HHS Public Access

Author manuscript

Life Sci. Author manuscript; available in PMC 2021 June 01.

Published in final edited form as:

Life Sci. 2020 June 01; 250: 117598. doi:10.1016/j.lfs.2020.117598.

Estrogen-Dependent Hypersensitivity to Diabetes-Evoked Cardiac Autonomic Dysregulation: Role of Hypothalamic Neuroinflammation

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Abstract

Aims: To investigate if autonomic dysregulation is exacerbated in female rats, subjected to diabetes mellitus (DM), via a paradoxical estrogen (E_2)-evoked provocation of neuroinflammation/injury of the hypothalamic paraventricular nucleus (PVN).

Main methods: We measured cardiac autonomic function and conducted subsequent PVN neurochemical studies, in DM rats, and their respective controls, divided as follows: male, sham operated (SO), ovariectomized (OVX), and OVX with E_2 supplementation (OVX/E_2).

Key findings: Autonomic dysregulation, expressed as sympathetic dominance (higher low frequency, LF, band), only occurred in DM E_2-replete (SO and OVX/E_2) rats, and was associated with higher neuronal activity (c-Fos) and higher levels of TNFα and phosphorylated death associated protein kinase-3 (p-DAPK3) in the PVN. These proinflammatory molecules likely contributed to the heightened PVN oxidative stress, injury and apoptosis. The PVN of these E_2-replete DM rats also exhibited upregulations of estrogen receptors, ERα and ERβ, and proinflammatory adenosine A1 and A2a receptors.

Significance: The E_2-dependent autonomic dysregulation likely predisposes DM female rats and women to hypersensitivity to cardiac dysfunction. Further, upregulations of proinflammatory mediators including adenosine A1 and A2 receptors, TNFα and DAPK3, conceivably explain the paradoxical hypersensitivity of DM females to PVN inflammation/injury and the subsequent autonomic dysregulation in the presence of E_2.

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Declaration of interest:

The authors declare no competing interests.

Keywords

Adenosine; Diabetes; Autonomic dysregulation; Estrogen; Hypothalamus

1. Introduction:

Our recent study (Leffler and Abdel-Rahman, 2019) is the first to replicate the exaggerated cardiac dysfunction in women, compared to men, with diabetes mellitus (DM) (Low Wang et al. , 2016, Regensteiner et al. , 2015). These studies implicated E_2 in the heightened cardiac dysfunction in diabetic females, at least partly, via the reductions in connexin-43 and the phosphorylated survival molecules, extracellular regulating kinases 1/2 and phosphorylated AKT, in the heart (Leffler and Abdel-Rahman, 2019). However, the previous studies did not consider the possible contribution of autonomic dysregulation to the sex/estrogen (E_2)-dependent hypersensitivity to DM-evoked cardiac dysfunction in females. This is important because regardless of the cause, autonomic dysregulation predisposes to cardiac dysfunction (Schwartz et al. , 1992). Further, it is not known if E_2 exaggerates DM-evoked injury in cardiac autonomic nuclei. This possibility is somewhat supported by the findings that E_2 loses its neuroprotective effect (Sasidharan et al. , 2013) or worsens neuronal injury (Santizo et al. , 2002) in diabetic animals. Importantly, these neuronal injuries were observed in non-cardiovascular regulating neurons, and no effort was made to investigate cardiac autonomic function in diabetic animals in these reported studies.

Oxidative stress/injury in brain stem autonomic nuclei, such as the rostral ventrolateral medulla (RVLM) and the nucleus tractus solitarius (NTS), contributes to sympathoexcitation, autonomic dysregulation and subsequent cardiac dysfunction in diabetic male rats (Fouda and Abdel-Rahman, 2017, Fouda et al. , 2018). Whether similar adverse effects occur in the hypothalamic paraventricular nucleus (PVN) of diabetic rats, in sex/E_2-dependent manner, has not been studied. In addition to regulating energy homeostasis (Williams and Elmquist, 2012), the PVN sends projections to brain stem autonomic nuclei (Morton et al. , 2006). Equally important, previous studies did not consider the possible role of interaction between ovarian hormones, particularly E_2, and central adenosine receptors in the neuroinflammation associated with DM.

The highly expressed adenosine A1 and A2a receptors in the brain (Fredholm et al. , 2005) mediate brain area-specific physiological and pathological effects (Gomes et al. , 2011, Mohamed et al. , 2016, Nassar and Abdel-Rahman, 2009). However, it remains unknown if upregulation of the proinflammatory adenosine A1 and A2a receptors occurs in brain nuclei that regulate autonomic function in DM rats and if the E_2-mediated upregulation of both receptors in human cancer cells (Mohamadi et al. , 2018) extends to PVN neuronal tissues in DM females.

Here, we first sought to determine if E_2 exacerbates cardiac autonomic dysregulation in diabetic female rats. Next, we discerned the neurochemical responses, which likely explain the exacerbated neuroinflammation/injury of the PVN, as underlying mechanism for this sex/E_2-dependent anomaly. Specifically, we hypothesized that E_2 paradoxically induces PVN oxidative stress via upregulation of the proinflammatory molecules TNFα, its

downstream effector, death associated protein kinase-3 (DAPK3) and adenosine A1/A2 receptors. Finally, we investigated the possibility that enhanced estrogen receptor signaling, via E_2 activation of upregulated ERα or ERβ in the PVN, likely contributed to these adverse neurochemical responses. The findings will advance our understanding of the exacerbated autonomic dysregulation, which predisposes to cardiac dysfunction in E_2-replete diabetic females and identify novel therapeutics targets for alleviating this clinically relevant problem.

2. Materials and methods

2.1. Animals

Female and male 8-week-old Wistar rats (initial weight, 170 −200 g; Charles River Laboratories, Raleigh, NC) were housed in pairs and allowed free access to water and chow (Prolab Rodent Chow; Granville Milling, Creedmoor, NC). Rats were fed, ad libitum, control (AIN-93G Growth Purified diet; 57W5 TestDiet) or high fat diet (DIO Rodent Purified diet with 45% energy from fat; 58V8 TestDiet; Granville Milling, Creedmoor, NC) based on reported studies (Brown and Panchal, 2011, Sasidharan et al., 2013, Srinivasan and Ramarao, 2007). The light-dark cycle was maintained, and the temperature and humidity were kept at 50 ± 10% and 23 ± 1ºC, respectively.

All animal related procedures were approved by the institutional animal care and use committee and conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institute of Health and the National Research Council Committee Update of the Guide for the Care and Use of Laboratory Animals, 2015.

2.2 Protocols and experimental groups

In the present study, we measured cardiac autonomic function (derived from heart rate variability) as detailed below. These new data were generated from blood pressure waveforms obtained from rats used in our recent study (Leffler and Abdel-Rahman, 2019); rats were divided into diabetic sham operated (SO), ovariectomized (OVX), OVX with E_2 supplementation (OVX/E_2), male rats and their corresponding controls (n=5 rats per group). We also conducted neurochemical studies on the brains of these rats to test the hypothesis that a paradoxical E_2-mediated exaggeration of the proinflammation milieu in the PVN underlies cardiac autonomic dysregulation in diabetic females. Briefly, the rats were injected with freshly prepared STZ (35 mg/kg; I.P.) in 0.1M citrate buffer (pH 4.0) or buffer (control) 4 weeks after starting the high fat or control diet regimen. One week after the first dose, a second STZ dose was given under the same conditions. OVX was performed at the beginning of the study and the rats were kept for two weeks after OVX to ensure ovarian hormones depletion in accordance with our established protocol (Yao and Abdel-Rahman, 2017). At this time, OVX rats received subcutaneously implanted E_2 or its vehicle in silastic tubing in the back of the neck as in our reported studies (Yao and Abdel-Rahman, 2017) and this procedure was repeated after 4 weeks to ensure the maintenance of physiological E_2 levels in OVX rats. Five weeks after STZ injection (9 weeks after starting the high fat or control diet), intravascular and left ventricular catheterization were performed to permit

generation of the hemodynamic data included in our previous studies (Leffler and Abdel-Rahman, 2019) and the spectral analyses included in this study (see below).

2.3. Heart rate variability (spectral analysis) studies

In the present study, we conducted spectral analysis of blood pressure data obtained from DM and control groups used in our previous study (Leffler and Abdel-Rahman, 2019), and recorded by ML870 (PowerLab 8/30; Colorado Springs, CO) and analyzed by LabChart (v.7) pro software (AD Instruments, Colorado Springs, CO). Frequency domain analysis using FFT algorithms of RR data series was followed (El-Mas and Abdel-Rahman, 2007). Spectra were integrated into 2 specific frequency bands, low-frequency (LF) (0.25–0.75 Hz) and high-frequency (HF) (0.75–3 Hz) bands. The data was expressed in normalized units (LFnu and HFnu), which reflect cardiac sympathetic and parasympathetic dominance, respectively. The LF/HF ratio was computed and taken as a measure of cardiac sympathovagal balance (El-Lakany et al. , 2018). At the end of hemodynamic measurements, rats were euthanized, and the tissues were excised, and flash frozen in 2-methylbutane (Sigma-Aldrich, St. Louis, MO) in dry ice. Tissue was stored at −80ºC until processed for biochemical studies.

2.4. Neurochemical studies

Hypothalamic coronal sections (30-μm-thick, −1.70 to −2.2 mm caudal to the Bregma) made by a freezing microtome (HM 505 E; Microm International GmbH, Walldorf, Germany) were used for neurochemical studies (Khazipov et al. , 2015, Paxinos et al. , 1980). In a separate group of rats (n=5), five serial coronal sections (100 μm) were cut at the same mentioned coordinates according to the Palkovits technique (Kleiber et al. , 2008, Palkovits, 1983). The PVN was bilaterally punched with an 18-gauge punch instrument (Stoelting Co., Wood Dale, IL) and homogenized with PBS (for ROS measurements; 50 Mm, pH 7.4) or with lysis buffer (for western blot analysis).

2.5. Quantification of neuronal injury (Fluorojade-C staining)

A fluorojade C staining kit was used in accordance with the manufacturer’s instructions (Biosensis, TR-100-FJ, Thebarton, South Australia) and our reported studies (Fouda and Abdel-Rahman, 2017, Fouda et al., 2018). Briefly, 0.06% potassium permanganate solution was used to incubate the PVN sections, on the slides, for 10 min followed by rinsing by distilled water then incubating in fluorojade C solution (1:25) for 30 min. The slides were then washed and mounted on coverslips with Vecta-shield mounting medium (Vector, Burlingame, CA, USA). A Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, New York), and a blue (450–490 nm) excitation light was used for the visualization of stained neurons and image acquisition (Yang et al. , 2015). For quantification, Zen Lite 2011 software was used to measure the fluorescence intensity and scored by a blinded investigator.

2.6. Immunohistochemical analysis

We followed the immunohistochemistry staining protocols described in our studies (Fouda and Abdel-Rahman, 2017, Fouda et al., 2018). Briefly, PVN sections were post-fixed for 30

min. in 4% paraformaldehyde in tris-buffered saline, and subsequently incubated in 0.3% H_2O_2 for 30 min to block endogenous peroxidase. Sections were then incubated with one of the following primary antibody: Rabbit polyclonal anti-c-Fos, anti-TNF-α, anti-A1 (adenosine A1) receptor, and anti-A2a (adenosine A2) receptor (1:200 dilution, Abcam, Cambridge MA), over night at 4°C using a modification of the avidin–biotin-complex method (ABC) kit (Vector Laboratories, Inc. Burlingame, CA). Positive profiles showed dark granular brown staining indicative of a 3, 3-Diaminobenzidine (DAB) reaction product. Specificity of the antibody was tested by processing side- by-side slices without incubation with the primary or the secondary antibody.

2.7. ROS measurement

Oxidative stress level was measured using 2', 7'-dichlorofluorescein diacetate (DCFH-DA), a detector of ROS (Korystov et al. , 2009). Briefly, a stock solution of DCFH-DA (20 mM, Molecular Probes, Grand Island, NY) in methanol and protected from light was freshly diluted with PBS to prepare a150 μM working solution. Afterwards, 10 μl of PVN homogenate supernatant was added in a 96-well plate to give a final concentration of 25 μM DCFH-DA to produce fluorescent 2′,7′-Dichlorofluorescein (DCF) in the incubation medium at 37°C. Fluorescence intensity was measured 30 min after the reaction initation using a microplate fluorescence reader set at excitation 485 nm/emission 530 nm. The ROS level was determined as relative fluorescence units (RFU) of generated DCF using standard curve of DCF (Fouda and Abdel-Rahman, 2017, Fouda et al., 2018).

2.8. Dihydroethidium staining for superoxide detection

In accordance with the recommendations of using two or more different methods for ROS levels measurement (Griendling et al. , 2016), PVN sections from diabetic and control rats (n = 5) were incubated with 10 μM dihydroethidium (DHE) (Molecular Probes, Grand Island, NY) at 37°C in the presence of 5% CO_2 in a moist chamber for 30 min. Images were visualized using Zeiss LSM 510 microscope. Image J Software (National Institutes of Health) was utilized for fluorscence quantification (Collin et al. , 2007, Fouda et al., 2018).

2.9. Western blot analysis

This procedure was conducted as described in our previous studies on other tissues (Fouda and Abdel-Rahman, 2017, Fouda et al., 2018). Punched PVN tissues were homogenized with lysis buffer containing 20 mM TRIS, Ph 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton x-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphateand 1 μg/ml leuptin with protease inhibitor cocktail (Roche diagnostics, Indianapolis, IN). After homogenization, a Bio-Rad protein assay system (Bio-Rad laboratories, Hercules, CA) was utilized for protein quantification. Protein (20 μg/lane) was separated using 4–12 % SDS/ PAGE gel (Invitrogen, Carlsbad, CA). After the transfer was done using nitrocellulose membranes, the proteins were revealed by immunoblotting using a 1:200 dilution of anti-ERα, anti-ERβ or anti p-DAPK3 polyclonal antibodies (Abcam, Cambridge MA) along with 1:200 dilution of anti-t-DAPK3 (for p-DAPK3) or 1:2000 anti-GAPDH (for ERs) (Abcam, Cambridge MA) at 4°C overnight. Afterwards, the membranes were washed, incubated for 60 min with mixture containing IRDye-680conjugated goat anti-mouse and IRDye800-conjugated goat anti-rabbit (1:15000; LI-COR Biosciences). The idetified proteins were

visulaized using Odyssey Infrared Imager and analyzed with Odyssey application software version 5.2 (LI-COR Biosciences). Data represents mean values of integrated density ratio of ERs or p-DAPK3 normalized to the corresponding housekeeping protein, GAPDH or t-DAPK3, and expressed as percent of non-diabetic male control. The representative bands were cropped from the same gel containg each of the targeted protein along with its corrseponding housekeeping protein for diabetic and control groups. The same contrast and background adjustments were uniformly applied to all bands on the gel.

2.10. Data analysis and statistics

Statistical analysis consisted of a two-way ANOVA (endpoint data) along with post hoc testing of significant findings along with F-test and Tukey's test to reduce family wise error rate was employed as appropriate using Prism 7 software (Graphpad Software Inc., San Diego, CA). Values are presented as mean ± SEM with probability levels less than 0.05 considered significant.

3. Results

3.1. Exacerbated autonomic dysregulation and PVN injury/inflammation in E_2 replete diabetic female rats

Mean arterial pressure (MAP), measured at the conclusion of the study, did not show significant differences amongst control (healthy) or DM rat groups (Fig. 1A). Spectral analysis of heart rate variability, derived from blood pressure, revealed cardiac autonomic dysregulation in DM rats only in the presence of ovarian hormones/E_2 (Fig. 1). Diabetic SO or OVX/E_2 rats exhibited higher ($P<0.05$) R-R oscillations in the LFnu range (0.25–0.75 Hz, Fig. 1B) and no change in R-R oscillations in the HFnu range (0.75–3 Hz; Fig. 1C) compared to their non-diabetic counterparts. On the other hand, diabetic male or OVX rats showed no significant difference in the LFnu or the HFnu range, compared to their non-diabetic counterparts (Fig. 1B and 1C). The resultant higher ($P<0.05$) LF/HF ratio, in only DM SO or OVX/E_2 rats, reflected an E_2-dependent shift in the cardiac sympathovagal balance toward sympathetic dominance in DM female, compared to DM male or OVX, rats (Fig. 1D). Importantly, control SO and OVX/E_2 female rats exhibited lower ($P<0.05$) LFnu and LF/HF ratio (lower sympathetic dominance), compared to male or OVX control rats (Fig. 1D).

PVN Neuronal injury (Fluorojade C staining intensity; Fig. 2), neuronal activity (c-Fos immunoreactivity; Fig. 3) and ROS levels (DHE fluorescence intensity and DCF kinetics; Fig. 4) were higher ($P<0.05$) in all diabetic groups, compared to their respective control. However, diabetic SO or OVX/E_2 rats exhibited greater ($P<0.05$) PVN injury (Fig. 2), neuroexcitation (Fig. 3) and ROS levels (Fig. 4), compared to diabetic male or OVX rats. Notably, control E_2-replete female (SO or OVX/E_2) rats exhibited lower ($P<0.05$) PVN ROS and neuronal injury than control male or OVX rats (Figs. 2–4).

3.2. DM-induced PVN upregulations of ER and adenosine A1/A2a receptors, and elevations of TNF-α and p-DAPK3 are exacerbated in E_2-replete rats

PVN ERα and ERβ were upregulated ($P<0.05$) in DM SO and OVX/E_2 rats, compared to control counterparts and DM OVX or male rats (Fig. 5). Notably, in control rats, PVN ERα expression was higher ($P<0.05$) in SO and OVX/E_2 rats than in male or OVX rats (Fig. 5A).

While DM increased ($P<0.05$) TNF-α (Fig. 6), and its downstream effector p-DAPK3 (Fig. 5C), the increases were most evident in the PVN of the E_2-replete (SO and OVX/E_2) DM rats (Figs. 5C, 6). It is noteworthy that the PVN of control SO and OVX/E_2 rats exhibited lower ($P<0.05$) TNF-α (Figs. 6) than control male or OVX rats.

PVN adenosine A1 receptor expression was lower ($P<0.05$) in control SO or OVX/E_2 than in control OVX or male rats (Fig. 7). However, irrespective of sex or ovarian hormonal status, A1 receptor expression increased ($P<0.05$) to similar levels in all DM groups (Fig. 7), indicating exaggerated PVN A1 receptor upregulation in E_2-replete DM rats, when compared to respective controls (Fig. 7). By contrast, while the PVN A2a receptor level was not affected by sex or ovarian homonal status in control rats, it only increased ($P<0.05$) in DM SO and OVX/E_2 rats (Fig. 8).

4. Discussion

Our recent preclinical study (Leffler and Abdel-Rahman, 2019), which replicated premenopausal women's hypersensitivity to DM-induced cardiac dysfunction (Juutilainen et al. , 2004), revealed sex/E_2-dependent cardiac molecular aberrations that likely explain this clinical problem. It remains unknown, however, if autonomic dysregulation, which contributes to cardiac dysfunction (De Angelis et al. , 2009), is exaggerated in E_2-replete DM rats. The present spectral analysis of inter-beat data revealed the first evidence of autonomic dysregulation only in the E_2-replete DM rats, which exhibited cardiac dysfunction in our recent study (Leffler and Abdel-Rahman, 2019). We then tested the hypothesis that a paradoxical E_2-mediated exaggeration of the proinflammation milieu in the PVN underlies the cardiac autonomic dysregulation in these diabetic females. Our findings support this hypothesis and implicate E_2-dependent upregulations of ERα/ERβ and adenosine A1/A2a receptors in PVN neuroinflammation and injury in DM rats. The present findings provide new insights into central mechanisms for the paradoxical E_2-dependent hypersensitivity to autonomic dysregulation, which likely contributes to cardiac dyfunction in diabetic females.

Our experimental design encompassed E_2-deficient (male and OVX) and E_2-replete (ovarian source; SO, and exogenous E_2, OVX/E_2) rats that were subjected to DM or served as (healthy) controls. This approach permitted critical assessment of the paradox of E_2 contribution to detrimental effects under pathological (DM), in marked contrast to its protective effects under healthy, condions.

Autonomic dysregulation caused by oxidative stress-evoked neuroactivation of autonomic nuclei, including the PVN, leads to cardiac sympathetic dominance (Kishi and Hirooka, 2012). These neuronal and cardiac sympathetic responses likely contribute to cardiac

dysfunction in models of human diseases including DM although most, if not all, of these studies were conducted in male animals (Ceriello and Motz, 2004, Fouda et al., 2018). Consistent with these findings, the exaggerated PVN oxidative stress and neuroactivation, higher c-Fos immunoreactivity (Figs. 3–4), likely explain the cardiac sympathetic dominance here (Fig. 1) and cardiac dysfunction (Leffler and Abdel-Rahman, 2019) in E_2-replete DM female rats. These preclinical findings likely explain the poorer cardiovascular health in DM premenopausal women, compared to age-matched DM men (Regensteiner et al., 2015).

We focused on the PVN neurobiology because it integrates neuroendocrine, metabolic and autonomic roles (Williams and Elmquist, 2012), sends projections to the RVLM, which maintains sympathetic dominance (Morton et al., 2006) and lesion-induced PVN injury (Deng et al. , 2015) causes autonomic dysregulation; the latter is linked to cardiac dysfunction in DM male rats (El-Sayed et al. , 2016). Based on this knowledge, the present findings support a role for the exacerbated PVN injury/oxidative stress (Figs. 2–4) in the autnomic dysregulation (Fig. 1) in E_2-replete DM rats. Further, the lower magnitude PVN injury/oxidative stress, in E_2-deficient (OVX and male) DM rats (Figs. 2–4), did not cause autonomic dysregulation here (Fig. 1) or cardiac dysfunction (Leffler and Abdel-Rahman, 2019) during the relatively short duration of our study. This conclusion is supported by the occurrence of cardiac dysfunction when male rats were subjected to the same DM regimen, adopted here, for a longer time period (Hoit et al. , 1999). Collectively, in addition to supporting DM-induced neurotoxicity in hypothalamus (Luo et al. , 2002), and other autonomic nuclei (Fouda et al., 2018) of male rats, the present findings demonstrate E_2-mediated hypersensitivity to DM-induced PVN neuronal oxidative stress and injury.

We hypothesized that a counterintuitive proinflammatory environment, triggered by E_2 activation of ERα, contributed to the exacerbated PVN neuroinflammation/injury in DM females. To this end, we interpreted our PVN and autonomic responses in DM rats with or without E_2, in relation to findings in other model systems. First, the feed-forward interaction between ROS and inflammatory responses, and the subsequent neuronal injury (Fischer and Maier, 2015), are drastically enhanced in E_2-replete DM rats in our study. Second, ROS transformation of E_2 into a proinflammatory hormone occurs in cardiovascular tissues (El-Mas and Abdel-Rahman, 2015, Leffler and Abdel-Rahman, 2019, White et al. , 2010), via upregulating ROS-induced TNF-α and its downstream effector DAPK-3, Third, the PVN levels of ROS (Fig. 4), TNF-α (Fig. 6) and phospho-DAPK-3 (Fig. 5) were substantially higher in E_2-replete DM, compared to their E_2-deficient counterparts (Fig. 4–6). Finally, our pharmacological loss or gain-in-function findings suggested a major roel for ERα in the E_2-dependent cardiac oxidative stress/dysfunction caused by alcohol (Yao and Abdel-Rahman, 2016, Yao and Abdel-Rahman, 2017).

Next, we considered the novel possibility that the PVN proinflammatory milieu is triggered by ERα mediated upregulations of the proinflammatory adenosine A1 and A2a receptors in E_2-replete DM. This premise is based on findings, in other cell types, that implicated E_2/ERα in elevations in cellular oxidative stress (Yao and Abdel-Rahman, 2016, Yao and Abdel-Rahman, 2017) and induction of proinflammatory adenosine receptors expression (Etique et al. , 2009, Mohamadi et al., 2018). Consistent with these findings, we demonstrate

upregulations of ERα/ERβ and adenosine A1/A2a receptors in the PVN of DM females in the presence of E_2 (Figs. 7, 8). It is logical to assume enhanced signaling of these four upregulated receptors by the higher levels of their ligands in E_2-replete DM rats because E_2 enhances adenosine release (Ribeiro, 2005). It is noteworthy that ERα, and to a lesser extent ERβ, blockade mitigated the exacerbation of ethanol-evoked oxidative stress and dysfunction in E_2-replete female rats (Yao and Abdel-Rahman, 2016) and adenosine A1 and A2 blockade mitigated cellular inflammation in other tissues (Phillis, 1995, Stockwell et al. , 2017).

Finally, our findings in healthy rats provide new insight into the physiological neuro- and cardioprotective roles of E_2 for these reasons. First, PVN ERα upregulation/activation by E_2 (Fig. 5A) agrees with physiological findings in other tissues of E_2-replete rats (Hao et al. , 2016, Steagall et al. , 2017). Second, the novel inverse relationship between ERα and reduced adenosine A1 receptor (Fig. 7) in the PVN likely contributes to reductions in central sympathetic tone (reduced PVN c-FOS immunoreactivity; Fig. 3) and in cardiac sympathetic dominance (Fig. 1); these responses underlie higher baroreflex sensitivity in healthy E_2-replete, compared to OVX or male rats (El-Mas and Abdel-Rahman, 1998, El-Mas and Abdel-Rahman, 2009). Third, the lower PVN ROS (Fig. 4) is expected to reduce adenosine level (Smith et al. , 2014), and its signalling via the downregulated A1 receptor in the E_2-replete (SO and OVX/E_2) healthy rats. Finally, A1 receptor blockade confers neuroprotection against brain insults (Phillis, 1995, Stockwell et al., 2017) via abrogation of inflammation and oxidative stress (Peleli and Carlstrom, 2017). Collectively, our findings highlight the importance of suppressed PVN adenosine A1 receptor expression in the E_2-dependent protective neurobiological/cardiovascular responses in healthy rats.

5. Conclusion

Estrogen upregulation and activation of ERα likely contributes to the paradoxical inflammatory milieu that caused PVN neuroinflammation/injury and the autonomic dysregulation in DM females. Building on evidence in other cell types, we show novel E_2/ERα-evoked upregulations of adenosine A1 and A2a receptors, which contributed, at least partly, to the PVN proinflammatory milieu in E_2-replete DM rats. Future studies are warranted to discern the molecular link between PVN adenosine A1/A2a receptors and the TNF-α-DAPK-3 cascade to better understand the mechanisms of the E_2-dependent hypersensitivity to autonomic dysfunction in diabetic females. Finally, the findings will help identify novel therapeutic targets for alleviating the neurotoxicity and the associated cardiovascular anomalies in diabetics, particularly in the presence of estrogen.

Acknowledgements

The authors thank Ms. Kui Sun for her technical assistance.

Funding sources

Partly supported by NIH grant 2R01 AA14441–12.

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Abbreviations:

DM	Diabetes mellitus, diabetic
E_2	Estrogen
PVN	Paraventricular nucleus
OVX	Ovariectomized
ROS	Reactive Oxygen Species
ER	Estrogen receptors

References:

Brown L, Panchal SK. Rodent models for metabolic syndrome research. Journal of Biomedicine and Biotechnology. 2011;2011:1–14.

Ceriello A, Motz E. Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. Arteriosclerosis, thrombosis, and vascular biology. 2004;24:816–23.

Collin B, Busseuil D, Zeller M, Perrin C, Barthez O, Duvillard L, et al. Increased superoxide anion production is associated with early atherosclerosis and cardiovascular dysfunctions in a rabbit model. Mol Cell Biochem. 2007;294:225–35. [PubMed: 16871360]

De Angelis K, Irigoyen MC, Morris M. Diabetes and cardiovascular autonomic dysfunction: application of animal models. Autonomic neuroscience : basic & clinical. 2009;145:3–10. [PubMed: 19054720]

Deng X, Feng X, Li S, Gao Y, Yu B, Li G. Influence of the hypothalamic paraventricular nucleus (PVN) on heart rate variability (HRV) in rat hearts via electronic lesion. Biomedical materials and engineering. 2015;26 Suppl 1:S487–95. [PubMed: 26406040]

El-Lakany MA, Fouda MA, El-Gowelli HM, El-Gowilly SM, El-Mas MM. Gonadal hormone receptors underlie the resistance of female rats to inflammatory and cardiovascular complications of endotoxemia. European journal of pharmacology. 2018;823:41–8. [PubMed: 29382531]

El-Mas MM, Abdel-Rahman AA. Estrogen enhances baroreflex control of heart rate in conscious ovariectomized rats. Can J Physiol Pharmacol. 1998;76:381–6. [PubMed: 9795746]

El-Mas MM, Abdel-Rahman AA. Intermittent clonidine regimen abolishes tolerance to its antihypertensive effect: a spectral study. Journal of cardiovascular pharmacology. 2007;49:174–81. [PubMed: 17414230]

El-Mas MM, Abdel-Rahman AA. Longitudinal assessment of the effects of oestrogen on blood pressure and cardiovascular autonomic activity in female rats. Clin Exp Pharmacol Physiol. 2009;36:1002–9. [PubMed: 19413598]

El-Mas MM, Abdel-Rahman AA. Estrogen modulation of the ethanol-evoked myocardial oxidative stress and dysfunction via DAPK3/Akt/ERK activation in male rats. Toxicology and applied pharmacology. 2015;287:284–92. [PubMed: 26111663]

El-Sayed SS, Zakaria MN, Abdel-Ghany RH, Abdel-Rahman AA. Cystathionine-gamma lyase-derived hydrogen sulfide mediates the cardiovascular protective effects of moxonidine in diabetic rats. European journal of pharmacology. 2016;783:73–84. [PubMed: 27138707]

Etique N, Grillier-Vuissoz I, Lecomte J, Flament S. Crosstalk between adenosine receptor (A2A isoform) and ERalpha mediates ethanol action in MCF-7 breast cancer cells. Oncology reports. 2009;21:977–81. [PubMed: 19287996]

Fischer R, Maier O. Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. Oxidative medicine and cellular longevity. 2015;2015:610813.

Fouda MA, Abdel-Rahman AA. Endothelin Confers Protection against High Glucose-Induced Neurotoxicity via Alleviation of Oxidative Stress. The Journal of pharmacology and experimental therapeutics. 2017;361:130–9. [PubMed: 28179472]

Fouda MA, El-Sayed SS, Abdel-Rahman AA. Restoration of Rostral Ventrolateral Medulla Cystathionine-gamma Lyase Activity Underlies Moxonidine-Evoked Neuroprotection and Sympathoinhibition in Diabetic Rats. The Journal of pharmacology and experimental therapeutics. 2018;364:170–8. [PubMed: 29133386]

Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM. Adenosine and brain function. International review of neurobiology. 2005;63:191–270. [PubMed: 15797469]

Gomes CV, Kaster MP, Tome AR, Agostinho PM, Cunha RA. Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. Biochimica et biophysica acta. 2011;1808:1380–99. [PubMed: 21145878]

Griendling KK, Touyz RM, Zweier JL, Dikalov S, Chilian W, Chen YR, et al. Measurement of Reactive Oxygen Species, Reactive Nitrogen Species, and Redox-Dependent Signaling in the Cardiovascular System: A Scientific Statement From the American Heart Association. Circulation research. 2016;119:e39–75. [PubMed: 27418630]

Hao F, Gu Y, Tan X, Deng Y, Wu Z-T, Xu M-J, et al. Estrogen Replacement Reduces Oxidative Stress in the Rostral Ventrolateral Medulla of Ovariectomized Rats. Oxidative medicine and cellular longevity. 2016;2016:2158971-.

Hoit BD, Castro C, Bultron G, Knight S, Matlib MA. Noninvasive evaluation of cardiac dysfunction by echocardiography in streptozotocin-induced diabetic rats. Journal of cardiac failure. 1999;5:324–33. [PubMed: 10634674]

Juutilainen A, Kortelainen S, Lehto S, Rönnemaa T. Gender Difference in the Impact of Type 2 Diabetes on Coronary Heart Disease Risk. Diabetes care. 2004;27:2898–904. [PubMed: 15562204]

Khazipov R, Zaynutdinova D, Ogievetsky E, Valeeva G, Mitrukhina O, Manent JB, et al. Atlas of the Postnatal Rat Brain in Stereotaxic Coordinates. Front Neuroanat. 2015;9:161. [PubMed: 26778970]

Kishi T, Hirooka Y. Oxidative stress in the brain causes hypertension via sympathoexcitation. Frontiers in physiology. 2012;3:335. [PubMed: 22934082]

Kleiber AC, Zheng H, Schultz HD, Peuler JD, Patel KP. Exercise training normalizes enhanced glutamate-mediated sympathetic activation from the PVN in heart failure. Am J Physiol Regul Integr Comp Physiol. 2008;294:R1863–72. [PubMed: 18385465]

Korystov YN, Emel'yanov MO, Korystova AF, Levitman MK, Shaposhnikova VV. Determination of reactive oxygen and nitrogen species in rat aorta using the dichlorofluorescein assay. Free radical research. 2009;43:149–55. [PubMed: 19204868]

Leffler KE, Abdel-Rahman AA. Estrogen-Dependent Disruption of Adiponectin-Connexin43 Signaling Underlies Exacerbated Myocardial Dysfunction in Diabetic Female Rats. The Journal of pharmacology and experimental therapeutics. 2019;368:208–17. [PubMed: 30523063]

Low Wang CC, Hess CN, Hiatt WR, Goldfine AB. Clinical Update: Cardiovascular Disease in Diabetes Mellitus: Atherosclerotic Cardiovascular Disease and Heart Failure in Type 2 Diabetes Mellitus - Mechanisms, Management, and Clinical Considerations. Circulation. 2016;133:2459–502. [PubMed: 27297342]

Luo Y, Kaur C, Ling EA. Neuronal and glial response in the rat hypothalamus-neurohypophysis complex with streptozotocin-induced diabetes. Brain research. 2002;925:42–54. [PubMed: 11755899]

Mohamadi A, Aghaei M, Panjehpour M. Estrogen stimulates adenosine receptor expression subtypes in human breast cancer MCF-7 cell line. Research in pharmaceutical sciences. 2018;13:57–64. [PubMed: 29387112]

Mohamed RA, Agha AM, Abdel-Rahman AA, Nassar NN. Role of adenosine A2A receptor in cerebral ischemia reperfusion injury: Signaling to phosphorylated extracellular signal-regulated protein kinase (pERK1/2). Neuroscience. 2016;314:145–59. [PubMed: 26642806]

Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. Nature. 2006;443:289–95. [PubMed: 16988703]

Nassar N, Abdel-Rahman AA. Brainstem adenosine A1 receptor signaling masks phosphorylated extracellular signal-regulated kinase 1/2-dependent hypotensive action of clonidine in conscious normotensive rats. The Journal of pharmacology and experimental therapeutics. 2009;328:83–9. [PubMed: 18849358]

Palkovits M Punch sampling biopsy technique. Methods in enzymology. 1983;103:368–76. [PubMed: 6366460]

Paxinos G, Watson CR, Emson PC. AChE-stained horizontal sections of the rat brain in stereotaxic coordinates. J Neurosci Methods. 1980;3:129–49. [PubMed: 6110810]

Peleli M, Carlstrom M. Adenosine signaling in diabetes mellitus and associated cardiovascular and renal complications. Molecular aspects of medicine. 2017;55:62–74. [PubMed: 28089906]

Phillis JW. The effects of selective A1 and A2a adenosine receptor antagonists on cerebral ischemic injury in the gerbil. Brain research. 1995;705:79–84. [PubMed: 8821736]

Regensteiner JG, Golden S, Huebschmann AG, Barrett-Connor E, Chang AY, Chyun D, et al. Sex Differences in the Cardiovascular Consequences of Diabetes Mellitus: A Scientific Statement From the American Heart Association. Circulation. 2015;132:2424–47. [PubMed: 26644329]

Ribeiro JA. What can adenosine neuromodulation do for neuroprotection? Current drug targets CNS and neurological disorders. 2005;4:325–9. [PubMed: 16101552]

Santizo RA, Xu HL, Ye S, Baughman VL, Pelligrino DA. Loss of benefit from estrogen replacement therapy in diabetic ovariectomized female rats subjected to transient forebrain ischemia. Brain research. 2002;956:86–95. [PubMed: 12426050]

Sasidharan SR, Joseph JA, Anandakumar S, Venkatesan V, Ariyattu Madhavan CN, Agarwal A. An Experimental Approach for Selecting Appropriate Rodent Diets for Research Studies on Metabolic Disorders. BioMed Research International. 2013;2013:1–9.

Schwartz PJ, La Rovere MT, Vanoli E. Autonomic nervous system and sudden cardiac death. Experimental basis and clinical observations for post-myocardial infarction risk stratification. Circulation. 1992;85:I77–91. [PubMed: 1728509]

Smith MD, Bhatt DP, Geiger JD, Rosenberger TA. Acetate supplementation modulates brain adenosine metabolizing enzymes and adenosine A(2)A receptor levels in rats subjected to neuroinflammation. Journal of neuroinflammation. 2014;11:99. [PubMed: 24898794]

Srinivasan K, Ramarao P. Animal models in type 2 diabetes research: An overview. Indian Journal of Medical Research. 2007;125:451–72. [PubMed: 17496368]

Steagall RJ, Yao F, Shaikh SR, Abdel-Rahman AA. Estrogen receptor α activation enhances its cell surface localization and improves myocardial redox status in ovariectomized rats. Life Sci. 2017;182:41–9. [PubMed: 28599865]

Stockwell J, Jakova E, Cayabyab FS. Adenosine A1 and A2A Receptors in the Brain: Current Research and Their Role in Neurodegeneration. Molecules (Basel, Switzerland). 2017;22.

White RE, Gerrity R, Barman SA, Han G. Estrogen and oxidative stress: A novel mechanism that may increase the risk for cardiovascular disease in women. Steroids. 2010;75:788–93. [PubMed: 20060403]

Williams KW, Elmquist JK. From neuroanatomy to behavior: central integration of peripheral signals regulating feeding behavior. Nature neuroscience. 2012;15:1350–5. [PubMed: 23007190]

Yang LY, Chu YH, Tweedie D, Yu QS, Pick CG, Hoffer BJ, et al. Post-trauma administration of the pifithrin-alpha oxygen analog improves histological and functional outcomes after experimental traumatic brain injury. Experimental neurology. 2015;269:56–66. [PubMed: 25819102]

Yao F, Abdel-Rahman AA. Estrogen receptor ERα plays a major role in ethanol-evoked myocardial oxidative stress and dysfunction in conscious female rats. Alcohol Clin Exp Res. 2016; 50:27–35.

Yao F, AbdelRahman AA. Estrogen Receptors α and β Play Major Roles in Ethanol-Evoked Myocardial Oxidative Stress and Dysfunction in Conscious Ovariectomized Rats. Alcoholism: Clinical and Experimental Research. 2017;41:279–90.

Highlights

- Diabetic (DM) female rats exhibit estrogen (E_2)-dependent autonomic dysregulation and heightened neurotoxicity in the hypothalamic paraventricular nucleus (PVN).
- E_2 exacerbates DM-evoked cardiac sympathetic dominance and elevations in PVN levels of TNFα and phosphorylated death associated protein kinase-3 (p-DAPK3).
- Paradoxical E_2-dependent neurochemical effects are corroborated by heightened PVN neuronal oxidative stress, apoptosis, and injury in DM females.
- Additive ERα and adenosine A1 and A2a receptors upregulations likely contribute to the PVN injury/neuroinflammation in DM females.

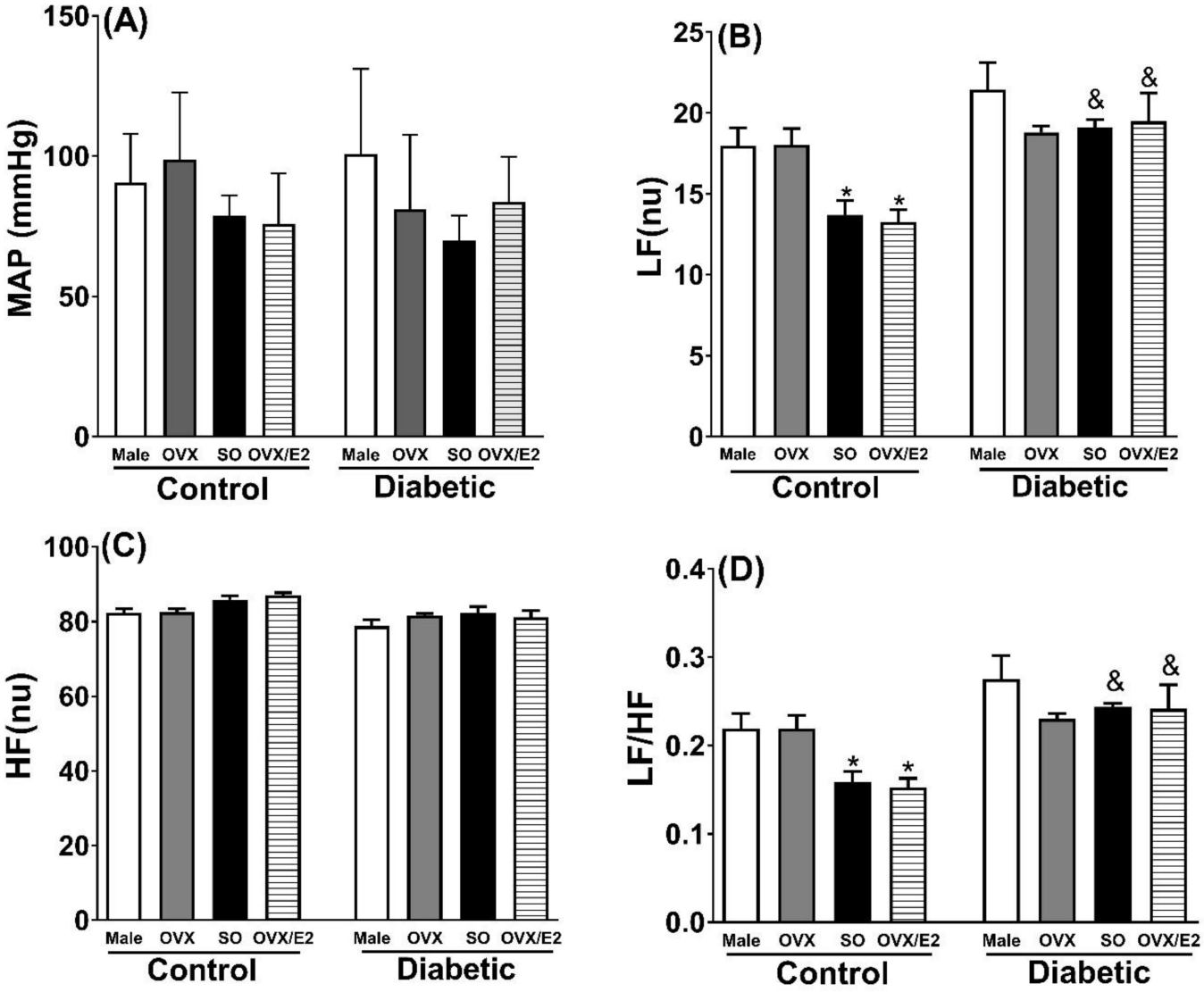

Fig. 1.

Mean blood pressure **(**MAP), measured at the conclusion of the study, along with spectral indices of heart rate variability in the following diabetic (DM) and respective control groups (n=5 rats each): male, ovariectomized (OVX), sham operated (SO), and OVX with E_2 supplementation (OVX/E_2). **(A)** MAP (mmHg)**. (B)** low-frequency (LFnu) (0.25–0.75 Hz) bands. **(C)** High-frequency (HFnu) (0.75–3 Hz) bands. **(D)** The LF/HF ratio depicting cardiac sympathovagal balance. Values are means ± SEM. *P < 0.05 vs. “male control” values. $^{\&}$P < 0.05 vs. SO control values.

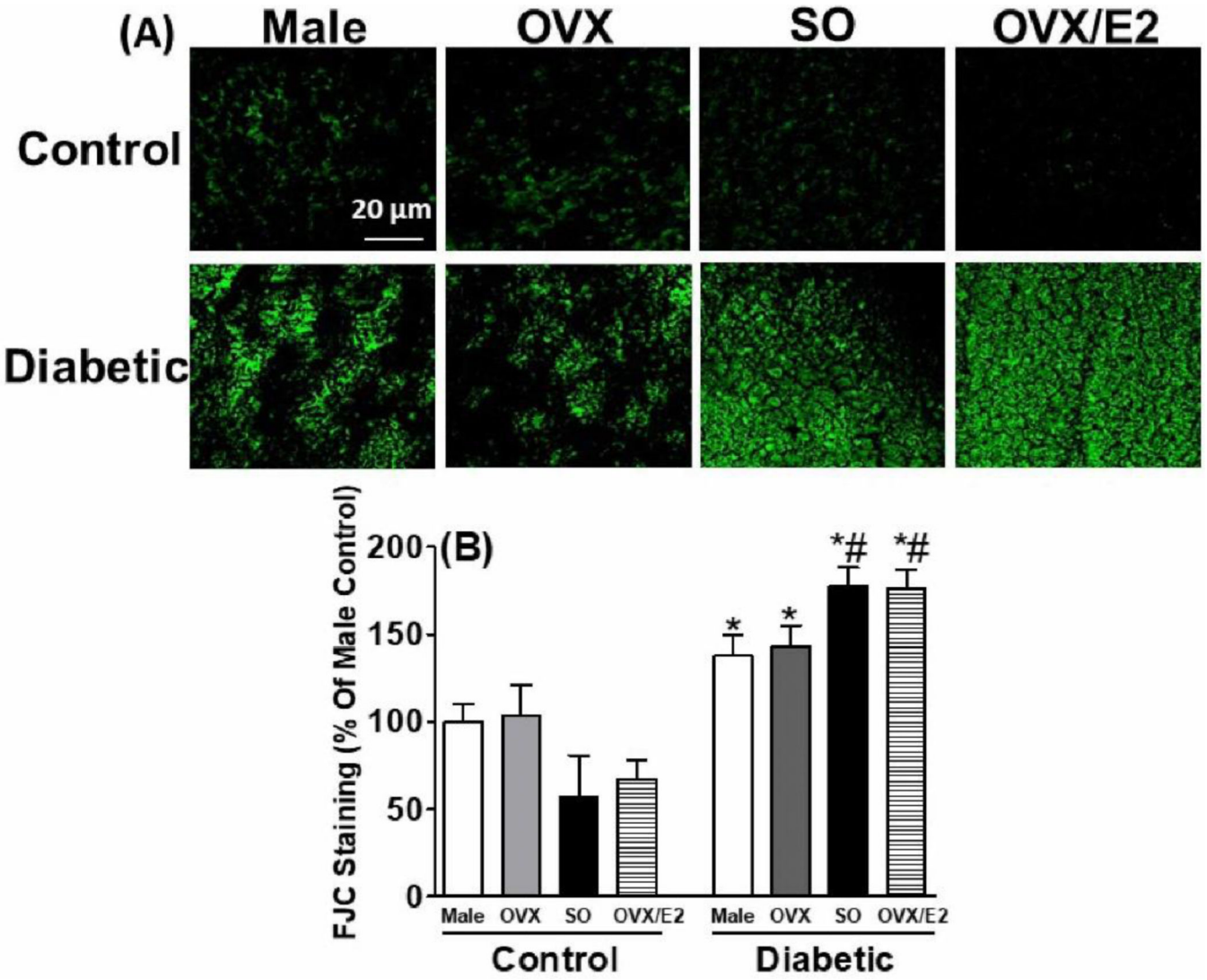

Fig. 2.

Representative images **(A)** and group data (**B**) of FluoroJade C (FJC) positive neurons, depicting neurodegeneration, in the hypothalamic PVN of the following diabetic (DM) and respective control groups (n=5 each): male, ovariectomized (OVX), sham operated (SO), and OVX with E_2 supplementation (OVX/E_2). Group data show the neurodegeneration level expressed as the number of FJC positive cells measured by NIH ImageJ analysis of confocal images. Values are expressed as means ± SEM. *P < 0.05 vs. male control values. $^{\#}$P < 0.05 vs. DM male values.

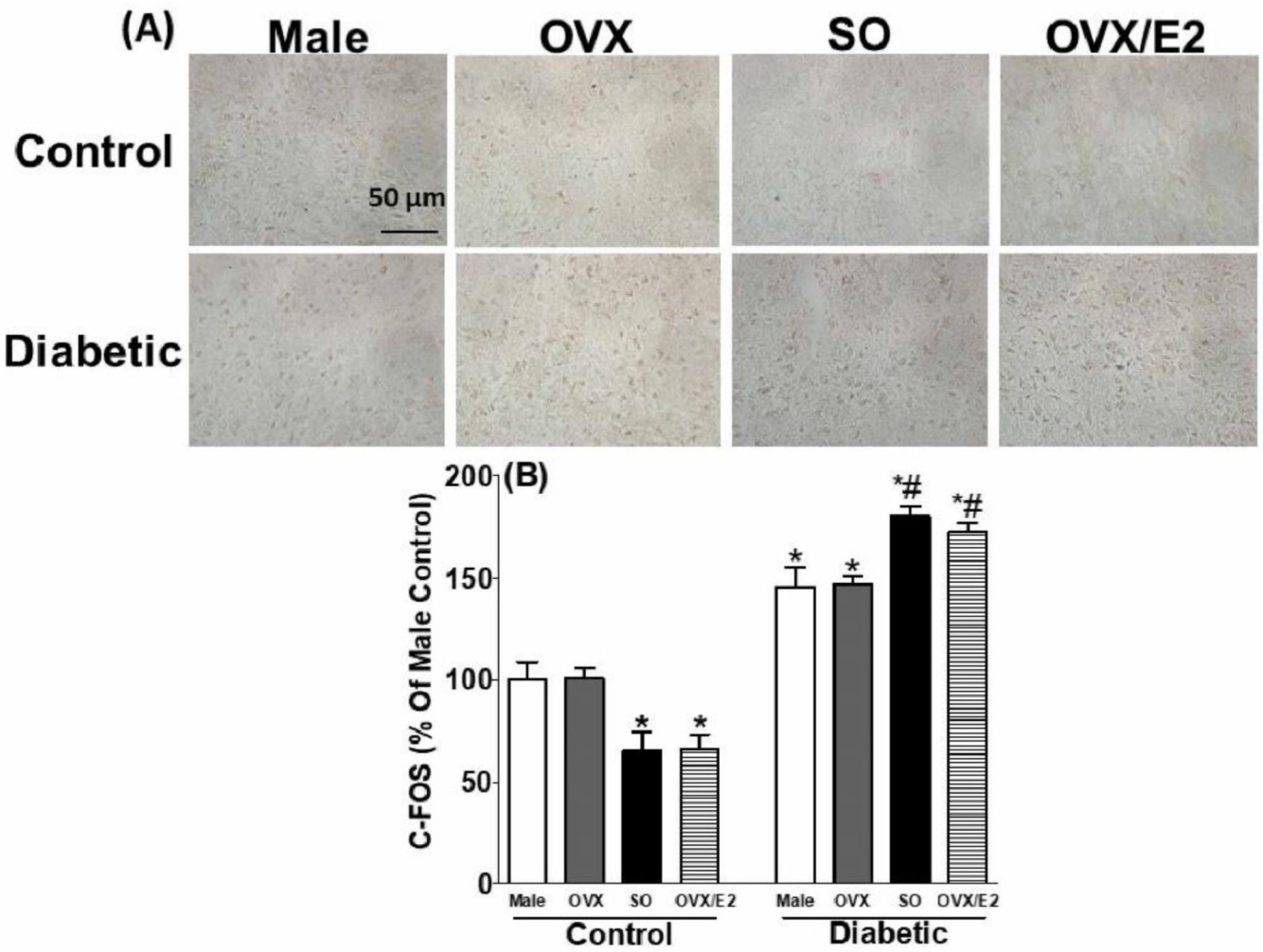

Fig. 3.

Representative immunohistochemical images **(A)** and group data (**B**) of c-Fos expression in the hypothalamic PVN of the following diabetic (DM) and respective control groups (n=5 rats each): male, ovariectomized (OVX), sham operated (SO), and OVX with E_2 supplementation (OVX/E_2). Group data showing the mean number of c-Fos expression measured by NIH ImageJ analysis of immunohistochemical images. Values are expressed as means ± SEM. *P < 0.05 vs. male control values. #P < 0.05 vs. DM male values.

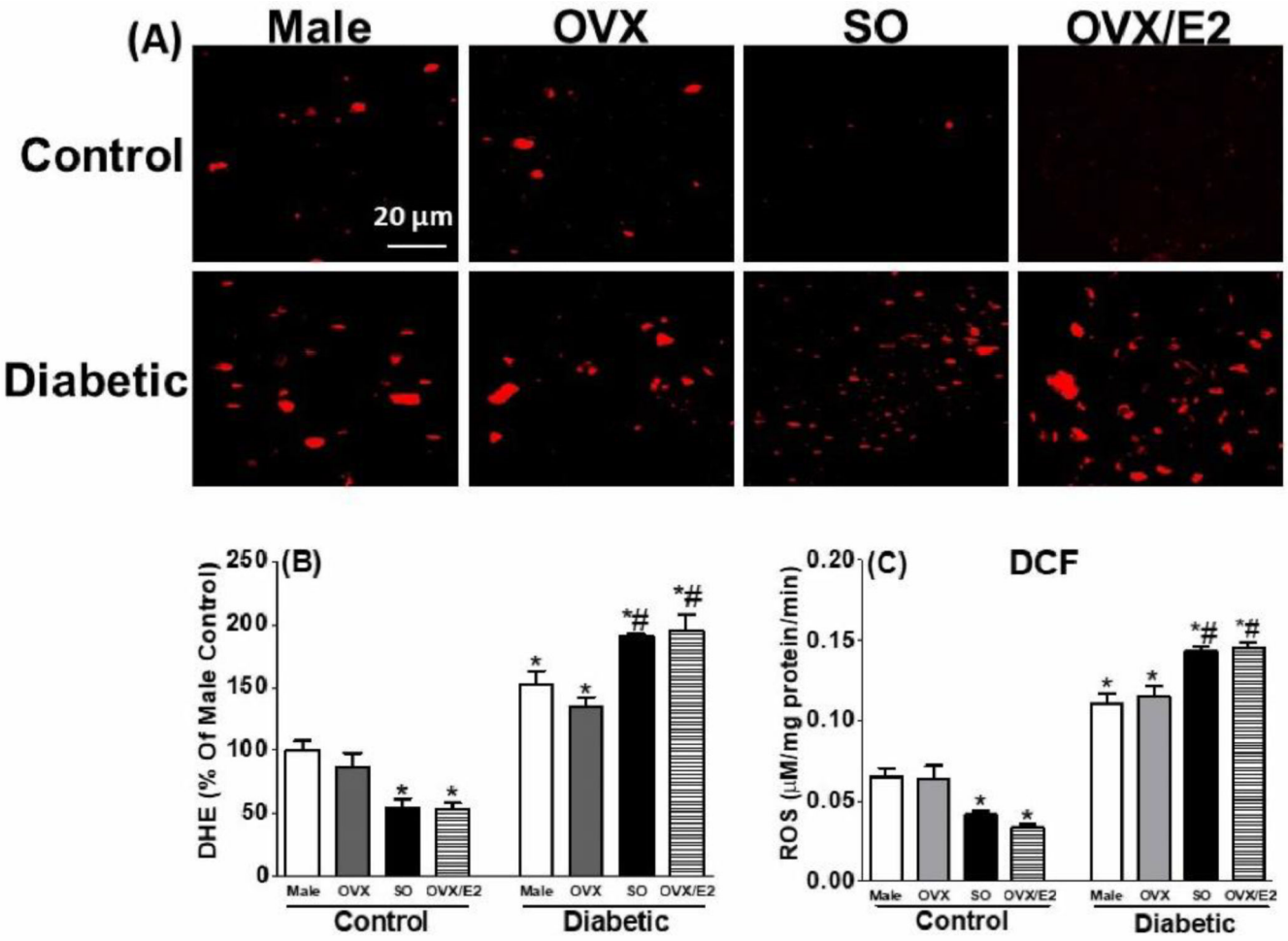

Fig. 4.

Representative confocal images **(A)** and group data (**B**) showing superoxide level measured by dihydroethidium (DHE) staining (red) as well as group data (**C**) showing the ROS level, measured by 2′,7′-dichlorofluorescein, in the hypothalamic PVN of the following diabetic (DM) and respective control groups (n=5 rats each): male, ovariectomized (OVX), sham operated (SO), and OVX with E_2 supplementation (OVX/E_2). Values are expressed as means ± SEM. *P < 0.05 vs. male control values. $^{\#}$P < 0.05 vs. DM male values.

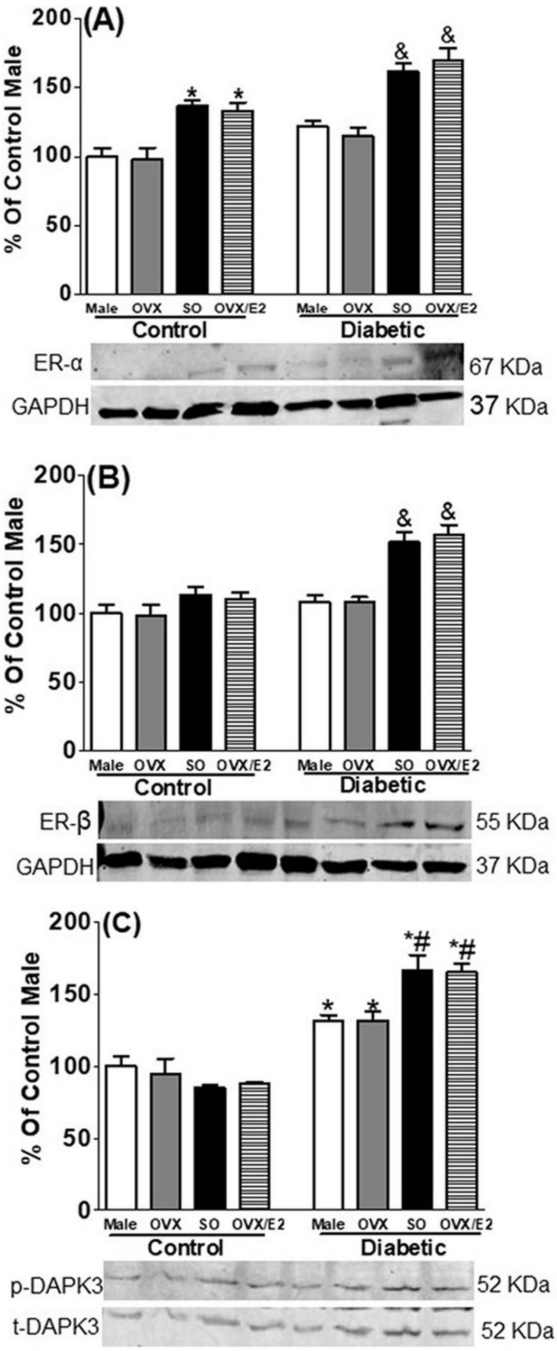

Fig. 5.

Western blots analysis showing ERα (**A**), ERβ (**B**) (both normalized to GAPDH) and phosphorylated death associated protein kinase-3, p-DAPK3 (**C**) (normalized to total DAPK3) expression in the hypothalamic PVN of the following diabetic (DM) and respective control groups (n=5 rats each): male, ovariectomized (OVX), sham operated (SO), and OVX with E_2 supplementation (OVX/E_2). Values are expressed as means ± SEM. *P < 0.05 vs. "male control" values. #P < 0.05 vs. DM male values. &P < 0.05 vs. SO control values.

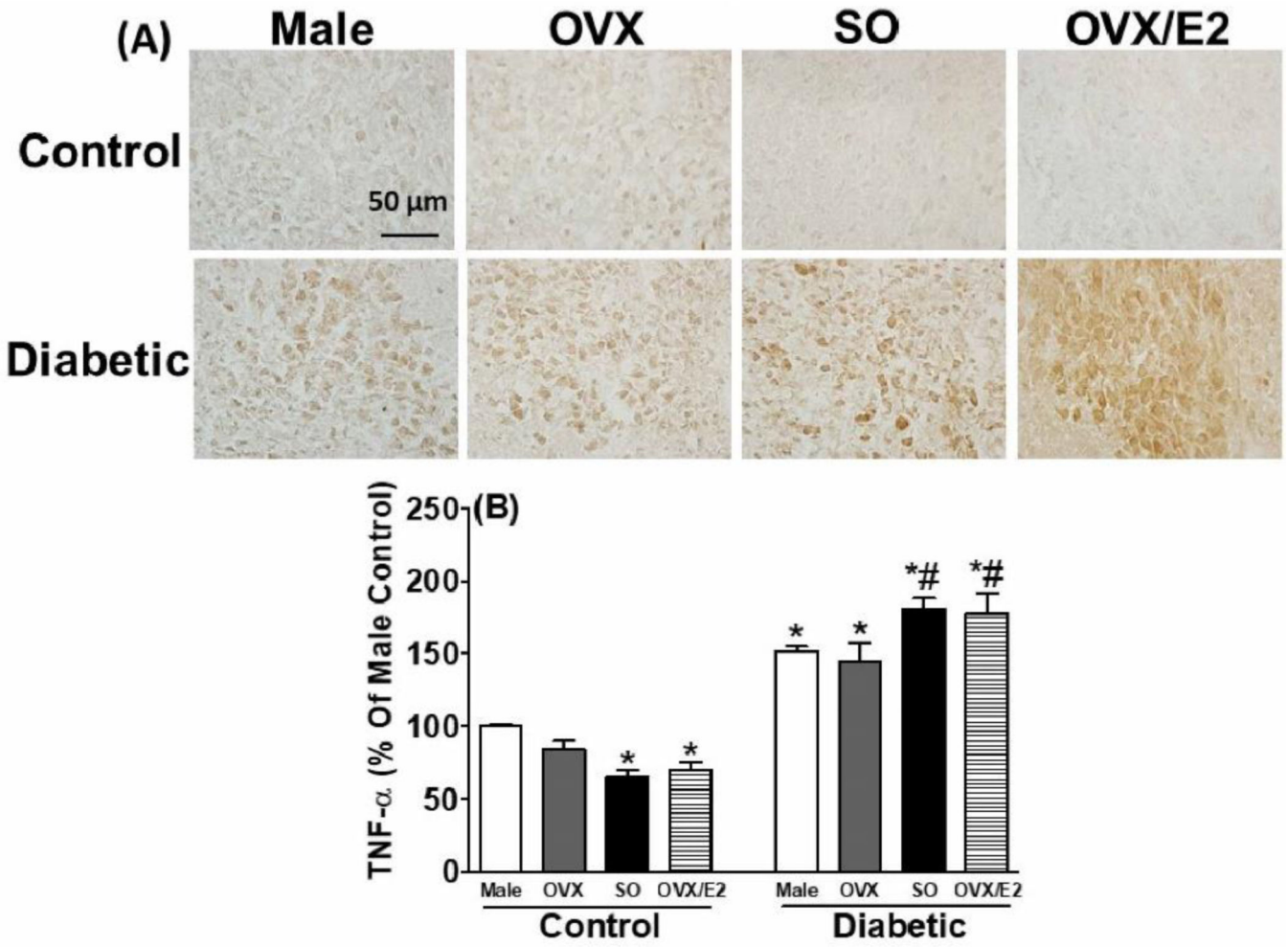

Fig. 6.

Representative confocal immunohistochemical images **(A)** and group data (**B**) of TNF-α levels in the hypothalamic PVN of the following DM and respective control groups (n=5 rats each): male, ovariectomized (OVX), sham operated (SO), and OVX with E_2 supplementation (OVX/E_2). TNF-α images were analyzed by NIH ImageJ. Values are means ± SEM. *P < 0.05 vs. male control values. $^{\#}$P < 0.05 vs. DM male values.

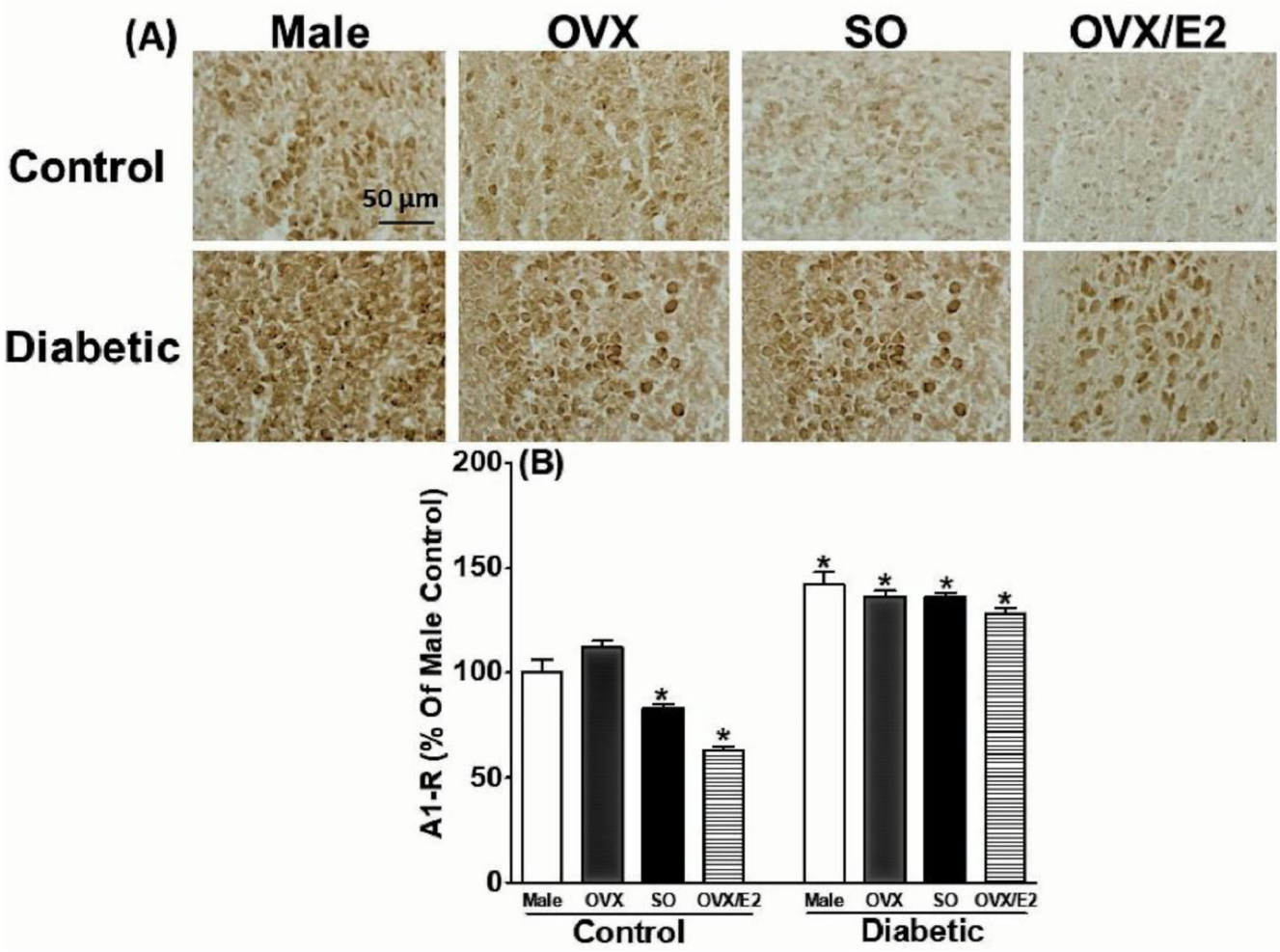

Fig. 7.

Representative immunohistochemical images **(A)** and group data (**B**) showing adenosine A1 receptor (A1-R) expression in the hypothalamic PVN of the following diabetic (DM) and respective control groups (n=5 rats each): male, ovariectomized (OVX), sham operated (SO), and OVX with E_2 supplementation (OVX/E_2). A1-R expression was quantified by NIH ImageJ analysis. Values are expressed as means ± SEM. *P < 0.05 vs. male control values.

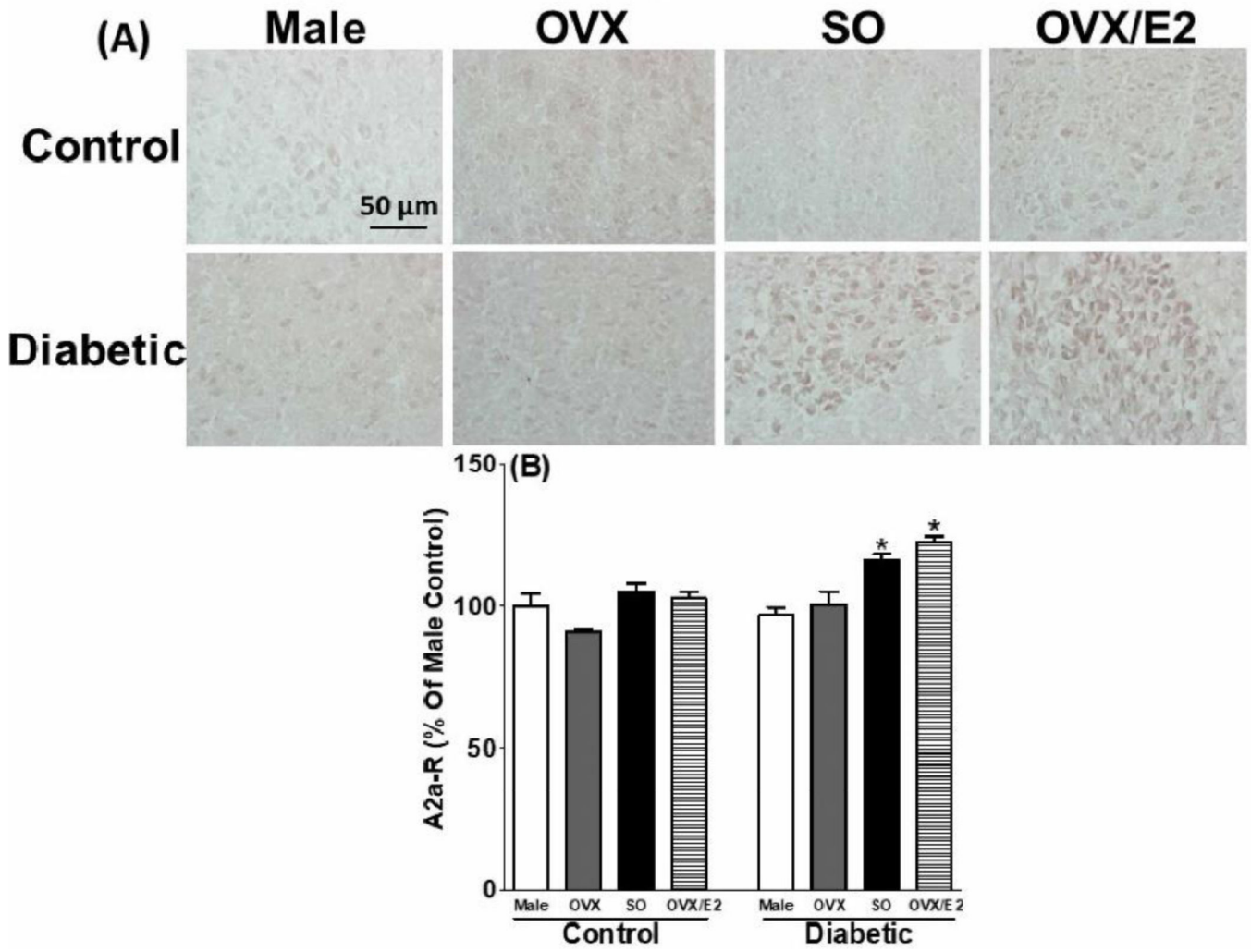

Fig. 8.

Representative immunohistochemical images **(A)** and group data (**B**) showing adenosine A2a receptor (A2a-R) expression in the hypothalamic PVN of the following diabetic (DM) and respective control groups (n=5 rats each): male, ovariectomized (OVX), sham operated (SO), and ovariectomized with E_2 supplementation (OVX/E_2). A2-R expression was quantified by NIH ImageJ analysis. Values are expressed as means ± SEM. *P < 0.05 vs. male control values.