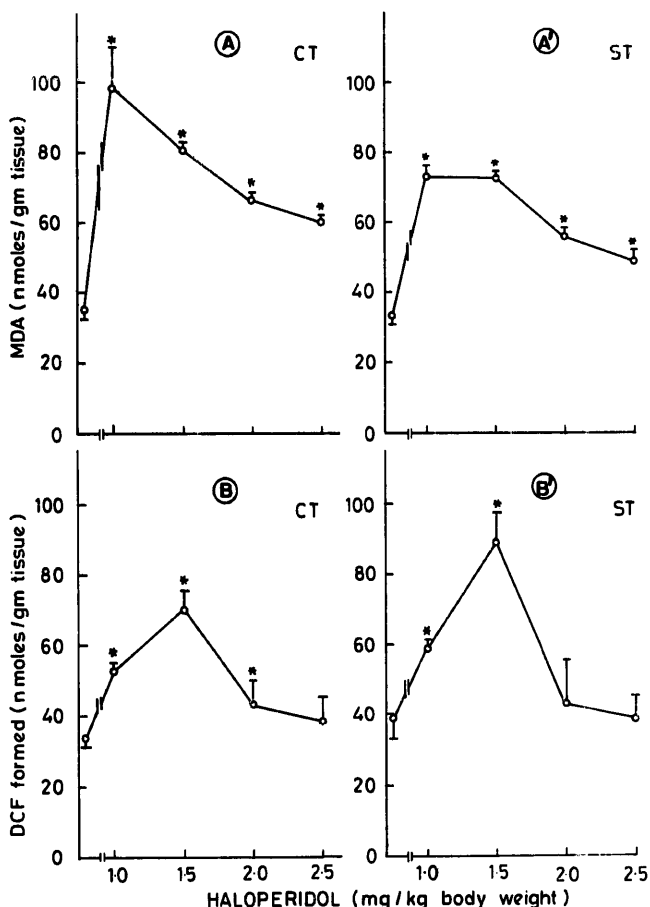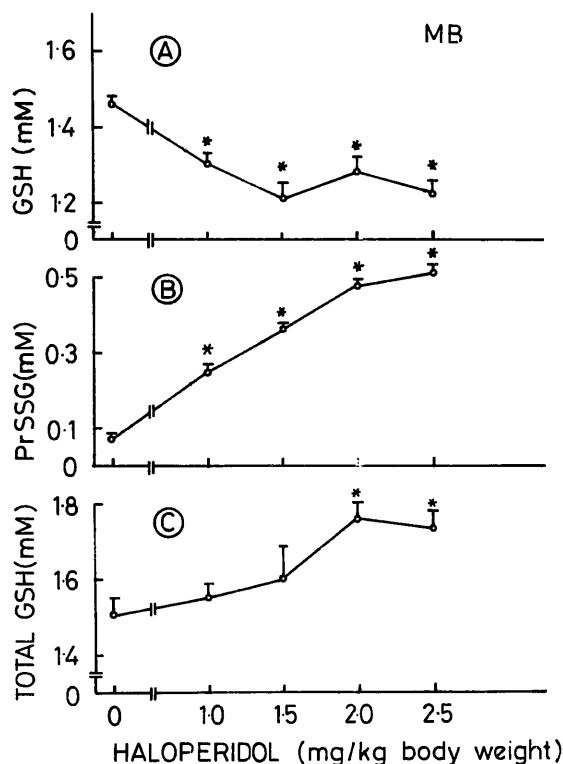

Fig. 3. The effect of haloperidol administration on cortical (CT) and striatal (ST) concentrations of MDA, (A and A′) and formation of reactive oxygen species (B and B′), following administration of varying doses of haloperidol. Rats were administered varying doses of haloperidol (1.0, 1.5, 2.0 and 2.5 mg/kg b.wt.) subcutaneously. Animals were sacrificed 4 h later following the dosage. Values are expressed as nmol/g tissue and are mean ± S.D. ($n = 4-8$, except for the dose of 1.0 mg/kg b.wt. of haloperidol wherein 3 animals were used). Asterisks indicate values significantly different from untreated controls ($P < 0.05$).

2.5 mg/kg b.wt. of haloperidol (Fig. 3B). The levels of MDA in the striatum were 223 and 221% of controls, following administration of 1.0 and 1.5 mg/kg b.wt. of haloperidol (Fig. 3A′). Following an increase in the dose (2.0 and 2.5 mg/kg b.wt.) the MDA levels decreased and were 170 and 149% of untreated controls. The generation of reactive oxygen species measured as the formation of the fluorescent 2′,7′-dichlorofluorescein increased with increasing doses of haloperidol up to 1.5 mg/kg b.wt. (150–225%). Further increase in the dose of haloperidol (2.0 and 2.5 mg/kg b.wt.) resulted in decreased production of reactive oxygen species (Fig. 3B′) and the levels were not significantly different from control.

In the midbrain of animals treated with haloperidol (Fig. 4A), maximal depletion of GSH was observed following a dose of 1.5 mg/kg b.wt. of haloperidol. The GSH levels observed were 83% of untreated controls. However, the levels of GSH did not decrease further following higher doses of haloperidol (2.0 and 2.5 mg/kg b.wt.) and were 88% and 83% of corresponding controls. The loss of GSH was accompanied by an

TABLE I

Glutathione disulfide (GSSG) concentration in rat striatum following treatment with haloperidol

Sprague-Dawley rats were administered a single dose of haloperidol (1.5 mg/kg b.wt.) subcutaneously. Animals were sacrificed after (A) 1 h or (B) 4 h and GSSG concentration in striatum was measured as described[25]. Values were mean ± S.D. ($n = 5$).

Expt.	Control	GSSG ($\mu mol/g$ tissue) haloperidol	Percent increase
A	0.017 + 0.002	0.035 + 0.003 *	206%
B	0.016 + 0.004	0.018 + 0.002	n.s.

* Indicates values significantly different from conrols ($P < 0.05$) and n.s. indicates values not significantly different from controls.

increase in the formation of Pr-SSG as shown in Fig. 4B. The Pr-SSG levels increased in a dose-dependent manner in the midbrain. The levels of Pr-SSG were 0.18 and 0.29 μmol of GSH equivalent/g tissue following doses of 1.0 and 1.5 mg/kg b.wt. of haloperidol. Increasing the dose (2.0 and 2.5 mg/kg b.wt.) led to increase in Pr-SSG levels (0.41 and 0.44 μmol of GSH equivalent/g tissue. The total GSH recovered as sum of GSH and Pr-SSG was compared with corresponding controls. The total GSH recovered was not significantly different from controls following administration of 1.0 and 1.5 mg/kg b.wt. of haloperidol. However, the total GSH recovered was higher than the controls in the midbrain (Fig. 4C) following administration of higher doses of haloperidol namely, 2.0 and 2.5 mg/kg b.wt. (116 and 114%, respectively).

Although GSH content decreased the GSSG levels did not increase significantly 4 h after haloperidol dosage as indicated in Table I. The GSSG concentration, however, increased in the striatum, 1 h after administration of haloperidol (1.5 mg/kg b.wt.) and was 206% of control as shown in Table I. The depleted GSH, recovered as GSSG, was 7% of the GSH lost at this time point. The GSH level in striatum 1 h after administration of 1.5 mg/kg b.wt. of haloperidol was 1.11 μmol of GSH/g tissue (data not shown) which indicated the depletion of 31% of the GSH levels present in untreated rat brain striatum.

DISCUSSION

Administration of increasing doses (1.0, 1.5, 2.0 and 2.5 mg/kg b.wt.) of haloperidol did not result in dose dependent decrease of GSH in striatum, cortex and midbrain. Maximal depletion (26%) was noted in the striatum following administration of 1.5 mg/kg b.wt. of haloperidol. The GSH lost was not recoverable as GSSG. The GSSG levels were not significantly different from untreated controls, 4 h after administration of

haloperidol (1.5 mg/kg b.wt.), although, GSSG levels were significantly increased (206%), 1 h following administration of haloperidol (1.5 mg/kg b.wt.). This is in concurrence with earlier report wherein GSSG levels were observed to be significantly higher following haloperidol administration to mice[5]. The GSSG increase was transient and was not observed 4 h after dosage although at this time enhanced oxidative stress was apparent as determined by increased MDA and Pr-SSG levels. The absence of elevation of GSSG levels may not always reflect lack of oxidative stress as demonstrated in the present study. It is therefore necessary to examine GSH and Pr-SSG levels in addition to determining GSSG concentration.

The depleted GSH was essentially recovered as Pr-SSG in all the regions of brain that were examined. This is similar to the observation made with brain mitochondria subjected to oxidative stress by treatment with t-butyl hydroperoxide[23]. The depleted GSH was recovered essentially as Pr-SSG and less than 5% of the depleted GSH was recovered as GSSG in the mitochondria. During severe hepatic oxidative stress when the NADPH levels are not sufficient to reduce all of the GSSG formed to GSH (through glutathione reductase), the GSSG is effluxed out of the cell, thus preventing the formation of excessive Pr-SSG and Pr-SS-Pr which can lead to membrane damage. Thus, in hepatocytes subjected to oxidative stress, the formation of Pr-SSG accounts for only 15% of the GSH depleted in liver[6]. In the brain, even under less severe oxidative stress, Pr-SSG formation accounts for over 90% of the depleted GSH. The formation of protein mixed disulfides may have far reaching consequences by affecting neuronal functions.

The Pr-SSG levels increased linearly with increasing doses of haloperidol in both striatum and midbrain. The total GSH recovered as GSH and Pr-SSG was calculated and compared with corresponding controls. The amount of total striatal GSH recovered (GSH + Pr-SSG) was not significantly different from controls following administration of 1.0 and 1.5 mg/kg b.wt. of haloperidol, but increased significantly (112 and 117% of control) after a dose of 2.0 and 2.5 mg/kg b.wt. of haloperidol. This indicates that a threshold level exists in the brain and depletion of GSH beyond that level results in increased recovery of GSH, probably due to increased synthesis of GSH. Similar threshold levels are also observed in liver, wherein depletion of about 80% hepatic GSH results in rebound of GSH due to increased synthesis[13,22]. However, in the brain increased recovery is observed following depletion of about 25% of the GSH present in brain. The highest recovery of GSH was observed in striatum and mid-

brain (sites of haloperidol action), while the recovery in the cortex was only 108% of corresponding levels of GSH in control.

The formation of Pr-SSG was accompanied by loss of protein thiols (Pr-SH). The amount of Pr-SH lost in striatum was significantly higher than the amount of Pr-SSG formed indicating the formation of protein mixed disulfide (Pr-SS-Pr). Concurrent with the increased recovery of GSH, the amount of Pr-SH lost also decreased.

A similar trend was observed in the levels of malondialdehyde. The MDA levels in striatum were 223 to 221% of control after 1.0 and 1.5 mg/kg b.wt. of haloperidol and they decreased substantially (150% of control) after administration of 2.5 mg/kg b.wt. of haloperidol. The measurement of reactive oxygen species also revealed an increase in the fluorescent derivatives with increasing doses of haloperidol up to 1.5 mg/kg b.wt. and thereafter they decreased and were not significantly different from the controls following administration of 2.0 and 2.5 mg/kg b.wt. of haloperidol. While the use of 2',7'-dichlorofluorescin diacetate gives a measure of reactive oxygen species, MDA measures the product of lipid peroxidation and this may be the reason that reactive oxygen species as measured by 2',7'-dichlorofluorescin diacetate were similar to controls after 2.0 and 2.5 mg/kg b.wt. of haloperidol, while MDA levels showed a more gradual decrease.

The present study demonstrates that administration of haloperidol results in production of oxidative stress as demonstrated by decreased GSH levels and increased Pr-SSG level and MDA levels, and reactive oxygen species generation. However, administration of higher doses of haloperidol results in increased recovery of GSH with concomitant decrease in oxidative stress. An inverse relationship is known to exist between the dose of haloperidol administered and the observation of extrapyrmidal symptoms. Thus, higher doses of haloperidol result in lower incidence of extrapyrimidal symptoms[28]. Whether this clinical phenomenon observed in human population is related to the changes in GSH homeostasis remains to be established. Nevertheless the present study clearly demonstrates that haloperidol administration generates oxidative stress. Hence, co-administration of antioxidants like vitamin E may help in overcoming this oxidative stress[15].

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