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Research Report

Oxidative stress: A potential recipe for anxiety, hypertension and insulin resistance

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ARTICLE INFO

Article history:

Accepted 27 August 2010

Available online 26 September 2010

Keywords:

Oxidative stress
Anxiety
Antioxidants
Hypertension
Insulin resistance
Diabetes
Antioxidants

ABSTRACT

We recently reported involvement of oxidative stress in anxiety-like behavior of rats. Others in separate studies have demonstrated a link between oxidative stress and hypertension as well as with type 2 diabetes/insulin resistance. In the present study, we have tested a putative role of oxidative stress in anxiety-like behavior, hypertension and insulin resistance using a rat model of oxidative stress. Oxidative stress in rats was produced by xanthine (0.1%; drinking water) and xanthine oxidase (5 U/kg; i.p.). X+XO-treated rats had increased plasma and urinary 8-isoprostane levels (a marker of oxidative stress) and increased malondialdehyde (MDA) levels in the hippocampus and amygdala as compared to control rats. Serum corticosterone (a systemic marker of stress and anxiety) levels also increased with X+XO treatment. Moreover, anxiety-like behavior measured via open-field and light–dark exploration behavior tests significantly increased in X+XO-treated rats. Mean arterial blood pressure measured in anesthetized rats increased in X+XO-treated compared to control rats. Furthermore, plasma insulin but not glucose levels together with homeostasis model assessment (HOMA), an index of insulin resistance, were higher in X+XO-treated rats. Our studies suggest that oxidative stress is a common factor that link anxiety-like behavior, hypertension and insulin resistance in rats.

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1. Introduction

An increased risk of psychiatric impairment is widely believed to be accompanied with a chronic illness (Simon et al., 1995). Substantial literature supports this clinically important association (Roy-Byrne et al., 2008). In particular, comorbidity of anxiety disorders with chronic medical conditions like hypertension and type-2 diabetes mellitus

is a topic of both clinical and policy interest (Roy-Byrne et al., 2008; Thomas et al., 2003). Interestingly, presence of anxiety is reported to contribute to an additional time of hospitalization due to the complications resulting from anxiety (Ball et al., 2002). Given the implications of comorbidity between anxiety, hypertension and type-2 diabetes mellitus, it is imperative to investigate whether there is a shared relationship underlying these conditions. Our studies point

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Abbreviations: HOMA, homeostasis model assessment; X+XO, xanthine and xanthine oxidase; MDA, malondialdehyde; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; OF, open-field; LD, light–dark

towards a common factor, oxidative stress that more than likely is a contributing factor in the occurrence of comorbidity between anxiety disorders, hypertension and insulin resistance/type-2 diabetes mellitus.

In general, evidence for the involvement of oxidative stress in human disease is frequently cited (Salustri et al., 2010; Dean et al., 2009; Ko et al., 2010; Liu et al., 2008). Increased oxidative damage is likely to occur in most if not all human diseases, although it probably plays a significant pathologic role only in few. Recent studies including our own have shown direct involvement of oxidative stress with anxiety-like behavior in rodents (Salim et al., 2010; Hovatta et al., 2005; Gingrich, 2005; Masood et al., 2008; Souza et al., 2007; Bouayed et al., 2007; de Oliveira et al., 2007). Recent work also has suggested correlation of oxidative stress with hypertension and type-2 diabetes mellitus (Ko et al., 2010; Liu et al., 2008). Occurrence of oxidative stress, anxiety-like behavior, hypertension and type-2 diabetes mellitus have never been examined in the same study. In this study, using Sprague–Dawley rats, we induced oxidative stress by xanthine plus xanthine oxidase treatment for one week. This treatment increased oxidative stress markers, 8-isoprostane (in serum and urine) and malondialdehyde in blood plasma, hippocampus and amygdala brain regions of rats. Moreover, X+XO treatment increased anxiety-like behavior of rats examined via light–dark and open-field anxiety tests, when compared to the vehicle treated control rats. Furthermore, X+XO treatment increased blood pressure, insulin resistance and corticosterone levels. This suggests that induction of oxidative stress results in elevated anxiety, hypertension and insulin resistance. It is likely that oxidative stress is the common link that is responsible for increased incidence of comorbidity in these three chronic illnesses. Even though, there is a large volume of evidence suggesting anxiety as a risk factor for hypertension and other cardiovascular diseases, as well as type-2 diabetes mellitus, there remains paucity of information linking oxidative stress, hypertension, type-2 diabetes mellitus and anxiety, all in the same model.

In this study, we have employed a rat model in which we have induced oxidative stress using X+XO to assess direct

involvement of oxidative stress in anxiety-like behavior, hypertension and insulin resistance.

2. Results

Oxidative stress was induced in rats by xanthine supplementation (0.1% in drinking water) and intraperitoneal injections at non-toxic dose of xanthine oxidase for 1 week. This treatment increased oxidative stress markers, 8-isoprostane (in serum and urine) and malondialdehyde in hippocampus and amygdala. The treatment also increased serum corticosterone levels, a generally considered marker of stress and anxiety (Kobayashi et al., 2009; Arranz et al., 2007). Moreover, X+XO treatment increased anxiety-like behavior of rats examined via light–dark and open-field anxiety tests, when compared to the control vehicle treated rats. Furthermore, X+XO treatment increased blood pressure of rats and insulin resistance as compared to vehicle-treated control rats (Fig. 1).

2.1. Effect of X+XO treatment on general body parameters

There were no significant changes observed in body weight, body temperature, food, or water intake habits in all groups of rats. All rats irrespective of treatment consumed similar amounts of rodent chow and tap water daily (Table 1).

2.2. Effect of X+XO treatment on indices of oxidative stress and corticosterone levels

Treatment with X+XO for 7 days caused significant increase in plasma (166%) and urinary (129%) 8-isoprostane as compared to vehicle-treated control rats (Fig. 2A, B). Also, treatment with X+XO for 7 days caused significant increase in markers of lipid peroxidation (MDA assay) in hippocampus (MDA: 153%) and amygdala (MDA: 101%) tissues (Fig. 2C). Serum corticosterone levels were examined for control (27.1 ng/ml) and X+XO treated (66.6 ng/ml) rats. X+XO treatment increased serum

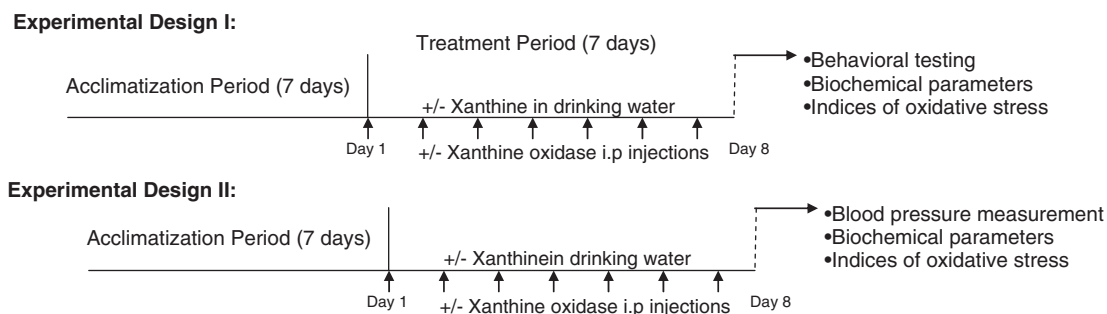


Fig. 1 – Schematic representation of the experimental plan. In experimental design I, the control group ($n=8-12$ rats) received vehicle injections (saline) and drinking water *ad libitum* for 7 days. Another group of rats ($n=8-12$ rats) received 0.2% xanthine supplementation in drinking water plus intraperitoneal injections at non-toxic dose of xanthine oxidase (5 Units/kg) once daily for 7 days. After 24 h of the last X+XO treatment, anxiety tests were conducted, brains harvested and biochemical and oxidative parameters measured. In experimental design II, rats were randomly selected into two groups (control and treated) as before. After 24 h of the last X+XO treatment, rats were anesthetized with inaction and blood pressure measurements were made, biochemical and oxidative parameters measured were analyzed in serum and urine.

Table 1 – X+XO treatment has no adverse effect on body weight, food or water intake and body temperature. Fifty grams of rodent chow (diet pellets) and 50 ml of tap water per rat was provided to the rats every day. The amount of food and water consumed was measured by weighing the left-over diet pellets and drinking water 24 h later. Data collected were averaged for each treatment group of rats on the last day of the experiment before behavior testing. Rectal temperature was manually recorded using animal rectal probe every day after 30 min of X+XO-treatment/vehicle injections. N=8–12 rats.

General parameters	Control	X+XO
Body weight (g)	225±1.0	223±2.1
Food intake (g)	22.4±4.0	21.6±3.8
Water intake (ml)	24±2.1	23±2.0
Body temperature (°C)	37.84±0.22	36.6±0.32

corticosterone levels by 143% as compared to vehicle treated control rats (Fig. 2D).

2.3. Effect of X+XO treatment on anxiety-like behavior of rats

Light-dark exploration and open-field activity tests were conducted to test for anxiety-like behavior. Open-field test demonstrated that X+XO treatment reduced center time (28.7 s) and rearing (22) (Fig. 3A, B) of treated rats as compared to center time (58.6 s) and rearing (37) exhibited by vehicle treated control rats. The light-dark exploration test results suggest that X+XO treated rats spent 75 s in the light compartment and control rats spent 127 s in the light compartment suggesting that X+XO treatment significantly decreased the time X+XO treated rats spent in lit area (Fig. 3C). After behavior assessments were made, rats were quickly sacrificed, blood (for serum preparation) and urine was collected and brains harvested to conduct oxidative stress marker measurements in serum, urine, hippocampus and amygdala regions of the brain.

2.4. Effect of X+XO treatment on blood pressure of rats

The mean arterial blood pressure of X+XO rats showed a significant increase as compared to vehicle treated control rats, with X+XO rats exhibiting 124 mmHg blood pressure while control rats exhibiting 109 mmHg, with ~14% increase in mean arterial blood pressure observed with induction of oxidative stress (Fig. 4).

2.5. Effect of X+XO treatment on general body parameters, indices of oxidative stress and corticosterone levels of rats

There were no significant changes observed in body weight, body temperature, food or water intake habits in control or X+XO treated groups of rats. All rats irrespective of treatment consumed similar amounts of rodent chow and tap water daily (Table 2). Treatment with X+XO for 7 days caused significant increases in markers of lipid peroxidation (MDA assay) with 80% increase in blood plasma, 103% increase in serum and 118% urinary 8-isoprostane levels as compared to vehicle treated controls (Table 2). X+XO treatment increased serum corticosterone by ~185%.

2.6. Effect of X+XO treatment on insulin resistance in rats

Plasma insulin but not glucose levels increased in X+XO-treated than in control rats. Homeostasis model assessment (HOMA), an index of insulin resistance, was ~3.5-fold high in X/XO-treated rats (Fig. 5).

3. Discussion

Earlier, we reported that sub-chronic oxidative stress induced by pro-oxidant drug L-buthionine-(S,R)-sulfoximine (BSO) increases anxiety-like behavior in rats (Salim et al., 2010). Separate studies have indicated that induction of oxidative stress with chronic BSO treatment increases blood pressure in rats (Banday et al., 2008; Banday and Lokhandwala, 2008a,b,

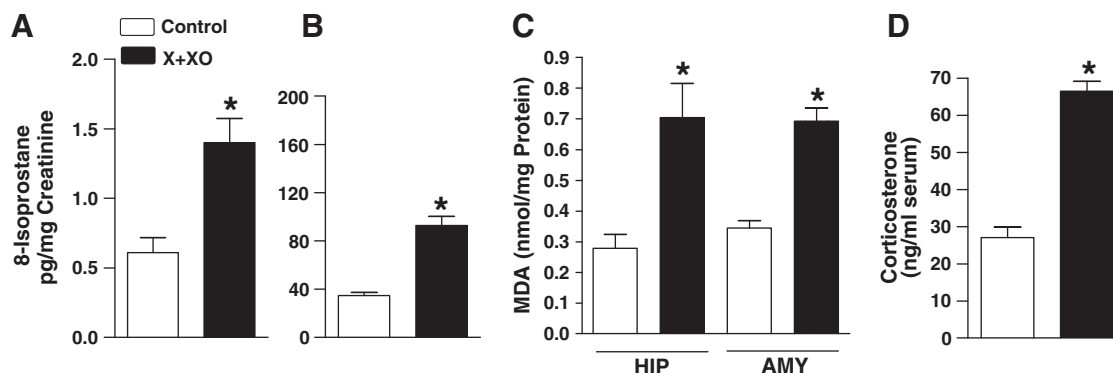


Fig. 2 – X+XO treatment increases oxidative stress and serum corticosterone levels. X+XO-treated rats had increased plasma and urinary 8-isoprostane levels (a marker of oxidative stress). X+XO treatment increased MDA levels in the hippocampus and amygdala as compared to control rats. Corticosterone levels measured by EIA (cat #500651, Cayman Chem. Co., Ann Arbor, MI) increased in X+XO treated rats as compared to control rats. *significantly different from control, $p < 0.05$, ANOVA, N=8–12 rats.

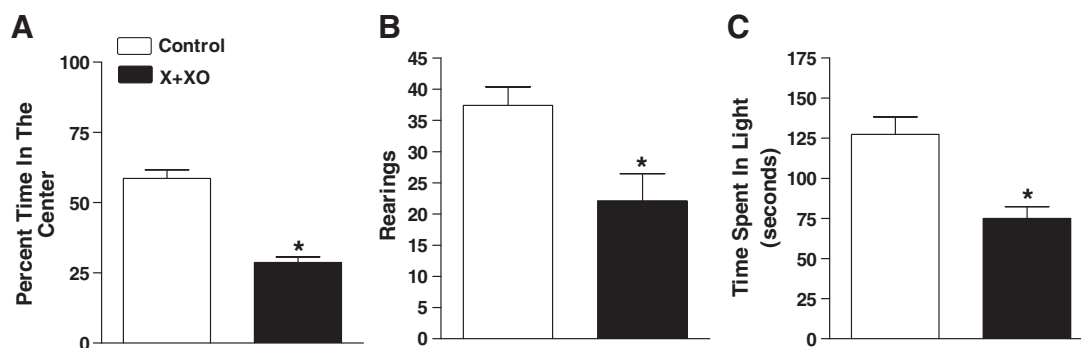


Fig. 3 – X+XO treatment increases anxiety-like behavior of rats as compared to controls. X+XO-treated rats displayed reduced center time (A), and rearing (B) in the open field-test and spent less time in lit areas than control (C). The total test time for light-dark test was 5 min and open-field test was 15 min. *significantly different from control treated rats ($p < 0.01$), using ANOVA Tukey's post-hoc analysis, $N = 12$ rats.

2007). Other studies have shown increased plasma insulin levels and HOMA in a rodent aging model associated with oxidative stress (Asghar et al., 2007). In our study, we have systematically examined the effect of inducing oxidative stress on anxiety-like behavior, blood pressure and insulin resistance, all in the same study, using the same oxidative stress inducer, X+XO.

X+XO treatment for 7 days increased anxiety-like behavior of rats assessed via two independent anxiety tests, open-field and light-dark tests. Display of increased anxiety-like behavior indicative from reduced time spent by the rats in the center versus the periphery of the open field arena and avoidance of lit area was not a result of sickness behavior that might be argued to have resulted from X+XO treatment as the body weight, food and water intake and body temperature of all groups of rats were similar. Fever, reduction of body weight and loss of appetite are common sickness responses to infection and inflammation (Skinner et al., 2009; Bluthé et al., 2006). Avoidance of lit areas by the rats was also not due to any sickness or eye inflammation as the eye reflexes were checked daily by placing a cotton swab close to ~1 in. of the eyes. The rats blinked in response to the

cotton swab and quickly moved aside. All rats responded to sounds and startle indicating that they were alert and active throughout the X+XO treatment period.

Our observation of increase in anxiety-like behavior with X+XO treatment is in agreement with our earlier findings of association of increased anxiety-like behavior with another oxidative stress inducer, BSO. These data suggest that two separate agents that cause oxidative stress, BSO and X+XO, both result in anxiety-like behavior of rats further supporting the involvement of oxidative stress in anxiety-like behavior. Moreover, consistent with previously reported oxidative stress-induced increase in serum corticosterones (Kobayashi et al., 2009), a known systemic marker of anxiety (Arranz et al., 2007), X+XO treatment increased serum corticosterone levels. Furthermore, X+XO treatment increased oxidative stress markers in serum, urine, and also increased oxidative stress in amygdala and hippocampus, brain regions implicated in anxiety response (Charney and Drevets, 2002). This also is in agreement with our earlier observations in which BSO treatment resulted in increased MDA levels in these brain regions (Salim et al., 2010). Therefore, rats with high levels of oxidative stress, exhibit higher anxiety evaluated via corticosterone levels and anxiety-like behavior.

As a second part of this study, we conducted blood pressure measurements on anesthetized rats, measured indices of oxidative stress, evaluated levels of corticosterones and plasma glucose and insulin levels (markers of insulin resistance) in rats that were treated with X+XO and compared our results with those of control rats. Body weight, food and water intake and body temperature was not adversely affected with X+XO treatment, suggesting that our observed increase in blood pressure is not a result of sickness but most likely a result of increase in oxidative stress. X+XO treatment for 7 days increased blood pressure and insulin resistance measured as HOMA in the same animals. Earlier studies conducted in rats have shown an increase in blood pressure with induction of oxidative stress with BSO treatment, however, insulin resistance parameters were not determined in those animals (Banday et al., 2008; Banday and Lokhandwala, 2008a,b). While one week X+XO treatment increased oxidative stress, insulin resistance and

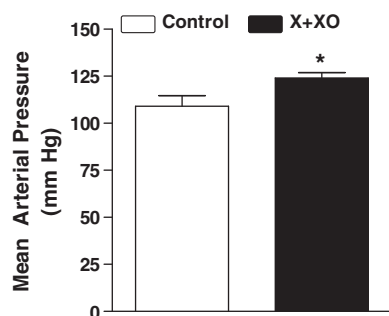


Fig. 4 – X+XO treatment increases blood pressure of rats as compared to controls. On the day of the experiment, the rats (fasted for 8–10 h) were anesthetized with Inactin (100 mg/kg; i.p.) followed by catheterization of carotid artery for blood pressure measurement. *significantly different from control, $p < 0.05$, ANOVA, $N = 8$ –12 rats.

Table 2 – X+XO treatment has no adverse effect on body weight, food or water intake and body temperature and X+XO treatment increases oxidative stress and corticosterone levels. Fifty grams of rodent chow (diet pellets) and 50 ml of tap water per rat was provided to the rats every day. The amount of food and water consumed was measured by weighing the left-over diet pellets and drinking water 24 h later. Data collected were averaged for each treatment group of rats on the last day of the experiment before behavior testing. Rectal temperature was manually recorded using animal rectal probe every day after 30 min of X+XO-treatment/vehicle injections. X+XO-treated rats had increased plasma and urinary 8-isoprostane levels (a marker of oxidative stress). X+XO treatment increased MDA levels in blood plasma as compared to control rats. *significantly different from control, $p < 0.05$, ANOVA, $N = 8-12$ rats.

General parameters	Control	X+XO
Body weight (g)	212±2.0	215±1.1
Food intake (g)	20.8±2.2	20.2±3.5
Water intake (ml)	23±2.2	24.2±2.0
Body temperature (°C)	36.5±0.31	37.2±0.33
<i>Indices of oxidative stress</i>		
8-isoprostane, urine pg/mg creatinine	0.711±0.01	1.552±0.02* $p < 0.05$
8-isoprostane, serum fg/mg creatinine	40.22±0.01	82.00±0.003* $p < 0.05$
MDA, serum nmol/mg protein	0.830±0.055	1.495±0.053* $p < 0.05$
<i>Index of stress/anxiety</i>		
Serum corticosterone (ng/ml)	33.58±0.22	96.02±0.21* $p < 0.05$

anxiety-like behavior, this treatment did not robustly elevate blood pressure. Probably some compensatory mechanisms come into play which restrict robust increase in blood pressure (for up to 7 days). Increasing the treatment period beyond 7 days with X+XO may further elevate blood pressure as has been shown with chronic BSO treatment (Banday et al., 2008).

Several limitations of our study deserve comment. Although we determined blood pressure and insulin resistance in the same rats, however, anxiety measurements were carried out in separate rats. The purpose of this approach was to harvest brains by quickly decapitating rats that underwent anxiety-like behavior measurements. The same rat could not be used for blood pressure measurements as blood pressure recording was done for 30 min under anesthesia, which would have delayed brain collection. Moreover, the risk of the involvement of non-specific effects due to longer exposure to anesthetic agent on biochemical parameters

analyzed in this study was avoided as this would have complicated our results confounding accurate data interpretation. However, future studies are needed for a better and more accurate analysis of contribution of oxidative stress to anxiety-like behavior, insulin resistance and hypertension where both blood pressure measurement by telemetry approach and anxiety-like behavior measurements are carried out in conscious freely moving animals. Nevertheless, our present data are suggestive of the fact that oxidative stress is a likely contributing factor towards increasing blood pressure, insulin resistance and anxiety-like behavior.

The question of whether oxidative stress precedes or follows anxiety, hypertension or insulin resistance is an interesting one. Recent studies have indicated that both hypertensive humans and animals have decreased antioxidant capacity and produce excessive amounts of reactive oxygen species and consequently have high oxidative stress

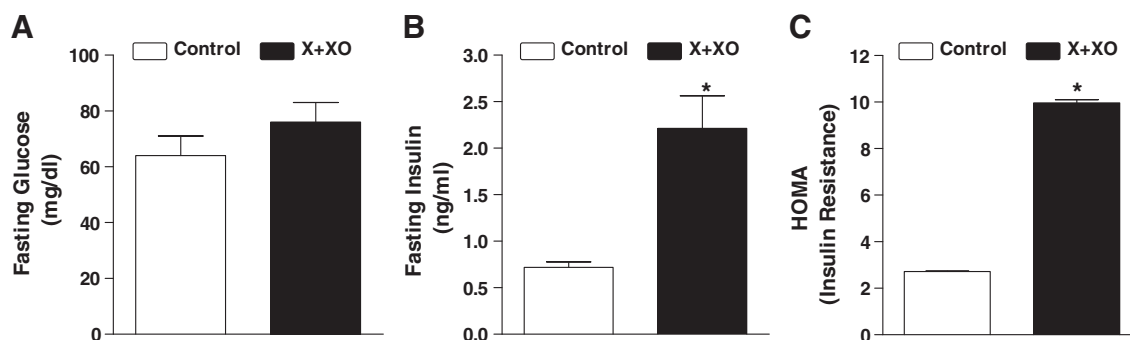


Fig. 5 – X+XO treatment increases insulin resistance in rats. On the day of experiment, the rats (fasted for 8–10 h) were anesthetized with Inactin (100 mg/kg; i.p.) followed by catheterization of carotid artery for blood sample collection. Glucose levels were measured with automated glucose analyzer (AccuCheck). Insulin level was determined by using radioimmunoassay kit employing manufacturer's protocol (Linco Research, St. Charles, Missouri). HOMA (homeostasis model assessment), an index of insulin resistance was calculated by using the formula, $[HOMA = \text{glucose (mM)} \times \text{insulin } (\mu\text{U ml}^{-1}) / 22.5]$ as described by Pikavance et al. (1999).

(Banday et al., 2008; Banday and Lokhandwala, 2008a,b). The cause and effect relationship between oxidative stress and hypertension is not clear, although there is evidence of a causal role of oxidative stress in hypertension in animal models (Banday et al., 2008; Banday and Lokhandwala, 2008a,b). Furthermore, there are some studies which suggest that oxidative stress leads to insulin resistance, while others indicate a reverse relationship. For example, the most direct evidence for oxidative stress-induced insulin resistance comes from the studies using antioxidant α -lipoic acid, which was reported to improve insulin sensitivity in an animal model of diabetes (Packer et al., 2001). Also, in obese Zucker rats, a model of insulin resistance exhibiting increased oxidative stress, antioxidant (tempol) supplementation reduced oxidative stress and insulin resistance (Banday et al., 2005). Furthermore, several clinical trials demonstrated that vitamins E and C or glutathione (all antioxidants) improve insulin sensitivity in insulin-resistant individuals and/or patients with type 2 diabetes (Packer et al., 2001; Evans et al., 2003). Moreover, results of longitudinal studies suggest association of anxiety with an increased risk for the development of type 2 diabetes (Pouwer et al., 2010a,b). Finally, direct involvement of oxidative stress in anxiety-like behavior in rodents also has been reported (Salim et al., 2010; Hovatta et al., 2005; Gingrich, 2005; Masood et al., 2008; Souza et al., 2007; Bouayed et al., 2007; de Oliveira et al., 2007). These observations clearly indicate that there is a need to identify mechanisms that result in these consequences and determining a causal relationship between oxidative stress, anxiety, hypertension and insulin resistance is of paramount significance. For example, whether preventing anxiety-like behavior rescues high blood pressure or insulin resistance remains to be seen. Such information is important as it will help in the development of novel drugs that can reduce/prevent comorbidity of hypertension, diabetes and anxiety disorders.

4. Experimental procedures

4.1. Animal model of oxidative stress

All experiments were conducted in accordance with the NIH guidelines using approved protocols from the University of Houston Animal Care Committee. Male Sprague–Dawley rats (200–250 g) between 7 and 8 weeks of age were acclimatized for one week before any treatment. Xanthine and xanthine oxidase, hereafter, referred as X+XO was administered to rats daily. Xanthine was supplied in drinking water *ad libitum* and xanthine oxidase (5 Units/kg) was injected intraperitoneally once daily for 7 days (Fig. 1). The control group received drinking water and saline injection as vehicle. After 24 h of last day of treatment, anxiety tests were conducted, following which rats were anesthetized with mild anesthesia and quickly decapitated as previously published by us (Salim et al., 2010). The abdomen was immediately opened and blood from aorta and urine from the bladder was collected. Brains were harvested at this time. Blood serum was collected after centrifugation at 3500 rpm at 4 °C. Oxidative stress parameters were measured in serum, urine and brain tissue homogenates. In the second set of experiment, the rats were similarly treated and anesthetized with Inactin (100 mg/kg) for blood pressure measurement.

4.2. General body parameters

Body weight, food and water intake were recorded manually every day. Fifty grams of rodent chow (diet pellets) and 50 ml of tap water per rat was provided to the rats per day. The amount of food and water consumed was measured by weighing the left-over diet pellets and drinking water 24 h later. Data collected were averaged for each group of rats on the last day of the experiment before behavior testing. Rectal temperature was determined in rats that had been gently restrained by wrapping in a towel using an animal rectal probe for adult rats (Physitemp-Clifton, NJ). The probe was held in place until the meter reading equilibrated which took less than 10 s. An initial temperature was taken on all rats to allow them to acclimate to the probe. This initial temperature reading was not used in the data analysis. Temperature was manually recorded every day after 30 min of X+XO/vehicle treatment.

4.3. Anxiety behavior tests

Animals were housed in groups of three to five in plastic cages (14×18×18 in.) and kept in a room on a 12-h light/dark schedule (lights on at 6:00 A.M.) at 23 °C, 60% humidity, with food and tap water available *ad libitum*. On the day of behavior testing, the rats were brought to the behavior room at least an hour before testing time and left undisturbed in a quiet setting. After each behavior test, the apparatus was cleaned with 70% ethanol, wiped with hand towels and allowed to air dry in between animal testing. At first open-field test was conducted following a rest period of 1 h, the same animals were tested in light–dark exploration test the same day.

4.3.1. Open field activity

The open field test was conducted 24 h after the last X+XO injection. The open field task was carried out in 60×40 cm open field surrounded by 50 cm high walled Plexiglas chambers in standard room lighting conditions (Salim et al., 2010; Paylor et al., 1998). The animals were placed in the center of the compartment and were left free to explore the arena for 15 min. Activity was quantitated using a computer-operated Opto-Varimex Micro Activity Meter v2.00 system (Optomax, Columbus Instruments; OH) that utilizes sensors containing 8 infrared light emitting diodes and 8 phototransistors that emit and detect modulated infrared light beams. Sensors were positioned to form two-dimensional cages each with rearing monitoring. Movement was detected by beam breaks and data from three test chambers were recorded simultaneously, one rat per chamber, collected in 3 min intervals over a 15-min test session. The program tabulated activity counts, zone entries, zone times, center time and the periphery time, distance travelled and rearing for every cage in the system. For center time analysis, an approximately 25 cm×25 cm square in the center of the open field arena was defined as the center zone for data analysis. Total time spent in the center of the arena and rearings were calculated for each group.

4.3.2. Light–dark exploration

The rats were subjected to light–dark exploration test. Rodents are nocturnal and prefer darker areas, so the decrease in the exploratory activity in a lighted area is believed to be indicative

of increased anxiety-like behavior (Salim et al., 2010) and the time spent in the light is considered as a measure of anxiety-like behavior (Salim et al., 2010; Crawley and Goodwin, 1980). The light–dark box consisted of a light compartment (27×27×27 cm) and a dark compartment (black colored surrounding walls and floor, 27×18×27 cm) separated by a partition with a single opening (7×7 cm) for passage from one compartment to the other as described in our previous publication (Salim et al., 2010) and as published by others (Ramos et al., 2008). The apparatus was situated within a screen enclosed area of behavior core facility room with only one experimenter/observer present in the room at the time of experiment under standard lighting conditions of approximately 700 lux as previously used by others (Salim et al., 2010; Paylor et al., 1998; Gangitano et al., 2009). A Microsoft Excel software program enabled the observer to record time by manually scoring the data as described in detail in our earlier publication (Salim et al., 2010). Thus, total time spent in the illuminated part was recorded by an observer blinded to treatment for 5 min. A rat was defined to have entered the lit or dark box when both front paws and shoulders were inside the respective compartment.

4.4. Blood pressure measurement

Blood pressure measurement was done as previously described (Asghar et al., 2009). Briefly, separate groups of rats (other than the ones used for anxiety-like behavior measurement) were anesthetized with Inactin (100 mg/kg ip). Tracheotomy was performed to facilitate breathing. To measure blood pressure and to collect blood samples, the left carotid artery was catheterized with PE-50 tubing. This tubing was connected to a pressure transducer, which was connected to an amplifier (GRASS, LP122). Blood pressure was continuously recorded for 30 min using GRASS PolyView Data Acquisition and Analysis Software systems (Astro-Med, GRASS Instrument Division, West Warwick, RI). After blood pressure measurement, aliquots of blood samples were withdrawn and plasma was isolated by centrifugation and kept frozen until further use.

4.5. Brain dissections

Experimental and control rats were anesthetized using mild anesthesia (Isoflurane, #57319-479-06, Phoenix Pharmaceuticals) immediately after anxiety behavior tests. The brains were quickly removed and rapidly frozen over dry ice and stored at -80 °C until analysis. Hippocampus and amygdala identified according to the atlas of Paxinos and Watson (1986) were grossly dissected out using a brain slicer (Zivic instruments, PA). Homogenates of the brain regions were prepared in buffer containing 20 mM Tris-HCl, 4 mM EDTA plus protease inhibitors, 100 µg/ml PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin (Salim and Dessauer, 2004). The lysates were examined for protein concentration using Pierce's protein detection kit (Pierce, Rockford, IL) (Smith et al., 1985) and used for analysis of oxidative stress marker measurements.

4.6. Indices of oxidative stress

Serum and urine 8-isoprostane levels were measured using EIA kit (Cayman, Ann Arbor, MI). Malondialdehyde (MDA) was

measured as published (Salim et al., 2010; Schacter et al., 1994; Urchiyama and Mihara, 1978) in serum and in brain homogenates prepared from amygdala and hippocampus. Briefly, protein was diluted to 1–2 mg/ml with 1.15% KCl and boiled with 2 ml of 15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.25 N HCl for 15 min. The sample was cooled, centrifuged at 1,000 g for 10 min and the color was read at 535 nm on a spectrophotometer. MDA was quantified using molar extinction coefficient, $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

4.7. Plasma glucose and insulin measurement

Insulin level was determined by using radioimmunoassay kit employing manufacturer's protocol (Linco Research, St. Charles, Missouri). HOMA (homeostasis model assessment), an index of insulin resistance was calculated by using the formula, $[\text{HOMA} = \text{glucose (mM)} \times \text{insulin } (\mu\text{U ml}^{-1}) / 22.5]$ as described by Pikavance et al. (1999).

4.8. Data analysis

Data are expressed as mean±SEM. Significance was determined by one way ANOVA applying Tukey's post-hoc test (GraphPad Software, Inc. San Diego, CA). A value of $p < 0.05$ was considered significant.

Acknowledgments

Funding for this research was provided by University of Houston GEAR grant awarded to S.S and NIH/NIA AG29904 to M.A. We would like to acknowledge Prof. Bhagwan S. Jhandyala for his valuable intellectual input and advice.

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