EFFECTS OF ANTIPSYCHOTIC DRUG ADMINISTRATION ON ANTIOXIDATIVE DEFENCE ENZYMES IN MALE RAT KIDNEY

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RUNNING TITLE:

EFFECTS OF ANTIPSYCHOTICS ON RENAL ANTIOXIDANT ENZYMES

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ABSTRACT

The use of atypical antipsychotic drugs (APD) was reported to be associated with adverse effects on the kidneys. Thus the aim of this study was to examine whether APD exerted their adverse effects by interfering with the renal antioxidant defence system. Male 3-month-old Wistar rats were treated for 28 days with ziprasidone (ZIP), clozapine (CLO) or sertindole (SER) using a daily dose recommended for antipyschotic therapy. The expression and activities of antioxidant enzymes superoxide dismutase (SOD) type 1 and 2, catalase (CAT), glutathione reductase (GR) as well as glutathione-S-transferases (GST) activity were measured in the kidneys. Changes in the kidneys were also evaluated histologically. Ziprasidone, CLO and SER reduced renal SOD type 1 and 2 activities. Decreased CAT activity was observed only in SER-treated rats. An inhibition in GR activity and increased activity of GST was found only after treatment with CLO. Histological analysis showed dilatation of proximal tubules in kidneys with all 3 drugs. In conclusion, data indicate that redox disturbances may contribute to renal morphologic alterations in proximal tubules in rats treated with all APD.

KEY WORDS: Ziprasidone, Clozapine, Sertindole, antioxidative enzymes, kidney.

ABBREVIATIONS: APD, Atypical antipsychotic drugs; CAT, Catalase; GSH, Gluthatione; GPx, Glutathione peroxidase; GR, Glutathione reductase; GSTs, glutathione-S-transferases; ROS, reactive oxygen species; SOD 1, Copper-zinc superoxide dismutase; SOD 2, Manganese superoxide dismutase.

INTRODUCTION

Millions of patients worldwide are prescribed atypical antipsychotic drugs (APD) annually. To date APD were found to be effective in the treatment of schizophrenia. The benefits of APD medication in treating acute psychotic episodes are widely accepted and documented (Baldessarini et al., 1988; Davis et al., 1993). In recent times APD have increasingly been used to treat non-psychotic disorders, from acute mania to treatment-resistant depression and dementia (Fineberg et al., 2006). However, there are safety concerns about the use of these drugs due to an increasing number of reports regarding their side effects.

Acute kidney injury (defined as sudden loss of kidney function) as a consequence of taking APD was noted in several case reports (Fraser and Jibani, 2000; Parekh et al., 2014; Moledina and Perazella, 2015) but adverse renal events are poorly characterized. There have been several single case reports of acute renal failure (ARF) attributed to interstitial nephritis which developed in patients with treatment-resistant chronic schizophrenia following initiation of treatment with clozapine (CLO) (Hunter et al., 2009; Au et al., 2004; Southall and Fernando, 2000; Elias et al., 1999).

Evidence suggests that APD effects are associated with increased oxidative stress originating from damaged mitochondria (Baig et al., 2010; Zhang et al., 2006). As kidney mitochondrial dysfunction plays an important role in the pathogenesis of renal diseases (Che et al., 2014), the aim of this study was to determine whether 28-day daily administration of APD promoted oxidative stress and damage in kidneys.

MATERIALS AND METHODS

Materials

Ziprasidone (Zeldox) (ZIP) was provided by Pfizer (Vienna, Austria), Clozapine (CLO) was provided by Alvogen (Remedica Ltd, CITY, Cyprus) and Sertindole (Serdolect) (SER) was obtained from H. Lundbeck (Valby, Denmark). Copper-zinc superoxide dismutase (SOD1), manganese superoxide dismutase (SOD2), glutathione reductase (GR), and glutathione peroxidase (GPx) levels were detected using antibodies from Abcam (ab13489, ab13533, ab16801 and ab22604, respectively), CAT using an antibody from Abgent (AP8623-c) and β-actin using an antibody from Sigma (AC-15). Polyvinylidenedifluoride (PVDF) membrane, enhanced chemifluorescent (ECF) Western blotting reagent pack, containing anti-mouse (IgG+IgM) and anti-rabbit IgG alkaline phosphatase linked whole antibodies, and the alkaline phosphatase substrate were obtained from Amersham Pharmacia Biotech, UK. All other chemicals were purchased from Sigma.

Animals and drug treatment

Thirty-two adult male Wistar albino rats (three months old, weighing 300-350g) were randomly divided into 4 experimental groups with 8 rats/group. Animals were provided with standard chow and drinking water *ad libitum.* The procedures complied with directive 2010/63/EU regarding protection of animals used for experimental and other scientific purposes. The study was approved by the Ethical Committee (decision No. 3-10/13) for the use of lab animals at the Institute for Biological Research “Siniša Stanković”, University of Belgrade. Rats were kept under standard conditions at 22°C with a 12-hr light/dark cycle.

All drugs were prepared (water suspension of pulverized tablets) and administered daily in the morning via a gastric tube to ensure that no drug loss occurred. Rats were dosed according

to the drug calculation formula (Shanon et al., 2007). Rats were exposed daily to: water (control), ZIP (20 mg/kg/day), CLO (45 mg/kg/day) or SER (2.5 mg/kg/day) for 4 weeks.

Tissue collection

After 28 days following overnight fasting rats were sacrificed by decapitation. The right kidneys were immediately excised, frozen in liquid nitrogen and kept at -80^{o}C until further analysis. The left kidney was excised, fixed in 4% paraformaldehyde solution for 24hr, dehydrated using increasing concentrations of ethanol and xylene and used for histopathological examination. After embedding in Histowax (Histolaboduct AB, Göteborg, Sweden), each tissue block was sectioned at 5 µm thickness on a rotary microtome (RM2125 RT Leica Microsystems, Wetzlar, Germany).

Tissue preparation and determination of antioxidant enzyme activities

For preparation of whole tissue extracts the right kidney was homogenized in 10 volumes (wt/vol) of 50 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, pH 7.4 and sonicated 3×10 sec at 10 MHz (Sonopuls, Bandelin) on ice followed by 60 min of centrifugation at 4°C and 105,000x*g* (Beckman L7-55 Ultracentrifuge). The supernatants were used as whole tissue extracts.

Total SOD activity was determined by the adrenaline method (Misra and Fridovich, 1972). One SOD unit was defined as the amount of the enzyme necessary to decrease the rate of adrenaline auto-oxidation by 50% at pH 10.2. For determination of SOD2 activity, the assay was performed after pre-incubation with 8 mM potassium cyanide. SOD1 activity was calculated as difference between total SOD and SOD2 activities. CAT activity was determined according to Beutler (1982). One unit of CAT activity was defined as the amount of the enzyme that decomposes 1 mmol H_2O_2 per min at 25°C and pH 7. The activity of GPx was determined by the glutathione reduction of t-butyl hydroperoxide, using a modification of the assay described by

Paglia and Valentine (1967). One unit of GPx activity was defined as the amount of the enzyme needed to oxidize 1 μmol NADPH per min at 25°C and pH 7. GR activity was determined using the method of Glatzle and colleagues (1974). One unit of GR activity was defined as the amount of the enzyme needed to oxidize 1 μmol NADPH per min at 25°C and pH 7.4. For the measurement of total glutathione-S-transferases (GST) activity, 1-chloro-2,4-dinitrobenzene (CNDB) was used as the substrate (Habig at al., 1974). One unit of GST activity is defined as the amount of enzyme needed to conjugate 1 μmol of CNDB with glutathione (GSH) per min at 25°C. All enzyme activities are expressed as units (U) per mg of protein. The protein concentration was determined by the Lowry et al **(**1951) method using bovine serum albumin as a standard.

SDS-Polyacrylamide gel electrophoresis and immunoblotting

Proteins from whole tissue extracts were resolved using 12% SDS-polyacrylamide gels and then transferred to a polyvinylidene difluoride (PVDF) membrane. Unbound sites on the membranes were blocked with 1% nonfat dry milk for 1.5 hr. After blocking membranes were incubated with primary antibody followed by alkaline phosphatase conjugated secondary antibody. Relative optical density of immunoreactive bands was determined by the enhanced chemifluorescent method using STORM scanner (Amersham) and ImageQuant software (GE Healthcare). For gel-to-gel comparison, one randomly chosen control sample was employed as internal reference that was run on each gel as described in Ristić et al. (2015). The intensity of each analyzed immunospecific band was normalized both to corresponding β-actin band and to internal reference on the same blot.

Light microscopy

Tissue sections were de-paraffinized in xylol, dehydrated using decreasing concentration of alcohol and stained with Periodic Acid Schiff (PAS) reagent. Digital images of kidney tissue were captured using a DM RB Photomicroscope (Leica, Wetzlar, Germany) with a DFC 320 charged coupled device camera (Leica).

Statistical analyses

Statistical analyses were performed according to protocols described by Hinkle and co-workers (1994). Each assay was performed in triplicate for each tissue sample. Statistical significance was tested by one–way analysis of variance (ANOVA) followed by Tukey's multiple comparison *post-hoc* test. A probability level of $p<0.05$ was considered statistically significant.

RESULTS

Daily food and liquid intake was measured during the treatment period and did not differ markedly between all groups (data not shown). Total body mass and relative kidney mass (expressed as tissue mass per total body mass) remained significantly unaltered.

All APD produced a reduction in both SOD1 and SOD2 activities. Clozapine produced the greatest decrease in SOD1 activity (approximately 43%), followed by ZIP (28%) and SER (28%); as illustrated in Figure 1. Sertindole produced a significant 52% fall in SOD2 activity followed by CLO and ZIP at 30%. As shown in Figure 1 CAT activity was significantly diminished only in the SER-treated group (58%). The activity of GR was significantly decreased only in CLO-administered rats (23%) while GPx activity was not markedly affected by any treatment (Figure 1).

The protein expression levels of antioxidant enzymes in response to APD treatment showed the same trend as their activities with a reduction compared to control. However, the results from Western blotting failed to reach significance (Figure 2). Data indicated that there was inhibition of antioxidant enzyme activity, most likely associated with APD-mediated increased ROS production.

Histopathological analysis demonstrated differences between control and ADP-treated rats, specifically in the kidney cortex. Proximal tubules in all ADP-administered animals showed dilatation compared with control (black arrows in Figure. 3). The most pronounced dilatation was observed in CLO-treated rats (Figure 3, panel C).

DISCUSSION

Raha et al (2012) postulated that the renal effects of APD are linked to increased oxidative stress. Oxidative stress occurs when enhanced reactive oxygen species (ROS) production is not accompanied by a proportional rise in endogenous antioxidant defence system activity. Such findings are in agreement with our previous observation that CLO effects in schizophrenic patient blood markers were partly due to oxidative stress (Miljević et al., 2010). Raha et al (2012) proposed that oxidative stress occurs as a consequence of elevated production of toxic dopamine metabolites which inhibit mitochondrial respiration and lead to disturbed oxidative balance, and that oxidative damage of proteins is associated with cellular energy metabolism (Baig et al., 2010). Increased GSH conjugation of the CLO nitrenium ion or differential toxicity of GST isoform-specific GSH conjugates (Vredenburg at al., 2013) may also account for enhanced ROS-induced damage in kidney cells.

Kidneys are high energy demanding organs, and Abraham et al (2013) demonstrated that proximal tubules have a high ATP requirement for the active reabsorption of filtered nutrients

and ions. Thus, damage to the proximal tubule might lead to tubular dysfunction as evidenced here by APD-induced dilatation. Moreover, superoxide formation was localized in proximal tubules (Forbes et al., 2012), damage normally prevented by adequate SOD activity (Nishikawa et al., 2001). Our data indicate that the antioxidative defence system failed to respond to the actions of APD where decreased activities of mitochondrial SOD2 and cytosolic SOD1 were found. Similarly Pillai et al (2007) reported that ZIP produced significant reduction in both SOD1 and SOD2 protein expression levels in rat brain. Interestingly, a significant inhibition in genes encoding SOD1 and SOD2 was noted by Schmidt et al (2009) in a study concerning the effects of APD on human neuroblastoma cells.

Oxidative metabolism is regulated by appropriate antioxidative defence strategies in subcellular compartments (Halliwell and Gutteridge, 1990; Matés, 2000; Ghio et al., 2012). As a consequence of decreased SOD2 activity, flow of hydrogen-peroxide into the cytosol may be diminished. Reduced cytosolic SOD1 activity also indicates a low level of endogenous hydrogen peroxide, which basal GPx activity might be able to handle since no marked changes in GPx activities were found. Considering different subcellular localization and substrate responsiveness of GPx and CAT (Halliwell and Gutteridge, 2007) our results suggest that APD affected the intensity of oxidative metabolism differentially within various compartments. Other studies reported no marked effects of APD on GPx activity, whereas significant reductions in both CAT activity and protein expression levels after treatment with APD (one of the tested was ZIP) was detected by Pillai et al (2007). In contrast Agostinho and colleagues (2007) found no marked changes in SOD and CAT activities in the hippocampus, cortex and striatum after chronic treatment with CLO.

CONCLUSIONS

Our findings of reduced antioxidative defence in kidneys may provide insight into understanding morphological changes induced by APD. Chronic atypical antipsychotic treatments need to be used with caution with periodical monitoring of kidney function.

FUNDING

This research was supported by grant (No. 173014) from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. David R. Jones from United Kingdom for the language editing and proof-reading of the manuscript.

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FIGURE LEGENDS:

FIGURE 1. Kidney tissue antioxidant enzyme activities and glutathione-S-transferases (GST). Data are presented as means ± S.E.M (n=8 per group) in units per milligram of total protein. Significant differences between controls and treated groups (one-way ANOVA followed by Tukey test) are presented by asterisks: * $p<0.05$. (ZIP-Ziprasidone; CLO-Clozapine; SER-Sertindole).

FIGURE 2. Kidney tissue protein expression levels of antioxidant enzymes. Kidney whole tissue extract (50 μg protein per lane) was subjected to SDS-PAGE and Western blotting. Immunopositive bands were visualized by enhanced chemifluorescence using STORM scanner. β-Actin was used as the loading control. Representative Western blots (above) and relative quantification of enzyme protein levels (below) are shown (Con-Control; ZIP-Ziprasidone; CLO-Clozapine; SER-Sertindole). Quantitative analysis was done by the ImageQuant software. The intensity of each analyzed immunospecific band was normalized both to corresponding β-actin band and to internal reference on the same blot. Relative integrated optical densities of bands are expressed in arbitrary units (AU) as mean ± S.E.M (n=8 per group). Statistical significance was tested by one-way ANOVA followed by Tukey test.

FUGURE 3. Upper: Histological features of the kidney cortex in adult male rats treated with APD obtained by light microscopy and PAS staining. Kidney cortex of: A) Control-treated rat shows normal architecture of kidney tubules; B) Ziprasidone-, C) Clozapine- and D) Sertindole-treated rats. The proximal kidney tubules (indicated by black arrows) show dilatation compared to control.. Bar = 40μm. Lower: Histogram represent diameter of proximal kidney tubules in controls and ZIP-Ziprasidone-, CLO-Clozapine- and SER-Sertindole-treated rats. Data are

presented as means ± SEM (n=8). Comparison between controls and treated groups were made by un-paired Student's t-test. * $p<0.05$.

FIGURS:

FIGURE 1:

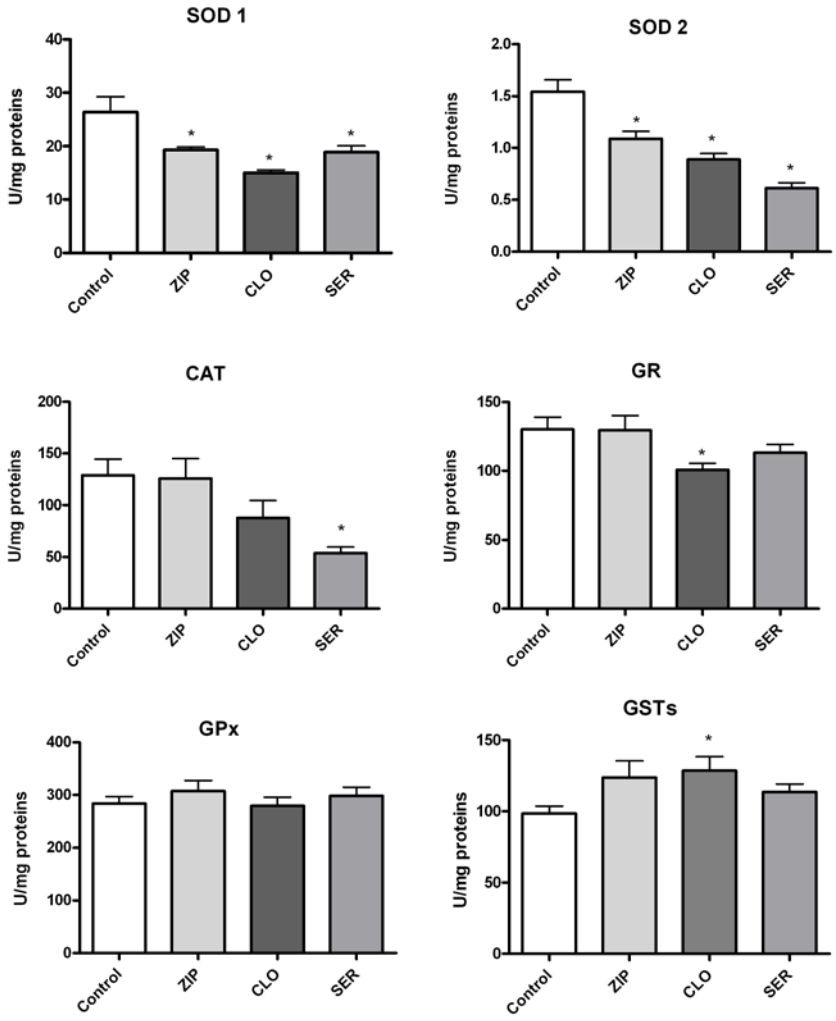
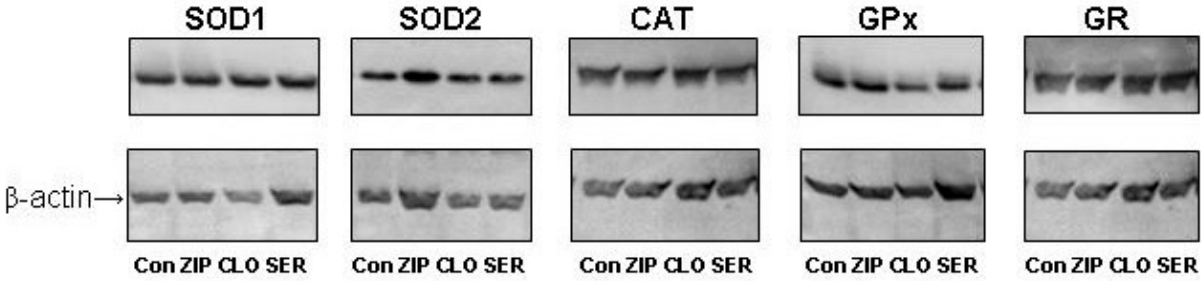


FIGURE 2:



	Control	Ziprasidone	Clozapine	Sertindole
SOD1 (AU)	0.90 ± 0.10	0.78 ± 0.14	0.82 ± 0.15	0.76 ± 0.03
SOD2 (AU)	0.91 ± 0.05	0.82 ± 0.13	0.82 ± 0.12	0.71 ± 0.12
CAT (AU)	1.25 ± 0.18	1.13 ± 0.17	1.12 ± 0.21	0.98 ± 0.24
GPx (AU)	1.40 ± 0.19	1.38 ± 0.20	1.23 ± 0.12	1.25 ± 0.16
GR (AU)	1.12 ± 0.11	0.96 ± 0.06	1.00 ± 0.13	0.99 ± 0.22

FIGURE 3: