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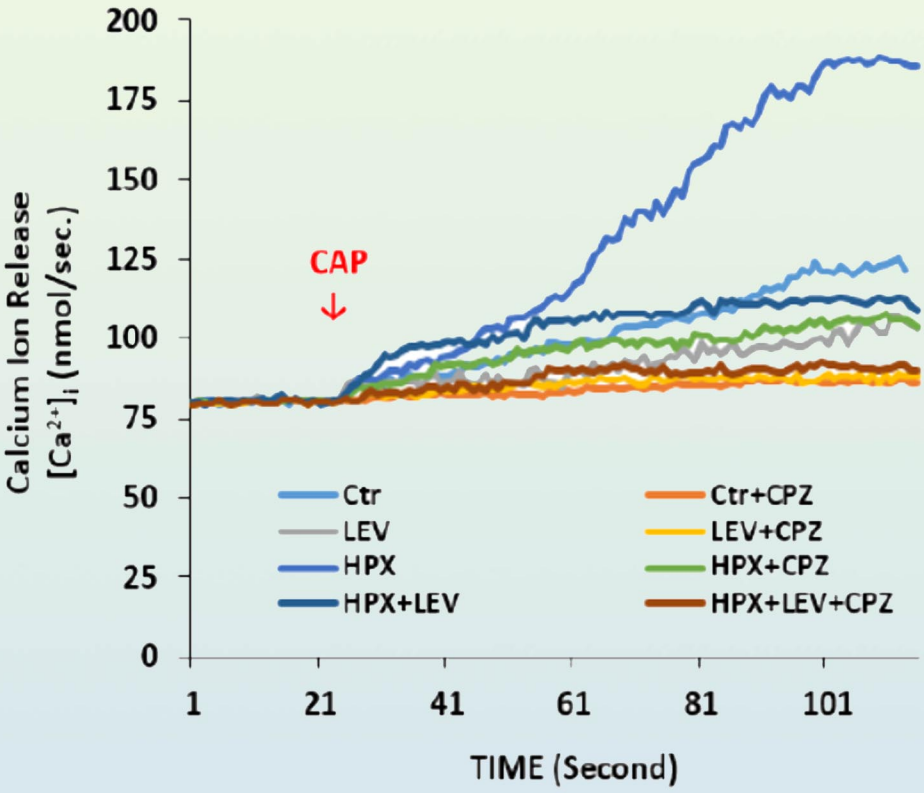

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AIM AND SCOPES
Journal of Cellular Neuroscience and Oxidative Stress is an online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

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A- Ion Channels (Na^+- K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

D- Gene and Oxidative Stress
(Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

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Biophysics	Biochemistry
Biology	Biomedical Engineering
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Cardiology	Neurology
Oncology	Psychiatry
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Keywords
Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide, ageing, antioxidants, neuropathy, traumatic brain injury, pain, spinal cord injury, Alzheimer's Disease, Parkinson's Disease.

RESEARCH ARTICLE

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Levetiracetam modulates hypoxia-induced inflammation and oxidative stress via inhibition of TRPV1 channel in the DBTRG glioblastoma cell line

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Abstract

Hypoxia (HPX) induces mitochondrial oxidative stress, inflammation and apoptosis in brain and neurons. Ca^{2+} permeable TRPV1 channel is gated by capsaicin and reactive oxygen species (ROS), although its activity was decreased in neurons by antioxidants. LEV has been used as antiepileptic drug in the treatment of epilepsy. LEV inhibited voltage gated calcium channels via its antioxidant property in neurons. Hence, it may modulate HPX-induced ROS, inflammation and apoptosis via inhibition of TRPV1 in the DBTRG cells.

The DBTRG glioblastoma cells were divided into four groups as control, LEV (100 µM and 24 hours), and HPX and LEV+HPX. HPX in the cells was induced by using $CoCl_2$ (200 µM and 24 hours).

HPX-induced intracellular Ca^{2+} response to TRPV1 activation was increased in the cells from capsaicin, although it was reduced by the LEV and TRPV1 blocker (capsazepine). LEV treatment improved intracellular Ca^{2+} responses, mitochondrial function, suppressed the generation of cytokine (IL-1β, and TNF-α) and ROS in the cells. Apoptosis, lipid peroxidation level, caspase -3 and -9 activities were increased in the cells exposed to the HPX, although glutathione peroxidase activity and reduced glutathione level were decreased by the HPX. However, they were modulated in the cells by LEV treatment.

In DBTRG neuronal cells exposed to HPX conditions, the neuroprotective effects of LEV were shown to be exerted via modulation of oxidative stress, inflammation, apoptosis and TRPV1 channel. LEV could be used as an effective agent via modulation of TRPV1 in the treatment of neurodegeneration exposure to HPX.

Keywords: Apoptosis; Hypoxia; Inflammation; Levetiracetam; Oxidative stress; TRPV1.

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List of Abbreviations;

$[Ca^{2+}]_c$, cytosolic free Ca^{2+}concentration; **Ca^{2+}**, calcium ion; **CAP**, capsaicin; **CPZ**, capsazepine; **DHR123**, dihydrorhodamine- 123; **GSHPx**, glutathione peroxidase; **HPX**, hypoxia; **IL-1β**, interleukin 1-beta; **JC1,** 5**'**,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide; **LEV**, levetiracetam; **MTT**, 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl- 2-tetrazolium bromide; **rGSH**, reduced glutathione; **ROS**, reactive oxygen species; **TNF-*α***, tumor necrosis factor-alpha; **TRPV1**, transient receptor potential vanilloid 1; **VGCC**, voltage gated calcium channel

Introduction

Cerebral ischemia and hypoxia (HPX) in the brain is induced by blood hypoperfusion of the brain tissue. There is a low oxygen flow and changes of ATP-driven bioenergetics in the ischemic and hypoxic neuron and brain. Mitochondria of brain and neurons are affected by HPX, because ATP generation bioenergetic requires oxygen consumption in mitochondria (Thornton et al. 2017). Mitochondria is a main source of reactive oxygen species (ROS) generation (Zhao et al. 2019). Accumulating evidence indicates that the hypoxia and ischemic conditions result in excessive ROS generation, inflammation and apoptosis through the increase of membrane depolarization in mitochondria of neurons (Sun et al. 2014; Yang et al. 2019).

Calcium ion (Ca^{2+}) signaling is implicated in different aspects of neuron biology. Basal neuronal Ca^{2+} changes control important aspects of neuronal homeostasis such neurotransmitter release and transmission (Nazıroğlu 2007). On the other hand, during HPX, neurons enhance intracellular Ca^{2+} influx (Kumar et al 2014) and activate a pathway of factors such as inflammatory cytokines and ROS (Zhao et al. 2019) that when produced in excess can lead to neurodegeneration and neuronal death. Ca^{2+} passes cell membrane via well-known calcium channels such as voltage gated Ca^{2+} channel (VGCC) and ligand channels. Hence, modulation of the channels decreases inflammation, ROS, neurodegeneration and neuronal death in several neurons. Besides the well-known calcium channels, the transient receptor potential (TRP) superfamily with 28 members and 6 subgroups in mammalian were discovered (Clapham 2003). A member of the TRP superfamily is TRP vanilloid 1 (TRPV1) calcium permeable cation channel. It is activated by acidic pH, heat and hot chili pepper component (capsaicin, CAP) (Caterina et al. 1997). In addition to the activators, TRPV1 in neurons such as dorsal root ganglion (DRG) and hippocampus is activated by nitric oxide and ROS (Ibi et al. 2008; Ogawa et al. 2016). Involvement of TRPV1 on the HPX-induced oxidative stress (Sun et al. 2014), inflammation (Yang et al. 2019) and apoptosis (Akpınar et al. 2016) in several neurons was recently reported. In addition, antioxidant treatments via inhibition of the TRPV1 in the neurons decreased HPX-induced oxidative stress, inflammation and apoptosis (Akpınar et al. 2016).

Levetiracetam (LEV) is a second-generation antiepileptic drug (Lukyanetz et al. 2002). LEV inhibited excitatory transmission was inhibited in CA1 neurons of hippocampus through inhibition of VGCC (Meehan et al. 2011). LEV via its antioxidant action inhibited apoptosis and oxidative stress in a neonatal rat model of hypoxic-ischemic brain injury (Komur et al. 2014) and a rat model of middle cerebral artery occlusion (Abd El Motteleb et al. 2018). In addition, LEV decreased lipopolysaccharide-induced cell death, tumor necrosis factor-alpha (TNF-α) and caspase activity (Stettner et al. 2011) and protected neurons against oxidative stress in a glutamate-based oxidative stress model (Abdel-Wahab et al. 2015). Therefore, the antioxidant LEV may reduce HPX-induced oxidative stress, inflammation and apoptosis via modulation of TRPV1 channel and the subject should have clarified in DBTRG neuronal cells.

There is no report the modulator action of LEV against the neurotoxicity via modulation of TRPV1 channel in the DBTRG neurons following HPX. In the present study I investigated whether LEV exerts neuroprotection against in vitro HPX-induced Ca^{2+} influx, oxidative stress and inflammation. Moreover, I have correlated the possible neuroprotective effects of the drug with their ability to modulate TRPV1 channel. One of the well-known hypoxia mimetic agents through inhibition of cellular oxygen uptake in cell line models is cobalt chloride ($CoCl_2$) (Muñoz-Sánchez and Chánez-Cárdenas 2019). Hence, I used $CoCl_2$ incubation in the DBTRG cell line model for induction of HPX.

Materials and methods

Cell Culture

Recently presence of TRPV1 channel in the DBTRG neuronal cells was reported by Western blot expression results (Nazıroğlu et al. 2019). For the reason, I used DBTRG cell line in the current study. 90% of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Istanbul, Turkey) was used for growing the cells. Remaining 10% of the medium was fetal bovine serum (FBS, Gibco, Istanbul, Turkey). The cells were kept in a humidified atmosphere in 5% CO_2 at 37°C. Casy Modell TT model automatic cell counter (Roche, Germany) was used for counting the cells.

Groups

The DBTRG cells were mainly divided into four groups as follows;

***Control group*:** The cells were kept in under the same cell culture medium and conditions for 48 hours without TRPV1 channel blocker (CPZ), LEV and $CoCl_2$ treatments.

***LEV group*:** After keeping 24 hours in the cell same culture condition without treatment, the cells in the group were pre-incubated with LEV (100 μM) for 24 hours as described in previous studies (Costa et al. 2006; Vogl et al. 2012).

***Hypoxia (HPX) group*:** After keeping 24 hours in the cell same culture condition, the cells were incubated with $CoCl_2$ (200 μM) for 24 hours as described in a previous study (Deveci et al. 2019).

***LEV+HPX group*:** After pre-incubation with $CoCl_2$ (200 μM) for 24 hours, the cells in the group were incubated with LEV (100 μM) for a further period of 24 hours.

The CAP, CPZ and LEV were purchased from Sigma-Aldrich Inc, (Istanbul, Turkey). Stock solutions of CAP and CPZ were dissolved in DMSO (1%). LEV was dissolved in sterile serum physiologic solution.

The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) determination of the cells in a spectrofluorometer

The cells were incubated with 2 μM fura-2 (Molecular Probes) for 45 min, washed twice with standard extracellular buffer with 1.2 mM Ca^{2+} (Ertilav et al. 2018). The fluorescence intensity of Fura-2-loaded cells was monitored with a spectrofluorometer (Carry Eclipse, Varian Inc, Ankara, Turkey). The fluorescence intensities of the cells collected at each excitation wavelength were processed using the spectrofluorometer, to provide ratios of Fura-2 fluorescence from excitation at 340 nm to that from excitation at 380 nm (F340/F380). The integral changes in the $[Ca^{2+}]_c$ was recorded in a computer by using a specific program (Carry Eclipse) for 200 seconds after addition of CAP (1 μM). The $[Ca^{2+}]_i$ concentration is expressed as nanomolar (nM) taking a sample every second as previously described (Ertilav et al. 2018).

Assay of cell viability (MTT)

Viability assays were performed by measuring mitochondrial reductase activity with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, Istanbul, Turkey) as described in previous studies (Ataizi et al. 2019). Absorbance in a microplate reader (Infinite pro200; Tecan Inc, Groedig, Austria) was read at 550 nm. The data are presented as percentage (%)-increase over the pretreatment level.

Assays of apoptosis level, caspase 3 and 9 activities

For detecting the apoptosis level, I used Cell-APOPercentage apoptosis assay commercial kit (Biocolor Ltd., Northern Ireland) and the analyses were performed in a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Details of the apoptosis analyses were given in instructions of Biocolor Ltd. and elsewhere (Ataizi et al. 2019; Ertilav et al. 2019).

To determine caspase-3 and -9 activity, the cells in the five groups were incubated with 2 ml of substrate solution for 1 h at 37 °C as previously described (Ertilav et al. 2019). For assay of caspase-3 and -9 activities, cleavages of fluorogenic substrates (AC-DEVD-AMC for caspase -3 and ACDEVD-AMC for caspase-9) (Bachem, Bubendorf, Switzerland) were used. The substrate cleavages were measured with the automatic microplate reader (Infinite pro200) with excitation wavelength of 360 nm and emission at 460 nm. The data were presented as % of control.

Measurement of mitochondrial membrane potential (ΔΨm) formation in the microplate reader

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes, Eugene, OR, USA) accumulates in mitochondria according to ΔΨm level and is present either as monomer or reversible J-aggregate (Keil et al. 2011). Details of the ΔΨm formation analyses were given in previous studies (Joshi and Bakowska 2011; Ertilav 2019). Briefly, the cells ($1x10^6$) in the microplate reader analyses were incubated with 5 μM JC-1 at 37 ^{0}C for 30 min.

In the microplate (Infinite Pro200) JC-1 analyses, the green (excitation; 485 nm and emission; 535 nm) and red (excitation; 540 nm and emission; 590 nm) JC-1 signals were measured in the cell line as described in a previous study (Ataizi et al. 2019). The data are presented as the fold-increase over the pretreatment level.

Measurement of intracellular reactive oxygen species (ROS) formation in the microplate reader

2′,7′-Dichlorofluorescin Diacetate (DCFH-DA) is an oxidation-sensitive stain. It is a non-fluorescent compound, but it is converted to a fluorescent form in the

cytosol of cell, when it was taken up into cells (Keil et al. 2011). ROS formation was monitored using the microplate plate reader (Infinite pro200) described previously (Ataizi et al. 2019). Briefly, the cells ($1x10^6$) were incubated with 10 μM DCFH-DA at 37 °C for 30 min. The fluorescence increase, which is due to the hydrolysis of DCFH-DA to DCF by nonspecific cellular esterase and its subsequent oxidation by peroxides, was measured at 488 nm (excitation)/525 nm (emission) by the microplate plate reader (Infinite pro200).

Assay of lipid peroxidation (LipPX), reduced glutathione (rGSH) level and glutathione peroxidase (GSHPx) activity:

The LipPX and rGSH levels in DBTRG neuronal cells (10^6 cells in per ml) were spectrophotometrically (UV-1800, Shimadzu, Kyoto, Japan) measured at 532 and 412 nm by using the methods of Placer et al. (1966) and Sedlak and Lindsay (1968) as described in a previous study, respectively (Ertilav et al. 2018; Ataizi et al. 2019). The rGSH level was expressed as μg/g protein.

For assaying GSHPx activity, a spectrophotometric method of Lawrence and Burk (1976) was used in the cells as described in previous studies (Ertilav et al. 2018; Ataizi et al. 2019). The results of GSHPx activity was expressed as international unit (IU) of rGSH oxidized/min/g protein. The total protein content in the cell suspension was spectrophotometrically (Shimadzu UV-1800) assessed using Lowry's reagent.

Cytokine assays

Following extraction and quantification of the DBTRG cell lysates, interleukin 1-beta (IL-1β) and TNF-α concentrations were determined using the commercially available ELISA kits according to the manufacturer's protocols (R&D Systems, Istanbul, Turkey) as described in a previous study (Ataizi et al. 2019). Each cytokine sample was analyzed in duplicates and the mean cytokine concentration was calculated. The activities of IL-1β and TNF-α were indicated as ng/10^6 cells.

Statistical analyses

All data were indicated as means ± standard deviation (SD). To assess the differences between treatment groups for each treatment, we used the one-way ANOVA. We used a post hoc test only when an ANOVA gave a statistically significant difference. We performed a Kruskal-Wallis in all data except Table 1 of the current study. We used a Student's t test when comparing two groups (in the Table 1). The $p \leq 0.05$ value was accepted statistically significant.

Results

HPX-induced increase of $[Ca^{2+}]_c$ was diminished via inhibition of TRPV1 by LEV

The increases of mitochondrial membrane depolarization and mitochondrial ROS productions were induced by overload Ca^{2+} influx via activation of several Ca^{2+} channels, including activation of TRPV1 (Ertilav et al. 2018). In the current study, I investigated involvement of LEV via inhibition of TRPV1 channel response to HPX treatment. The effect of HPX on $[Ca^{2+}]_c$ was detected by detection of $[Ca^{2+}]_c$ using the TRPV1 (CAP) channel activator and blocker (CPZ). In the Fura-2 results of the spectrofluorometer analyses (Figure 1a) and column (Figures 1b), the $[Ca^{2+}]_c$ was increased in HPX group by CAP stimulation (activation of TRPV1) ($p \leq 0.001$) and its concentration was higher in the HPX group than in the Ctr, Ctr+CPZ, LEV and LEV+CPZ groups. However, the HPX-induced increase of $[Ca^{2+}]_c$ was decreased by the LEV and CPZ treatments and its concentration were significantly ($p \leq 0.001$) lower in the HPX+CPZ, HPX+LEV and HPX+LEV+CPZ groups as compared to HPX group.

LEV treatment modulated HPX-induced mitochondrial membrane depolarization (JC-1) and cytosolic ROS (DCFH-DA) generation changes in the DBTRG cells.

The electron transport system of mitochondria induces loss of mitochondrial membrane depolarization in the mitochondria resulting in excessive ROS generation (Joshi and Bakowska 2011). For this reason, mitochondrial membrane depolarization is an important parameter of mitochondrial function and it was used as an indicator of ROS generation in neurons. The results of plate reader analyses of JC-1 (Figure 2a) and DCFH-DA (Figure 2b) levels are shown in Figure 2. The JC-1 and DCFH-DA levels in the cells were higher in the HPX group than in the control and LEV groups ($p \leq 0.001$). However, the JC-1 and DCFH-DA levels were lower in the HPX+LEV and HPX+CPZ groups as compared to HPX group ($p \leq 0.001$).

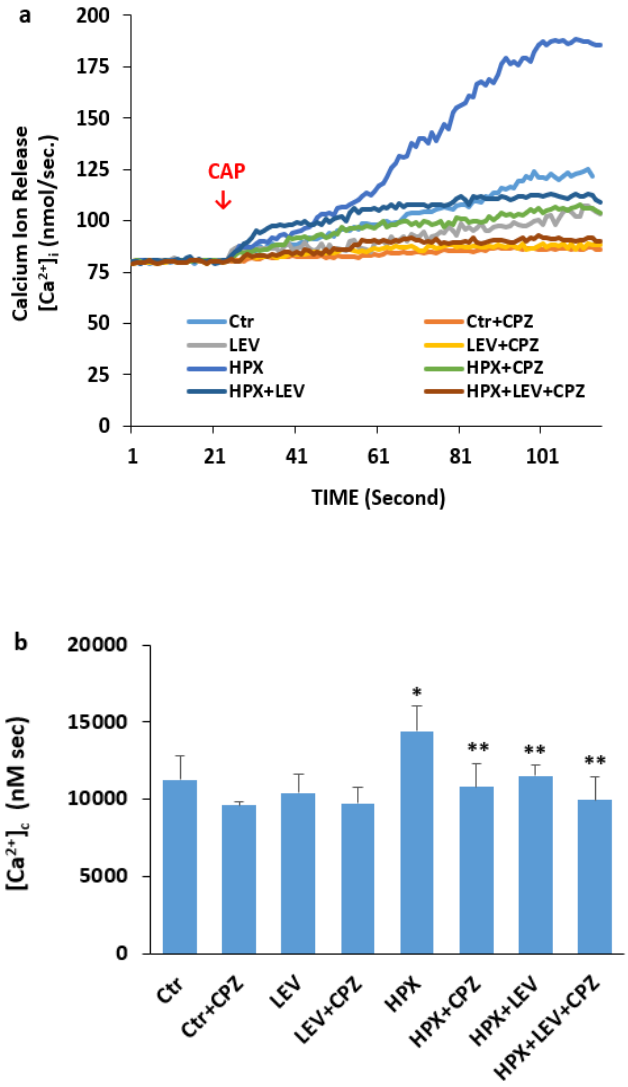

Figure 1. HPX-induced increase of $[Ca^{2+}]_c$ was decreased via modulation of TRPV1 channel in the DBTRG cells by the LEV treatment. (Mean±SD and n=3). *After incubation of the DBRTG cells with $CoCl_2$, LEV and their combination, they were stained with Fura-2 calcium dye. Then they were stimulated by CAP (1 μM), although they were inhibited by LEV and CPZ (10 μM) after loading with Fura-2 for 120 seconds. (*p ≤ 0.001 vs. Ctr, Ctr+CPZ, LEV and LEV+CPZ groups. **p ≤ 0.001 vs. HPX group).*

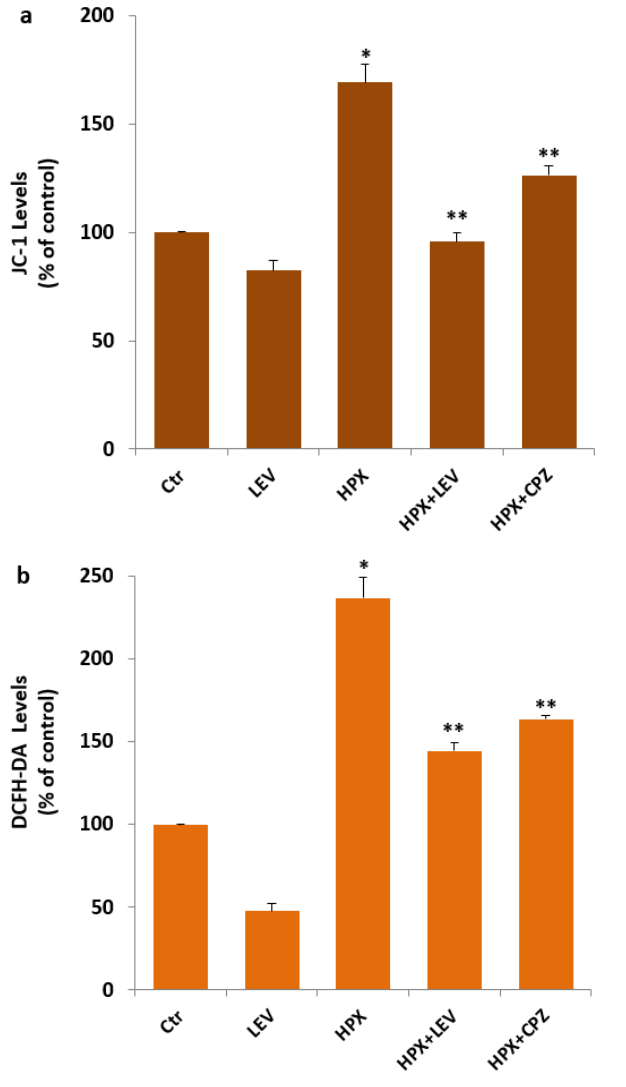

Figure 2. LEV and TRPV1 blocker (CPZ) modulated HPX-induced changes of mitochondrial membrane depolarization and cytosolic ROS production in the DBTRG cells. (Mean±SD and n=3). *The DBRTG cells of four group were separately incubated with JC-1 and DCFH-DA stains in the cell culture conditions. After analyzing in the microplate reader (Infinite pro200), they were stimulated by CAP (1 μM), although they were inhibited by CPZ (10 μM). Then they were analyzed in the microplate reader again. The JC-1 and DCFH-DA values were expressed fold increase (% of control). (*p ≤ 0.001 vs. Ctr and LEV groups. **p ≤ 0.001 vs. HPX group).*

LEV treatment modulated HPX-induced cell viability (MTT), apoptosis, caspase -3 and caspase -9 values in the DBTRG cells

In addition to the excessive cytosolic ROS production, the loss of mitochondrial membrane depolarization in the mitochondria results in apoptosis via increases of caspase- -3 and -9 activations (Keil et al.

2011; Nazıroğlu 2012; Ureshino et al. 2019). For this reason, mitochondrial membrane depolarization is an important parameter of mitochondrial function and it was used as an indicator of caspase activity and apoptosis in the neurons. The results of microplate reader analyses of MTT (Figure 3a), apoptosis (Figure 3b), caspase -3 (Figure 3c) and caspase -9 (Figure 3d) values are shown in the Figure 3. The apoptosis level, caspase -3 and caspase –9 activities in the cells were higher in the HPX group than in the control and LEV groups, although MTT level was lower in the HPX group than in the control and LEV groups ($p \leq 0.001$). However, the apoptosis level, caspase -3 and caspase -9 activities were lower in the HPX+LEV and HPX+CPZ groups as compared to HPX group, although MTT level was increased in the HPX+LEV and HPX+CPZ groups by the LEV and CPZ treatments ($p \leq 0.001$).

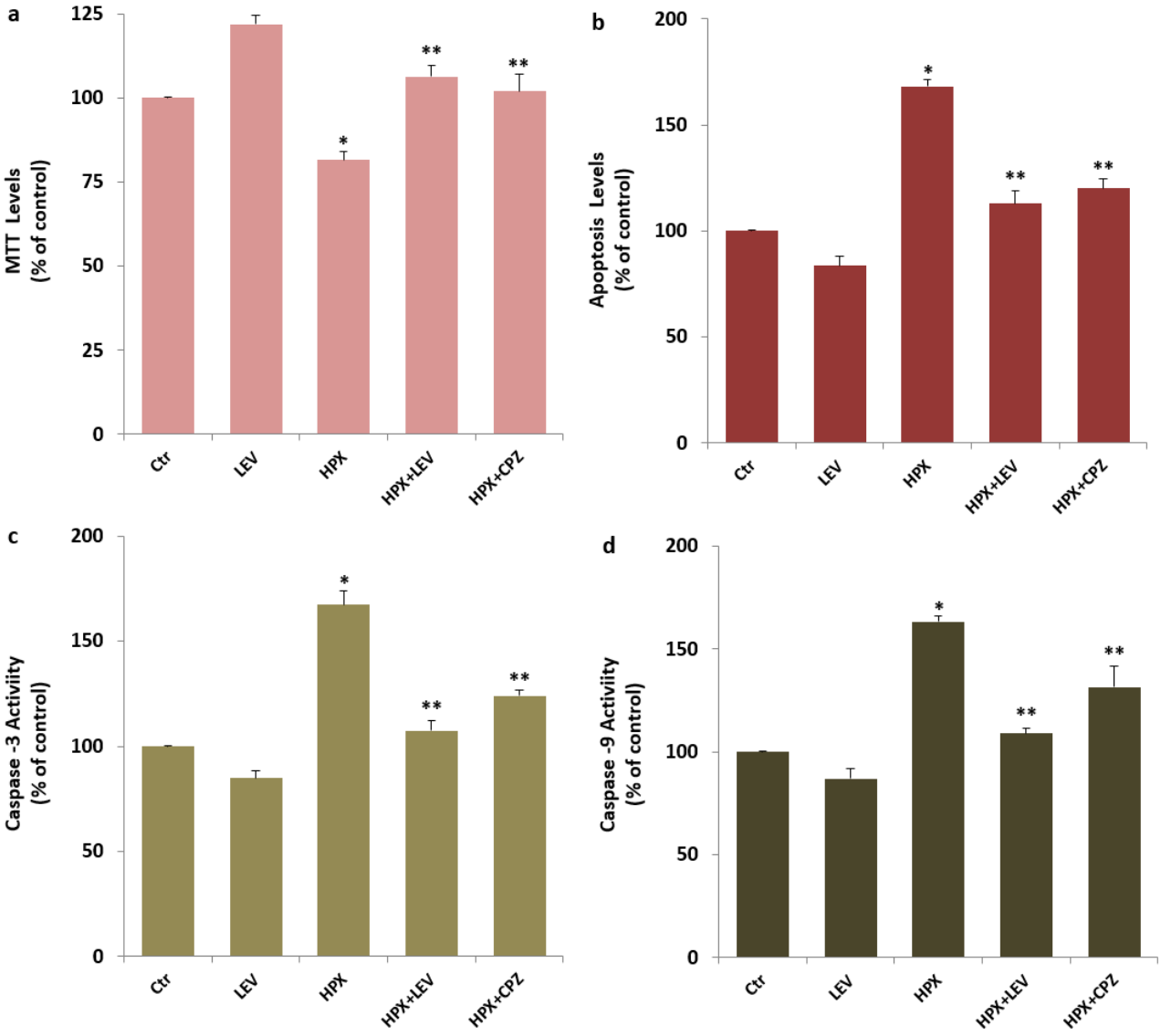

Figure 3. LEV and TRPV1 blocker (CPZ) modulated HPX-induced changes of MTT, apoptosis, caspase -3 and -9 values in the DBTRG cells. (Mean±SD and n=3). *The DBRTG cells of four group were separately incubated with MTT, Cell-APOPercentage apoptosis kit, caspase -3 (AC-DEVD-AMC) and caspase -9 (ACDEVD-AMC) substrates in the cell culture conditions. After analyzing in the microplate reader, they were stimulated by CAP (1 μM), although they were inhibited by CPZ (10 μM). Then they were analyzed in the microplate reader again. The four values were expressed as fold increase (% of control). (*p ≤ 0.001 vs. Ctr and LEV groups. **p ≤ 0.001 vs. HPX group).*

Lipid peroxidation (LipPX), rGSH level and GPx results

MDA, rGSH concentrations and GSHPx activity results are shown in Table 1. The rGSH concentration and GSHPx activity were lower in the HPX group than in the control and LEV groups, although the LipPX level was high in the HPX group ($p \leq 0.001$). However, the GSHPx activity and rGSH level were increased in the HPX+LEV group by the LEV treatment, although LipPX level was decreased in the group ($p \leq 0.001$). These results clearly indicated that HPX induced increase of LipPX is decreased in the DBTRG neuronal cells by LEV treatment through upregulation of rGSH level and GSHPx activity.

Values	Control	LEV	HPX	HPX+LEV
LipPx (µmol/ g protein)	18.20 ± 1.31	17.10 ± 1.19	31.10 ± 1.16*	20.06 ± 3.91**
rGSH (µmol/ g protein)	10.80 ± 1.22	11.40 ± 0.78	8.30 ± 0.48*	13.60 ± 1.43**
GSHPx (IU/ g protein)	22.30 ± 0.95	24.50 ± 0.99	15.80 ± 2.11*	22.10 ± 1.13**

* $p \leq 0.001$ versus control and LEV groups. ** $p \leq 0.001$ versus HPX group.

The data were analyzed by Student's t test.

Table 1. *Effect of HPX ($CoCl_2$ and 50 µM) and levetiracetam (LEV and 100 µM) on lipid peroxidation (LipPx), reduced glutathione (rGSH) level and glutathione peroxidase (GSHPx) activity in the DBTRG neuronal cells (mean ± SD and n=6).*

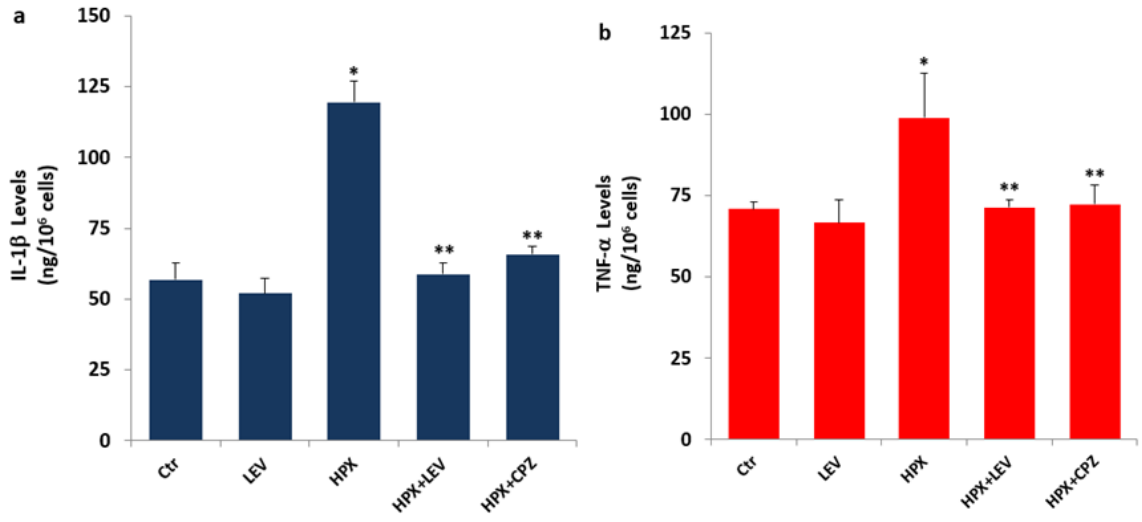

Figure 4. LEV and TRPV1 blocker (CPZ) modulated HPX-induced changes of IL-1β and TNF-α levels in the DBTRG cells. (Mean±SD and n=3). *The IL-1β (a) and TNF-α (b) levels were assayed in the ELISA by using the commercial ELISA kits. The levels of IL-1β and TNF-α in the five groups were expressed as ng/10^6 cells. (*p ≤ 0.001 vs. Ctr and LEV groups. **p ≤ 0.001 vs. HPX group).*

Oxidative-redox shift causes divergent immune responses in the DBTRG neurons

It is well known that HPX has a major role in the induction of inflammation. The IL-1β and TNF-α are the main indicators of cytokine productions (Kaur et al. 2013). Accumulating evidences indicated that increase of TRPV1 activity induces an increase of cytokine production in hippocampus (Ataizi et al. 2019), astrocytes (Yang et al. 2019) and H9C2 cardiomyocyte cell line (Sun et al. 2014). After observing increase of $[Ca^{2+}]_c$ via TRPV1 activation, I set to evaluate the effect of HPX and LEV treatment via TRPV1 modulation on cytokine productions in DBTRG cells. To determine changes in the level of cytokines for each treatment regimen, values are expressed as fold change over the corresponding control. Exposure to HPX upregulated the levels of inflammatory cytokines (IL-1β and TNF-α) in the cells, when compared to control (Figure 4a and b) ($p \leq 0.001$). In contrast, LEV and CPZ treatments markedly downregulated the levels of IL-1β and TNF-α in the cells, in the presence of HPX ($p \leq 0.001$).

Discussion

There are currently no effective protective agents against the injury action of HPX. Recently, LEV as an VGCC blocker and effective antiepileptic drug has attracted attention as a potential therapeutic agent based on its potential antioxidant or anti-apoptotic actions (Stettner et al. 2011; Abdel-Wahab et al. 2015). In the present study, I observed that LEV incubation ameliorated HPX-induced deleterious effects via modulating the intracellular Ca^{2+} hemostasis, inflammation, mitochondrial oxidative stress, cell viability and apoptosis pathways, as well as up-regulating the rGSH and GSHPx molecular antioxidant pathways. Based on our results, I propose that the beneficial effects of LEV on the HPX-induced neurotoxicity via activation of TRPV1 were based on promoting rGSH concentration and GPx activity and reducing mitochondrial oxidative stress, and restoring inflammation in the DBTRG cells.

TRPV1 within TRP superfamily members was mostly expressed in neurons such as DRG and hippocampus, because it has a main role in the homeostasis of the neurons (Clapham 2003). Brain and neurons are very sensitive to ROS production, because they have high oxygen consumption and poly unsaturated fatty acid but they have low antioxidant level (Nazıroğlu 2007). In addition to CAP, ROS also induce activation of the TRPV1 channel via promoting oxidation of cysteine group in the structure of the channel (Ogawa et al. 2016). Consistent with the reports, I observed Ca^{2+} influx and excessive ROS production through the TRPV1 activation in the DBTRG cells by the HPX-induced mitochondrial oxidative stress. HPX-induced ROS was diminished via upregulation of antioxidant enzymes, but down-regulation of TRPV1 channel activity in H9C2 cardiomyocyte cell line (Sun et al. 2014). TRPV1 is activated in DBTRG cell line by depletion of rGSH and GSHPx (Nazıroğlu et al. 2019), although treatment with antioxidants acted TRPV1 channel blocker action through inhibition of oxidative stress in the DRG and hippocampal neurons (Kahya et al. 2017). LEV acted as a potential therapeutic VGCC blocker agent based on its potential antioxidant action (Stettner et al. 2011; Abdel-Wahab et al. 2015). In the present study, I observed modulation of the TRPV1 channel via inhibition excessive ROS generation and increase of thiol redox cycle antioxidants (rGSH and GSHPx) in the HPX-induced DBTRG cells by the LEV and TRPV1 blocker (CPZ) treatments.

In the current study, I observed increase of apoptosis, mitochondrial membrane depolarization and ROS levels via stimulation of TRPV1 in the DBTRG cells by HPX induction, although the levels were decreased in the cells by the LEV treatment. Mitochondria act major actions in neuronal ATP metabolism, energy homeostasis, the oxidative stress and apoptosis (Joshi and Bakowska 2012; Keil et al. 2011). Regulation of mitochondrial Ca^{2+} uptake could counteract HPX-induced mitochondrial membrane depolarization suppression, stimulate recovery of mitochondrial homeostasis, reduce neuronal oxidative injury and apoptosis, and enhance tissue repair and regeneration in the DBTRG neuronal cells (Cheng et al. 2017; Khan et al. 2017; Deveci et al. 2019). It was reported that LEV modulates mitochondrial biogenesis and attenuates mitochondrial dysfunction, apoptosis and cell death in the HPX-induced oxidative stress of H9C2 cardiomyocyte cells (Sun et al. 2014). In the present literature data, there is no report on the protective action of LEV against TRPV1 activation in neurons during the HPX. I examined whether TRPV1 blocker (CPZ) mediates the effects of HPX and LEV on mitochondria in the DBTRG cells. Consistent with present findings, the mitochondria membrane depolarization, ROS generation

and apoptosis via stimulation of $[Ca^{2+}]_c$ increase and TRPV1 activation were upregulated in the cells exposed to HPX; importantly, these were totally reversed by CPZ treatment. These data indicate that LEV may preserve neuronal function by modulating mitochondrial homeostasis via $[Ca^{2+}]_c$ and TRPV1 function.

Apoptosis and inflammation through TRPV1 in cardiomyocyte cell line and rat hippocampus has been implicated in HPX-induced IL-1β, TNF-α, caspase -3 and -9 activations (Dai et a. 2008; Sun et al. 2014). The IL-1β, TNF-α, caspase -3 and -9 of current study confirmed that TRPV1 activation-induced apoptosis in the DBTRG cells was increased by HPX induction. LEV induced several antioxidant actions against apoptosis in hypoxic brain of experimental animals (Komur et al. 2014). It was reported that lipopolysaccharide-induced increase of TNF-a and caspase levels in the DRG rats was decreased by the antioxidant property of LEV (Stettner et al. 2011). Consistent with the literature results, I observed modulator role of LEV on the apoptosis and inflammation via inhibition of IL-1β, TNF-α, caspase -3 and -9 activations in the DBTRG neuronal cells.

In summary, the present study provides the first evidence that LEV treatment in the DBTRG cells can ameliorate the mitochondria ROS, caspase -3, caspase -9, apoptosis and inflammation induced by HPX by modulating TRPV1 activation, and protecting rGSH and GSHPx thiol redox system. These effects resulted from an increase in the restoration of mitochondrial function via modulation of TRPV1-induced calcium signaling. The current findings suggest a new treatment target via for preventing HPX-induced neurodegeneration, TRPV1 activation and oxidative injury.

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Conflict of Interest Statement

Dr. Kemal Ertilav declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Statement

The author confirms that human and animal samples did not use in the current study. The study was performed in the commercial DBTRG cell line.

Competing Interest Statement

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