HHS Public Access

Author manuscript

Biometals. Author manuscript; available in PMC 2019 October 18.

Published in final edited form as:

Biometals. 2018 August ; 31(4): 561–570. doi:10.1007/s10534-018-0104-8.

Iron overload prevents oxidative damage to rat brain after chlorpromazine administration

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Abstract

The hypothesis tested is that Fe administration leads to a response in rat brain modulating the effects of later oxidative challenges such as chlorpromazine (CPZ) administration. Either a single dose (acute Fe overload) or 6 doses every second day (sub-chronic Fe overload) of 500 or 50 mg Fe-dextran/kg, respectively, were injected intraperitoneally (ip) to rats. A single dose of 10 mg CPZ/kg was injected ip 8 h after Fe treatment. DNA integrity was evaluated by quantitative PCR, lipid radical (LR) generation rate by electron paramagnetic resonance (EPR), and catalase (CAT) activity by UV spectrophotometry in isolated brains. The maximum increase in total Fe brain was detected after 6 or 2 h in the acute and sub-chronic Fe overload model, respectively. Mitochondrial and nuclear DNA integrity decreased after acute Fe overload at the time of maximal Fe content; the decrease in DNA integrity was lower after sub-chronic than after acute Fe overload. CPZ administration increased LR generation rate in control rat brain after 1 and 2 h; however, CPZ administration after acute or sub-chronic Fe overload did not affect LR generation rate. CPZ treatment did not affect CAT activity after 1–4 h neither in control rats nor in acute Fe-overloaded rats. However, CPZ administration to rats treated sub-chronically with Fe showed increased brain CAT activity after 2 or 4 h, as compared to control values. Fe supplementation prevented brain damage in both acute and sub-chronic models of Fe overload by selectively activating antioxidant pathways.

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Keywords

Brain; Oxidative stress; Fe; Chlorpromazine; Antioxidant; Electron paramagnetic resonance

Introduction

Chlorpromazine (CPZ) is a typical or first generation antipsychotic drug widely used to treat schizophrenia and other psychotic disorders such as bipolar disorder, severe anxiety, psychotic aggression, and others (Brunton et al. 2010; Breitbart et al. 1996). Although second generation antipsychotic drugs were developed, and are frequently used in clinical treatment, CPZ is still widely used. Even though meta-analyses of clinical trials in participants with chronic schizophrenia have suggested a limited advantage of the newer agents in terms of efficacy (Crossley et al. 2010), none of them have surpassed the effectiveness of CPZ (Ban 2004; Lieberman et al. 2005). However, it was recognized that CPZ may lead to metabolic disorders due to changes in lipid and glucose metabolism, which are associated with increasing body weight, higher risk for diabetes, hypertension and cardiovascular disease (Henderson 2004; McIntyre et al. 2001; Newcomer 2004).

Iron (Fe) plays a fundamental role in the brain, participating in neurotransmission, myelination and cell division (Moos et al. 2007). Fe homeostasis is maintained through highly regulated mechanisms of uptake, storage and secretion (Mills et al. 2010). The accumulation of transition metals in the nervous system seems to be involved in different neurodegenerative pathologies such as Alzheimer's and Parkinson's diseases, and neuroferritinopathies, among others (Salvador et al. 2011). The deficit in Fe availability may also contribute to cause neurological disease (Salvador et al. 2011). Even though increasing evidence points out towards the participation of Fe in oxidative stress-damaging events to macromolecules and protein aggregation, oxidative stress triggered by Fe overload was recently reported to be followed by beneficial effects (Piloni et al. 2016). This pattern of response could be described as a hormetic effect.

The alterations to cellular oxidative metabolism triggered by Fe critically depend on its administration pattern. On this topic, Table 1 briefly summarizes data from our laboratory describing the differential effects in brain of Fe-dextran administration, both in an acute (one dose of 500 mg Fe-dextran/kg body weight ip) or sub-chronic (6 doses of 50 mg Fe-dextran/kg body weight ip over a 12 days' treatment) regimen. Significant Fe overload was achieved by both treatments, with an increase in the labile Fe pool (LIP) and the activity of the antioxidant enzyme CAT, although a kinetic difference was seen regarding the time required to get the maximum response. Moreover, the effect of Fe overload on the rate of generation of LR and on the nuclear factor kappa B (NF-κB) DNA binding capacity was different in both models of Fe administration.

The hypothesis tested here was that the pattern of administration of Fe leads to a specific response by the antioxidant network in the rat brain that could modulate the effect of later oxidative challenges (such as CPZ) on cells. To test this hypothesis, the effect of both acute and sub-chronic Fe overload treatment on rat brain DNA integrity, and LR generation rate

was studied. In addition, the effect of CPZ supplementation after Fe overload on LR generation rate and antioxidant capacity in the brain was characterized.

Materials and methods

Animal preparation

Male Sprague–Dawley rats (180 ± 10 g, 45 ± 5 days old), from the Animal Facility of the School of Pharmacy and Biochemistry of the University of Buenos Aires, were used. The animals were housed under standard conditions of light, temperature and humidity with unlimited access to water and food. Acute Fe overload was developed by injecting a single dose of 500 mg Fe-dextran/kg body weight ip. Sub-chronic Fe overload was achieved by 6 doses of 50 mg Fe-dextran/kg body weight ip injected every second day. In both protocols, control rats were sham-injected ip with saline solution.

A single dose of 10 mg CPZ/kg body weight (Sigma Aldrich) was injected ip either 8 h after the single Fe-dextran dose or the 6th dose of Fe-dextran in the acute and sub-chronic Fe treatment, respectively. At specific time points after CPZ administration, animals were anesthetized in a CO_2 chamber, and the whole brain was rapidly removed and immediately frozen and stored under liquid N_2. Experimental animal protocols and animal procedures complied with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 6–23, revised 1985) and were carried out according to the principles and directives of the European Communities Council Directives (86/609/EEC). The procedures also received approval from the Institutional Animal Care and Use Committee-Facultad de Farmacia y Bioquímica (CICUAL-FF_yB, RES N°81037).

Total Fe content

Brain samples, dried in an oven at 60 °C until constant weight, were mineralized in HNO_3 according to Laurie et al. (1991). Fe content was spectrophotometrically determined after reduction with thioglycolic acid by measuring the absorbance (λ = 535 nm) in the presence of bathophenanthroline according to Brumby and Massey (1967).

Detection of LR generation rate by Electron Paramagnetic Resonance (EPR)

LR generation rate was detected by a spin trapping technique using N-t-butyl-α-phenyl nitrone (PBN). A 40 mM PBN stock solution was prepared in dimethyl sulfoxide (DMSO) immediately prior to use. Brain tissue was homogenized in DMSO-PBN (stock solution), incubated for 30 min and immediately transferred to a Pasteur pipette for LR detection. Instrument settings were as follows: modulation frequency 50 kHz, microwave power 10 mW, microwave frequency 9.75 GHz, centered field 3487 G, time constant 81.92 ms, modulation amplitude 1.20 G and sweep width 100 G, according to Lai et al. (1986). Quantification of the spin adduct was performed using TEMPO introduced into the same sample cell used for spin trapping. EPR spectra for both sample and TEMPO solutions were recorded at exactly the same spectrometer settings and the first derivative EPR spectra were double integrated to obtain the area intensity, then the concentration of spin adduct was calculated according to Kotake et al. (1996).

Determination of CAT activity in rat brain

Tissues were homogenized in 40 mM potassium phosphate buffer, 120 mM KCl (pH 7.4) and centrifuged at 6009g for 10 min to obtain the supernatant. CAT activity was assayed spectrophotometrically (k = 240 nm) by the decomposition of hydrogen peroxide (H_2O_2) in a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 (Aebi 1984). Protein was determined as described by Lowry et al. (1951).

DNA damage

Genomic DNA was extracted from frozen brain tissue using the DNEasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Genomic DNA concentration was determined by fluorescence labeling using the Pico Green dsDNA kit (Life Technologies, Grand Island, NY). Mitochondrial and nuclear DNA damage was determined by quantitative PCR (qPCR) as described in Santos et al. (2002). Briefly, genomic DNA was amplified in three separate PCR reactions, one amplifying a short 211 bp fragment of mitochondrial DNA, another amplifying a long13.4 kb fragment of mitochondrial DNA, and another amplifying a 12.5 kb long fragment of nuclear DNA. mtDNA integrity was determined by calculating the ratio of long mitochondrial PCR product to short mitochondrial PCR product, and normalizing it to the ratio obtained from control brains, which was set to 100%. Nuclear DNA integrity was determined by normalizing the amount of long nuclear DNA product to that of control brains, which was set at 100%.

Statistical analyses

Unless otherwise indicated, data in the text and tables are expressed as mean ± standard error of the media (SEM) of three to five independent experiments, with two replicates in each experiment. Statistical tests were carried out using Graph InStat, Student t test or one-way ANOVA followed by the Newman–Keuls test.

Results

Both total Fe content upon acute and sub-chronic Fedextran treatment were assessed in rat brain to confirm the efficiency of the treatment. Data in Table 2 show that Fe incorporation to the brain upon the initial 8 h after administration of Fe-dextran was in agreement with previous data (Piloni et al. 2013, 2016). Maximal total Fe content increased 7.7-and 7.6-fold after acute and sub-chronic Fe overload, respectively. In the acute Fe overload, the maximum significant increase in total brain Fe was detected after 6 h, while upon sub-chronic Fe treatment, the maximum significant increase in total Fe was detected after only 2 h of the 6th dose. Thus, Fe was effectively uptaken by the tissue in both protocols reaching approximately the same values at the maximum effect, but with a quite different kinetic profile.

Considering these differences in kinetics, the degree of damage to DNA integrity was studied as a function of time post-Fe administration following the acute or sub-chronic protocols. Quantitative PCR assays show that after 6 h of acute Fe overload (corresponding to the time to maximal Fe content), mitochondrial DNA integrity decreased by 65%, while nuclear DNA integrity decreased by 41% (Table 3). However, after sub-chronic Fe overload,

mitochondrial DNA integrity decreased by only 25% after 2 h post administration of the 6th Fe dose (corresponding to the time to maximal Fe content), and by 23% after 8 h, while no significant effects were seen on nuclear DNA integrity at any time point (Table 3).

The presence of LR in rat brain was determined with the spin trap PBN that combines with LR to form adducts that give a characteristic EPR spectrum (Buettner 1987). Data in Fig. 1a (trace a) show a computer-simulated signal obtained using hyperfine coupling constants of a_N = 15.8 G and a_H = 2.6 G. Due to the similarity of the corresponding coupling constants, spin trapping spectra cannot readily distinguish between peroxyl, alcohoxyl and alkyl adducts, but these constants could be assigned to lipid-derived radicals. No PBN adduct was observed in the absence of brain tissues (Fig. 1a, trace b). The generation rate of the LR adducts by control brain homogenates (Fig. 1a, trace c) was increased 1 and 2 h post administration of CPZ, returning to control values after 4 h (Fig. 1a, trace d–f, respectively). Quantification of the recorded spectra of LR generation rate is shown in Fig. 1b, post-acute CPZ treatment. The LR generation rate increased by 57 and 65% after 1 and 2 h of CPZ administration, respectively. However, when CPZ was administered after 8 h of an acute or sub-chronic Fe overload, no modifications were observed in the LR generation rate (Fig. 2).

A single dose of CPZ in control rats did not affect the activity of the antioxidant enzyme CAT after 1–4 h of administration (Fig. 3a). In the same way, data in Fig. 3b show that a single dose of CPZ in rats after 8 h of acute Fe overload did not modify CAT activity. In contrast, a single dose of CPZ in rats after 8 h of sub-chronic Fe treatment induced a significant, time dependent increase in CAT activity (Fig. 3b).

Discussion

It is well known that oxidative stress is implicated in the pathophysiology of many neurological diseases, among others, where cellular components such as lipids, proteins and DNA, considered in the present study can be damaged. Not only reactive oxygen species, such as H_2O_2, but also the products of lipid peroxidation could damage these components (Dejanović et al. 2016). Damage to molecules can be prevented because antioxidant enzimes provide adequate protection against reactive oxygen and nitrogen species harmful effects on all kinds of biomolecules. According to Bubber et al. (2004), there is evidence that the oxidative stress and mitochondrial pathology may be the most critical concerns in the pathophysiology of schizophrenia. Changes in oxidative defense mechanisms derived from both conditions in patients with schizophrenia may be exacerbated by the treatment with antipsychotics having pro-oxidant properties (Mahadik et al. 2001; Dejanović et al. 2016). CPZ is still used because of its effectiveness in spite of the alternative of using other antipsychotics recommended for the treatment of schizophrenia. Common side effects of CPZ include tardive dyskinesia, extrapyramidal effects, hepatotoxicity, agranulocytosis, hyperprolactinemia, and skin and ocular disorders (Otręba et al. 2015; Parikh et al. 2003). In melanocytes, Otręba et al. (2015) found that CPZ in concentrations of $1 * 10^{-7}$ and $1 * 10^{-6}$ M caused, in a doses-dependent manner, cytotoxicity and an increase in the formation of free radicals, assessed by EPR. Moreover, the oxidative stress produced in brain due to CPZ seems to be important in producing motor defects (Sandhu and Rana 2013). On the other hand, Fe-dextran treatment has been recently characterized as a possible oxidative stress

trigger, followed by an antioxidant response as evidenced by the significant antioxidant response achieved in rat brain (Piloni et al. 2013, 2016). Moreover, Galleano et al. (2011) observed beneficial effects in rat liver after a sub-chronic Fe-dextran treatment. These findings can be observed in a doses-dependent manner.

The term hormesis has been most widely used in toxicology to describe a biphasic dose–response showing stimulation or beneficial effects at low doses and inhibitory or toxic effects at higher doses (Mattson 2008). On the other hand, in biology and medicine, hormesis is understood as an adaptive response of cells and organisms to a moderate stress (Mattson 2008). Hormetic responses signaling pathways typically involve the activity of enzymes, such as kinases and deacetylases, and transcription factors, such as nuclear factor erythroid-2-related factor 2 (Nrf-2) and NF-κB (Mattson 2008; Piloni et al. 2013).

Our previous data leads us to ask if it is possible to reduce the adverse effects derived from CPZ use, treating animals with Fe-dextran, previous to the administration of CPZ. The application of an acute or sub-chronic Fe-dextran treatment prior to acute CPZ treatment, triggered a hormetic effect that is supported by the following evidence:

a. Our results show that sub-chronic Fe overload occurred with lower DNA (mitochondrial and nuclear) damage than acute Fe overload (Table 3). It is known that Fe is involved in brain oxidative DNA damage; for example, Vasudevaraju et al. (2010) showed that in normal aging brain, there is a selective increase of single strand and double strand breaks in DNA, which can be triggered by endogenous and exogenous factors such as trace metals and oxidative stress. Fe induced oxidative DNA damage after intracerebral hemorrhage (Nakamura et al. 2006). Therefore, our results suggest that although brain Fe accumulation may cause DNA oxidation and damage, such events could be controlled or restricted by the appropriate administration protocol (such as sub-chronic administration). Steady state levels of oxidative modifications of DNA reflect the balance between the rate of generation of oxidative modifications (which itself depends on oxidative stress levels), and of nuclear DNA repair. The fact that sub-chronic Fe treatment decreased the steady state levels of oxidative DNA modifications might reflect a decrease in the rate of generation of these oxidative modifications (due to an antioxidant effect), and/or an increased rate of DNA repair. Further studies are necessary to evaluate these two possible mechanisms.

Fe plays a crucial role in many facets of mitochondrial metabolism. Therefore, the mitochondrion, a site of dynamically active electron transfer and redox activity, would possess adequate mechanisms for the safe trafficking and metabolism of Fe. However, until recently, knowledge of the Fe metabolism of the mitochondrion has been largely confined to the heme synthesis pathway (Galatro and Puntarulo 2007). In spite of cytosolic ferritins (Ft) in mammals being ubiquitous, mitochondrial ferritin (mtF) expression is restricted mainly to the testis, neuronal cells and islets of Langherans. Furthermore, compared to cytosolic Ft, Fe inserted in mtF is less available for chelation. Mitochondrial Fe level must be well regulated because an inadequate supply of Fe would impair the metabolic and respiratory activities of the organelle, whereas excess labile Fe in mitochondria would promote the generation of

harmful ROS, which are produced as a side reaction of mitochondrial electron transport (Cadenas and Davies 2000). It could be postulated that depending on the way Fe gets into the mitochondria (acute or sub-chronic), the role of mtF as an active sequestering agent for Fe could vary, and thus the intra-mitochondrial LIP could be modified following a different pattern. This different profile could be responsible for a different effect of Fe on the integrity of DNA after acute and sub-chronic administration. The evaluation of the subcellular distribution of Fe is planned for future mechanistic studies.

While oxidative modifications in DNA contribute to genomic instability of cells and are recognized as important for carcinogenesis, aging, and neurodegeneration, the site-specific mechanism of DNA damage by hydroxyl radical (OH) could explain either an amplification or a dampening of a damage caused by a number of OH radicals. If the metal-binding site is important for function or activity, or for special orientation or conformation, then the funneling effect will cause marked amplification of the biological damage, because all of the deleterious radicals will be produced and will react at this pertinent site. In contrast, if the metal-binding site is relatively unimportant, the funneling effect will direct the damage to the redundant site, and the recorded biological damage will be noticeably reduced. Moreover, a low incidence of single strand break on the DNA is usually efficiently tolerated, while when the two strands are broken at spatially nearby sites, a double-strand break is formed (Chevion et al. 1999). The exact correlation between levels of DNA damage and deleterious biological effects is expected to vary in complex ways with cell and tissue type, and the nature of the DNA damage. While this work evaluated brain DNA integrity relative to the control brain, the type of DNA damage and biological effects will be assessed in future mechanistic studies.

b. Both acute and sub-chronic iron overload prevented the lipid oxidative damage caused by CPZ. The molecular mechanisms by which neuroleptics increase O_2 free radical production are mostly unknown (Naidu et al. 2002). CPZ has been suggested to generate H_2O_2 by auto oxidation (Parikh et al. 2003). Neuroleptics act by blocking dopamine receptors that could result in an increase of dopamine turnover, which could conceivably lead to an increased production of H_2O_2, resulting in oxidative stress (Spina and Cohen 1989). Dopamine is primarily metabolized through oxidation by monoamine oxidase (MAO) to 3,4-dihydroxyphenyl-acetic acid (DOPAC) and H_2O_2. Dopamine is also metabolized by auto-oxidation yielding super-oxide radical (O_2^-). H_2O_2 can further react with Fe or Cu ions to produce the OH, which is the most toxic of free radicals. Increased dopamine turnover by neuroleptics could lead to excessive production of these potentially damaging free radicals (Elkashef and Wyatt 1999), which could explain the increased production of LR in the brain after 1 and 2 h of administration of CPZ (Fig. 1).

c. Sub-chronic Fe overload (but not acute Fe overload) induced the activity of the antioxidant enzyme CAT after CPZ administration. The fact that acute Fe overload did not induce CAT activity after CPZ administration suggests that the nature of the Fe supplementation protocol is a key factor to selectively activate the network of protective metabolic pathways triggered in the brain. In

agreement with this hypothesis, a differential effect of acute and sub-chronic Fe overload on the transcription factor NF-jB activation was reported (Table 1).

Oxidative stress, lipid peroxidation and changes of the antioxidant enzyme activity may be responsible for one of the molecular mechanisms of CPZ induced tissue damage, however, CPZ administration following Fe overload (acute or sub-chronic) might trigger protective antioxidant lipid signaling.

Conclusions

In conclusion, we propose that Fe triggers a hormetic protective response that limits oxidative damage to the brain, depending on the pattern of Fe administration (acute or sub-chronic).

The diagram in Fig. 4 briefly summarizes the sequence of events activated by Fe proposed here. Much progress still needs to be made in order to understand the nature and function of Fe and mostly of the labile Fe pool, as contributors to oxidative stress and disease, as well as role of Fe-dependent stress in the development of possible chemotherapeutic strategies.

Acknowledgements

This study was supported by grants from the University of Buenos Aires (UBACyT 20020130100383BA), ANPCyT (PICT-BID 845/12), CONICET (PIP 687), Arkansas INBRE (National Institutes of Health, National Institute of General Medical Sciences Grant P20 GM103429), and the Byrd Chemistry Research Fund Award from Hendrix College. S.P. is career investigator from CONICET.

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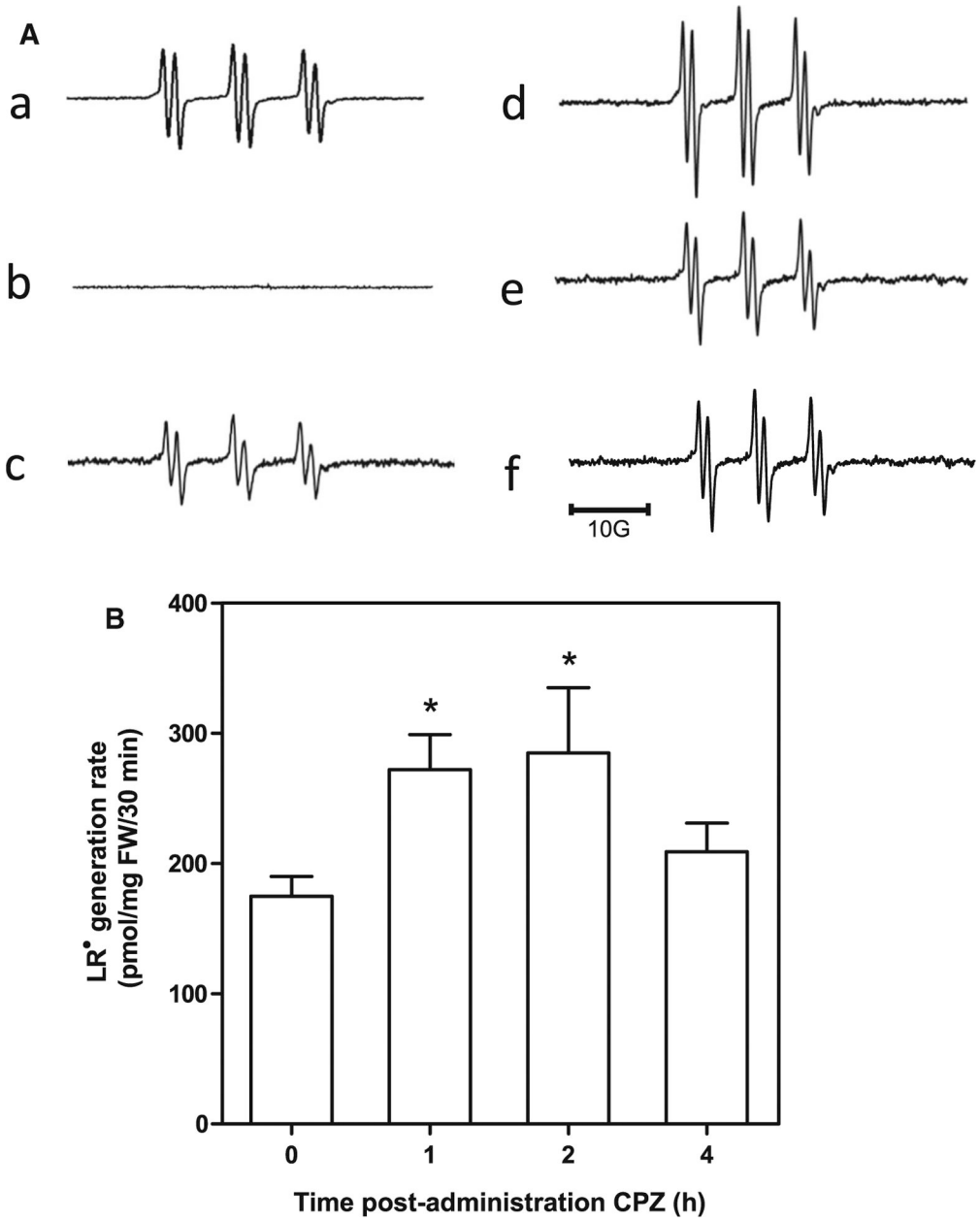

Fig. 1.

LR generation rate after administration of CPZ in rat brain. a EPR signal for LR in control rat brains(a) computer simulated spectrum employing the following spectral parameters: g = 2.005 and a_H = 2.6 G and a_N = 15.8 G,(b) PBN-DMSO alone,(c) control rat brain, and rat brain from control rats administered CPZ at (d) 1 h post-treatment, (e) 2 h posttreatment and (f) 4 h post treatment. b Quantification of LR generation rate after administration of CPZ in control rats. Brain homogenates were incubated in the presence of 40 mM PBN-DMSO for30 min. *Significantly different from control values ($p < 0.05$)

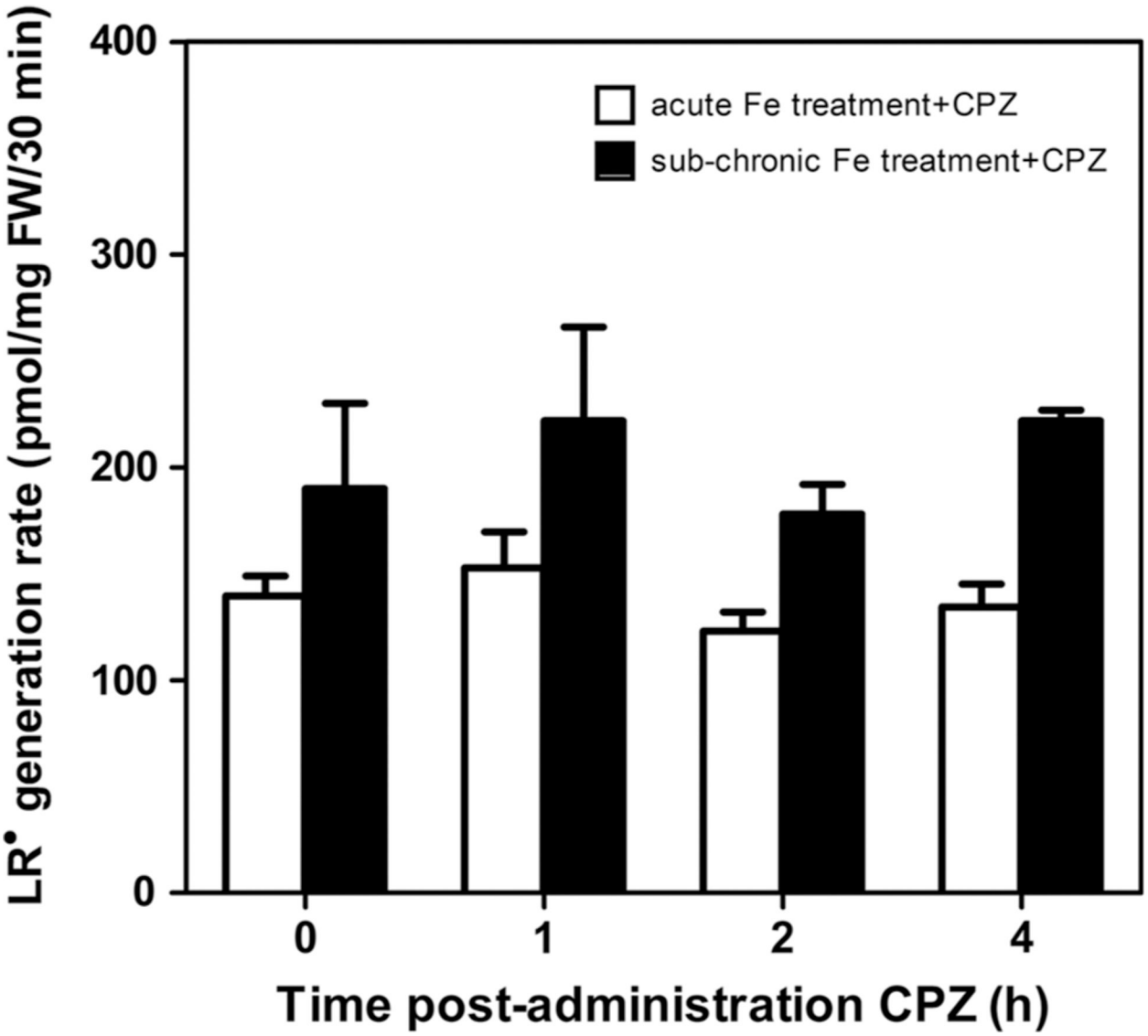

Fig. 2.

Quantification of LR generation rate post-administration of CPZ after acute Fe-dextran treatment (open square) or post-administration of CPZ after sub-chronic Fe-dextran treatment (filled square), as described under “Materials and Methods”. The time point 0 represents an average between values in brains from saline injected (Control group) animals and Fe-injected rats after recovery from Fe overload

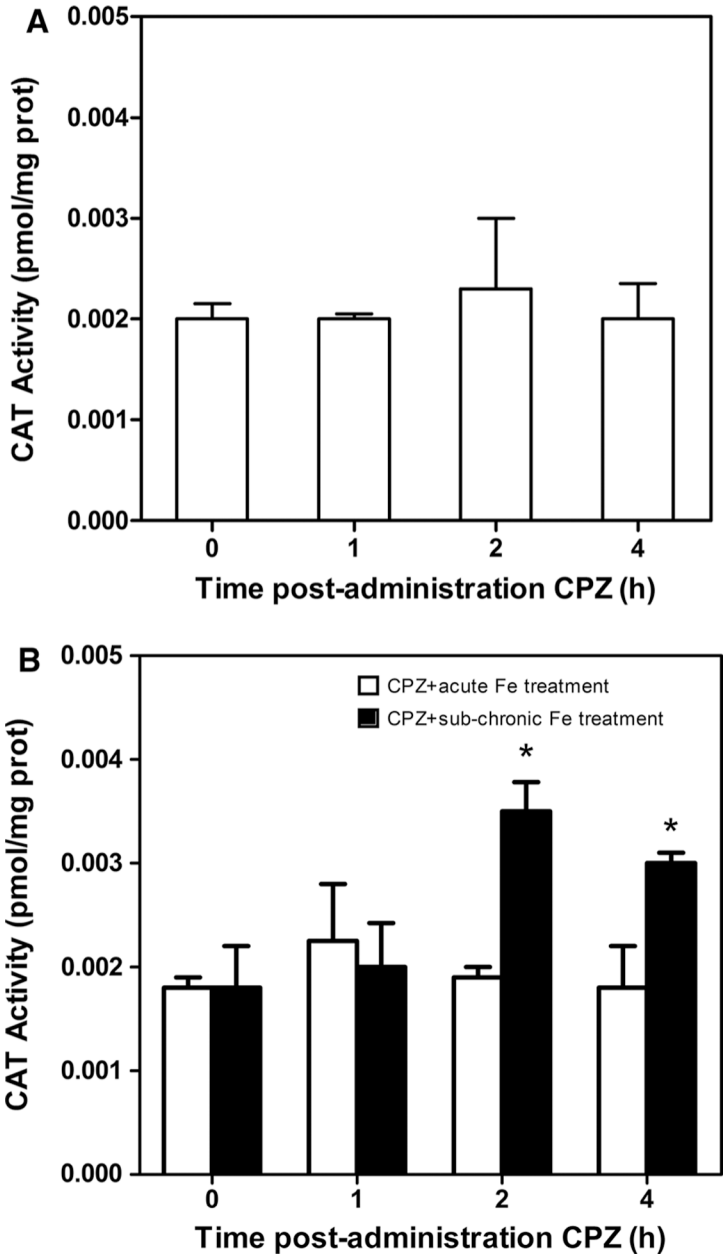

Fig. 3.

Kinetic study of CAT activity in rat brain a after CPZ administration to control rats. b Post administration of CPZ after acute (open square) and sub-chronic (filled square) Fe-dextran treatment, as described under “Materials and Methods”. *Significantly different from control values ($p<0.05$). The time point 0 represents an average between values in brains from saline injected (Control group) animals and Fe-injected rats after recovery from Fe overload

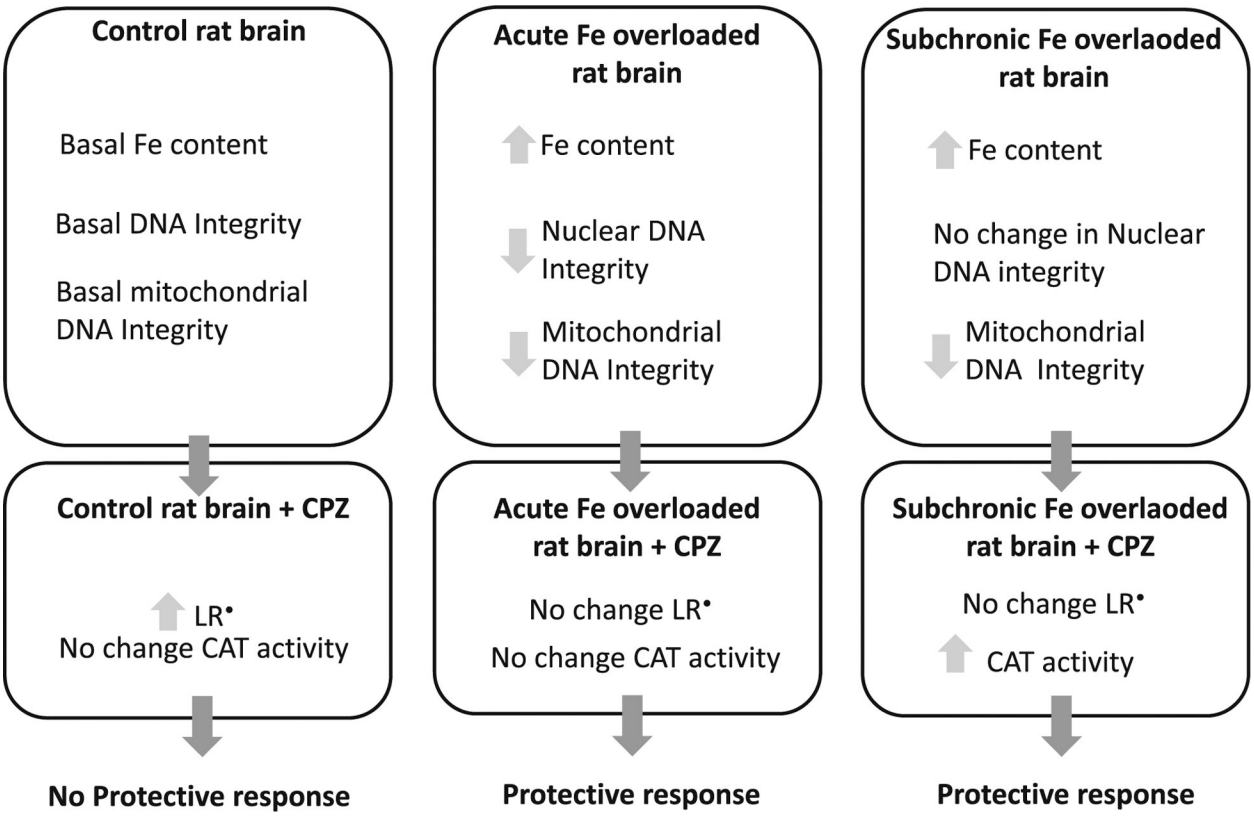

Fig. 4.
Integrative diagram of the reported data on CPZ effects in rat brain

Table 1

Parameters determined upon acute and sub-chronic Fe-dextran treatment

	Acute Fe-dextran	Sub-chronic Fe-dextran
Histopathology	No change[2,a]	Not determined
LIP	2.8-fold increase[2,c]	2.3-fold increase[1,b]
$LR^{\cdot}$	No change[2,c]	2.0-fold increase[1,b]
TBARS	No change[2,c]	No change
CAT	1.6-fold increase[4,c]	3.2-fold increase[3,b]
NF-κB	1.3-fold increase[3,c]	No change[3,b]

Measurements were listed at the following time points:

[1] at 2 h post injection (pi);

[2] at 6 h pi;

[3] at 8 h pi;

[4] at 21 h pi

[a] Hematoxylin and eosin were used as dyes (data not shown)

[b] Piloni et al. (2013)

[c] Piloni et al. (2016)

Table 2

Total Fe content upon Fe-dextran treatment

Time (h)	Acute Fe-dextran (pmol/mg FW)	Sub-chronic Fe-dextran (pmol/mg FW)
0	0.7 ± 0.1	0.85 ± 0.02
2	0.30 ± 0.02	6.50 ± 0.25 *
4	3.2 ± 1.4 *	0.60 ± 0.06
6	5.4 ± 1.5 *	1.20 ± 0.05
8	2.8 ± 0.8 *	0.71 ± 0.03

* Significantly different from control values($p<0.05$)

Table 3

Nuclear and mitochondrial DNA integrity upon Fe-dextran treatment

	Nuclear DNA integrity (%)	Mitochondrial DNA integrity (%)
Acute Fe overload		
0 h	100 ± 9	100 ± 10
6 h	59 ± 4 *	35 ± 11 *
Sub-chronic Fe overload		
0 h	100 ± 5	100 ± 10
2 h	85 ± 6	75 ± 5 *
8 h	92 ± 7	77 ± 6 *

* Significantly different from control values($p < 0.05$)