Effect of vanillic acid on pentylenetetrazole-kindled rats: Nrf2/HO-1, IGF-1 signaling pathways cross talk

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Vanillic acid (VA) exhibited antioxidant and neuroprotective properties in some neurodegenerative disorders. So, the current study examined the neuroprotective potential of VA as an antiepileptic agent in pentylenetetrazole (PTZ)-induced epileptic rats and the prospective role of Insulin like growth factor-1 (IGF-1) and nuclear factor-2 erythroid-related factor-2 (Nrf2)/heme oxygenase-1 (HO-1) pathway in this respect. Thirty male albino rats were equally subdivided into 3 groups; (1) normal control (NC) group, (2) PTZ-group: received PTZ (50 mg/Kg, i.p. every other day) for 14 days, and (3) PTZ + VA group: received PTZ and VA (50 mg/Kg daily for 2 weeks). The seizure score and latency were evaluated after PTZ injection. Also, the markers of oxidative stress (malondialdehyde (MDA), catalase, and reduced glutathione (GSH)), histopathological examination, the expression of glial fibrillary acidic protein (GFAP) (a marker of astrocytes) IGF-1, Nrf2, and HO-1 were assessed in the brain tissues by the end of the experiment. PTZ caused significant decrease in seizure latency and significant increase in seizure score by the end of the experiment (p < 0.01). This was associated with significant increase in MDA and GFAP with significant decrease in GSH, total antioxidant capacity (TAC) and IGF-1 in brain tissues compared to normal group (p < 0.01). On the other hand, treatment with VA caused significant attenuation in PTZ-induced seizures which was associated with significant improvement in oxidative pathological markers and downregulation in GFAP and upregulation of Nrf2, HO-1 and IGF-1 in CA3 hippocampal region (p < 0.01). VA showed neuroprotective and anti-epileptic effects against PTZ-induced epilepsy which probably might be due to its antioxidant properties and upregulation of Nrf2/HO-1 pathway and IGF-1.

Keywords
PTZ-induced epilepsy, Vanillic acid, IGF-1, Nrf2, HO-1

1. Introduction

Epilepsy is a serious neurological disorder that impacts about 1%-2% of the world’s population [1]. Epilepsy is accompanied with cognitive deficits which negatively influence life’s quality [2] and about one third of patients with epilepsy show resistance to the established lines of treatment [3]. So, looking into the possible mechanisms of the process of epileptogenesis is mandatory to develop new agents that regulate epileptic seizures during epilepsy. During the period of epileptogenesis, molecular events bring about various changes in the brain structure including loss of neurons, sprouting of mossy fibers, reorganization of synapses, astrocitosis, neurogenesis, etc. [4]. The process of epileptogenesis can be replicated through kindling. Kindling is a process through which we can ignite prolonged seizures with gradually increased duration and degree of behavioral disorder. Kindling is achieved by repetitive sub-threshold applications of any convulsing agent. Chemical kindling via pentylenetetrazole (PTZ) is one way to induce animal models for temporal lobe epilepsy, which is the most common symptomatic refractory form of epilepsy [5]. Oxidative stress is one of the major mechanisms which initiates epilepsy and leads to its progression next to a primary brain insult [6]. Oxidative stress in neurons during epilepsy results from excessive production of reactive oxygen radicals (ROS), apoptotic, inflammatory, immune changes and dysfunction of blood-brain barrier [7, 8]. So, the use of antioxidants as ascorbic acid [9], flavonoids [10], vitamin E [11], L-carnitine [12] etc., protected against epilepsy in animal models.

Nuclear factor-2 erythroid-related factor-2 (Nrf2) is a transcription factor that promotes antioxidant enzymes production such as heme oxygenase-1 (HO-1), superoxide dismutase (SOD) glutathione peroxidase (GPx), thus renders cells oxidative stress resistant [13]. Nrf2 is the principal transcription factor activated succeeding oxidative stress in epilepsy representing an endogenous adaptive mechanism that protect against the neuronal oxidant insult [14]. In case of temporal lobe epilepsy, Nrf2/antioxidant response element (ARE)-dependent HO-1/NAD(P)H dehydrogenase (quinone)-1 (NQO-1) production was found to ameliorate...
oxidative injury induced by glutamate. Nrf2/HO-1 pathway activation can be considered a potential therapeutic avenue in intractable epilepsy [13, 15]. Moreover, IGF-1 refines the central nervous system development, aids neurons to differentiate, mature and connect properly. It urges axons to grow and neurons to survive [16]. Insulin like growth factor-1 (IGF-1)/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway has been recently featured as a protective pathway against oxidative stress in astrocytes. It was also implicated in ameliorating neurodegenerative changes in Alzheimer’s [17]. Recently, it has been found that IGF-1 induces a transcriptional program for mitochondrial biogenesis through induction of Nrf2 expression [18]. So, we suggest that there would be a cross talk between IGF-1 and Nrf2/HO-1 pathways.

Plant phenolics including vanillic acid (VA), the major constituent of vanilla bean and pod extracts [19], are commonly used as a food flavour. It has been reported that they have neuroprotective properties and can be used in treatment of several disorders [20]. Also, vanillic acid demonstrated antioxidative, antihypertensive and anti-inflammatory properties [21–23]. Recently, VA was reported to activate the Nrf2/HO-1 pathway in neurodegenerative disorders as Alzheimer’s disease. Also, VA exerted its antioxidant effect through the protein kinase B (Akt)/glycogen synthase kinase 3/β (GSK3/β)/Nrf2 signaling pathway in mice brains [24]. We hypothesized that VA could attenuate epileptic seizures induced by PTZ in rats via Nrf2/HO-1 and IGF1 activation. So, the present study aimed to investigate the impact of VA on PTZ-induced epileptic seizure and to explore the role of Nrf2/HO-1 and IGF-1 in this possible neuroprotective of VA in rats.

2. Materials and methods

2.1 Experimental animals

Thirty male sprague-dawely rats weighs 170–190 g were accommodated in standard cages at Medical Experimental Research, Mansoura, Egypt. Animals fed on standard diet and water ad libitum. Animal care was done according to The Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, D.C. 20055, USA). The IRB-committee approved all experimental procedures (code #R.20.11.1098.r).

2.2 Study design

Rats were randomly (sealed envelopes) partitioned into 3 equal groups (10 rats in each) as the following: (a) Normal group: normal animals received 0.2 mL saline via intraperitoneal injection (i.p.) for 2 weeks, (b) PTZ group: rats were i.p injected with 50 mg/kg PTZ (purchased from Sigma Aldrich, USA) in 0.2 mL every other day for 2 weeks [25] and (c) VA + PTZ group: the same as PTZ group except that rats were pretreated with VA (purchased from Sigma Aldrich, USA) daily 50 mg/kg VA by oral gavage 30 min prior to PTZ for 2 weeks [22].

2.3 Animal model

Subsequent to each PTZ injection, rats were placed in transparent Plexiglas cages to record their 30 min convulsive behavior by a video camera after PTZ administration for 2 weeks or 7 records or trials (every other day). Latency to seizure onset (sec) and seizure score were recorded. Scoring of seizure severity was done based on Racine’s scale (0 = normal, non-epileptic activity, 1 = movements of mouth and face, hyperactivity, grooming, sniffing, scratching, wet dog shakes, 2 = head nodding, staring, tremor, 3 = forelimb clonus, forelimb extension, 4 = rearing, salivating, tonic clonic activity and 5 = falling, status epilepticus) [26].

2.4 Harvesting of brain tissues

At the end of the study, a high dose of Na+ thiopental (120 mg/kg) i.p. was used for rat sacrifice. Six rats were perfused with one hundred mL heparinized saline via cardiac catheter for brain collection for biochemical and molecular studies, while the rest of rats (4 rats in each group) were perfused with one hundred mL heparinized saline then one hundred and fifty-mL formalin (10%) through a cardiac catheter for collection of brains for histopathological and immunohistochemical studies. Details of collection and storing of brain tissues is described in our previous work [12].

2.5 Assay of markers of oxidative stress (MDA, catalase activity, total antioxidant capacity) in brain tissues

Hippocampal regions of the brain were homogenized in 1–2 mL cold phosphate buffered saline (50 mM in EDTA (1 mM) at pH 7.5. Then, centrifugation done at 4000 rpm at 4 ºC for 15 min. Colorimetric assay of these markers were done by using commercially available kits (Bio-Diagnostics, Dokki, Giza, Egypt) according to the manufacturer’s instructions.

2.6 Hematoxylin and eosin staining

Brain tissues (n = 4 rats in each group) staining with hematoxylin and eosin (H&E) was achieved based on our preceding work [12]. Neuronal loss and pyknotic nuclei were assessed by light microscopy in the CA3 region of hippocampus [12].

2.7 Assessment of Nf2 and HO-1 expression at the level of mRNA by real-time polymerase chain reaction (PCR)

The mRNAs encoding for antioxidant transcription factor, Nrf2 and heme oxygenase (HO)-1 were identified by real-time PCR in brain tissues. According to the manufacturer’s instructions, we isolated the total RNA from brain tissue specimens. RNA was quantified spectrophotometrically, and its quality was determined by agarose gel electrophoresis and ethidium bromide staining. cDNA was synthesized from 1 μg total RNA and then buffered in a volume of 25 μL. Then, the 25 μL cDNA was diluted in a total volume of 100 μL. All details of gene (Nrf2, HO-1 and GAPDH) amplification and detection were mentioned in our previous work [27].

2.8 Immunohistochemical examination for glial fibrillary acidic protein (GFAP), and IGF-1 in cornu ammonis (CA) CA3 and CA1 regions of the hippocampus

Sagittal section (40 μm) was obtained from brain tissue (n = 4 rats in each group) to perform immunohisto-
Fig. 1. Effects of VA on behavioral effects in PTZ-induced seizures. (A) seizure score. (B) seizure latency (Sec). Two-way ANOVA test, results were represented as mean ± SEM. **significant vs PTZ group $p < 0.001$ and *significant vs PTZ group $p < 0.01$.

Fig. 2. Effects of VA on oxidative stress markers in PTZ-induced seizures. (A) Malondialdehyde (MDA) (nmol/g brain tissues). (B) Catalase (CAT) enzyme activity (U/g brain tissues). (C) Total antioxidant capacity (TAC). One-way ANOVA test, results were represented as mean ± SEM. ***significant vs control group $p < 0.0001$, ###significant vs PTZ group $p < 0.0001$ and ##significant vs PTZ group $p < 0.001$. n = number of rats per group.

Chemical staining based on a prior work [28]. The primary rabbit anti-glial fibrillary acidic protein (GFAP) antibody (cat#SAB5600060, 1:200, Sigma, USA), and IGF-1 rabbit anti-rat monoclonal antibodies (Cat# sc-74116, 1:50, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (with final dilution 1:50) were used. The hippocampus was examined blindly by 2 expert pathologists using optika light microscope with optika B-10 (OPTIKA Microscopes, Italy) and the CA3 hippocampal region was analyzed for the immunostaining of these markers and the average of the number of GFAP-positive cells and area of interest of interest (ROI) of IGF-1 membranous immunostaining in 5 high power fields (HPF) were calculated.
2.9 Statistical analysis

GraphPad Prism version 5.0 (GraphPad Software Inc., CA, USA) was used for statistical analysis. Statistical significance in behavioral parameters was determined by two-way analysis of variance (ANOVA) with Bonferroni posthoc test. Also, one-way ANOVA with Tukey’s posthoc test was applied to find the statistical significance in biochemical, molecular, and histochemical parameters. Also, Pearson correlations between IGF-1 expression and other studied parameters were calculated. Statistical significance is determined when $p \leq 0.05$.

3. Results

3.1 VA effect on seizure latency and score in PTZ-induced epilepsy

In comparison with PTZ group, rats with VA administration displayed significant reduction in the PTZ-induced seizure score in trial 4 ($p < 0.01$), trial 5 ($p < 0.05$) and trial 7 ($p < 0.001$) (Fig. 1A). Also, the latency of epileptic seizures showed significantly longer values in the VA group compared to the PTZ group in trials (2, 6, 7) ($p < 0.05$) (Fig. 1B).

3.2 Effects of VA on MDA, catalase activity and total antioxidant capacity (TAC) in brain tissues of PTZ-induced epilepsy

The concentration of MDA in the brain tissues showed a significant increase in the PTZ group as opposed to the control group ($p < 0.0001$). However, the VA group exhibited a significant reduction in the MDA concentration in comparison to the PTZ group ($p < 0.01$). On the other hand, catalase enzyme activity revealed a significant reduction in the PTZ group as opposed to the control group ($p < 0.0001$), while rats with VA showed a non-significant rise in the catalase (CAT) activity as opposed to the ones received PTZ only. Also, the total antioxidant capacity (TAC) showed a significant drop in rats with PTZ in contrast with the control rats ($p < 0.001$) while, VA administration displayed significantly increased TAC in comparison with PTZ only ($p < 0.001$) (Fig. 2A–C).

3.3 VA effect on Nrf2 and HO-1 expression at the level of mRNA in rat hippocampus

In the hippocampus, the Nrf2 and HO-1 expression at the mRNA level showed non-significant increase in the PTZ rats as opposed to the control ones ($p > 0.05$). However, rats with VA exhibited significantly elevated Nrf2 and HO-1 in comparison to the ones with PTZ only ($p < 0.001$) (Fig. 3A–B).

3.4 VA effect on hippocampal histopathology

Control rats displayed normally shaped neurons with normal number in the hippocampal CA3 subfield with histopathological examination (Fig. 4A–B), while neurons in the CA3 region in rats with PTZ were irregularly arranged, of diminished number and showed pyknosis (darkly stained nucleus and cytoplasm) (Fig. 4C–D). However, VA expanded the territory of normal neurons and decreased the number of abnormal ones in the hippocampus CA3 region (Fig. 4E–F).

3.5 Effects of VA on GFAP and IGF-1 expression by immunohistochemistry in the hippocampus CA3 region

Fig. 5A displays a significant rise in the GFAP positive cells number (a marker of astrocytosis) in the hippocampus CA3 subfield in the PTZ group as opposed to the control one ($p < 0.0001$). In contrast, VA significantly lowered the positive cells number in comparison to the PTZ group ($p < 0.001$). Brain specimens from different groups showed mild expression of astrocytes by GFAP in the control rats (Fig. 5B), substantial expression of GFAP in rats with PTZ (Fig. 5C), and mild expression of GFAP in VA group (Fig. 5D).

Moreover, the ROI of IGF-1 positivity in the hippocampus CA3 region significantly decreased in the PTZ group as opposed to the control one ($p < 0.001$). Moreover, VA significantly elevated the IGF-1 expression in comparison to the PTZ group ($p < 0.01$) (Fig. 6A). Brain specimens obtained showed marked cytoplasmic expression of IGF-1 in the control group (Fig. 6B), mild cytoplasmic expression in the PTZ group (Fig. 6C), moderate cytoplasmic expression in the VA group (Fig. 6D).
3.6 Correlations between IGF-1 and other studied parameters

The expression of IGF-1 showed a negative significant correlation with the PTZ-induced epileptic seizure score \( (p = 0.0067) \) (Fig. 7A), a non-significant positive correlation with the seizure latency \( (p = 0.2401) \) (Fig. 7B). Also, the oxidative stress markers showed negative correlations between IGF-1 and MDA \( (p = 0.0016) \) (Fig. 7C), and positive correlation between IGF-1 and CAT enzyme activity \( (p < 0.0001) \) and TAC \( (p < 0.0001) \) (Fig. 7D–E respectively). Moreover, there is a negative correlation between IGF-1 and GFAP \( (p < 0.0001) \) (Fig. 7F), while the expression of Nrf2 and HO-1 showed positive correlations with IGF-1 and Nrf2 and HO-1 \( (p = 0.0026, 0.0172 \) respectively) (Fig. 7G–H).

4. Discussion

With an alternate day PTZ injection for two weeks, our study brought about a substantial increase in the PTZ-induced seizures, the GFAP expression, and the extent of neuronal loss. Also, PTZ worsened the oxidative state and decreased the IGF-1 level with insignificant changes in
Fig. 5. Effects of VA on GFAP (marker of astrocytes and astrogliosis) expression in CA3 hippocampal region. (A) Represents the mean of GFAP-positive cells per high power field (HPF) in the CA3 region in different groups. Brain specimens from (B) normal control group show normal expression (red arrows) of GFAP in CA3 region of hippocampus (400×). (C) PTZ group show marked GFAP expression (red arrows) in CA3 hippocampal region (astrogliosis) (400×). (D) VA + PTZ group show decreased GFAP expression (red arrows) in CA3 hippocampal region. One-way ANOVA with Tukey posthoc test, results were represented as mean ± SEM. ***significant vs control group p < 0.0001, **significant vs control group p < 0.01 and ###significant vs PTZ group p < 0.0001. n = number of rats per group.
Fig. 6. Effects of Stevia on IGF-1 expression in CA3 hippocampal region by immunohistochemistry. (A) Represents the mean area of interest (ROI) of IGF-1 expression per high power field (HPF) in different groups. Brain specimens from normal control group show (B) different regions of hippocampus (CA1, CA2, and CA3) at low power (100×). (C) Marked membranous expression (red arrows) of IGF-1 in CA3 region of hippocampus (400×). Brain specimens from PTZ group show (D) different regions of hippocampus (CA1, CA2, and CA3) at low power (100×). (E) Mild membranous IGF-1 expression (red arrows) in CA3 hippocampal region (400×) and brain specimens from VA + PTZ group show (F) different regions of hippocampus (CA1, CA2, and CA3) at low power (100×). (G) Moderate membranous IGF-1 expression (red arrows) in CA3 hippocampal region. One-way ANOVA with Tukey posthoc test, results were represented as mean ± SEM. **significant vs control group \( p < 0.0001 \) and *** significant vs PTZ group \( p < 0.0001 \). \( n \) = number of rats per group.
seizures by minimum effective doses inducing clonic-tonic convulsions and tonic extension and against maximal electroshock induced seizures in mice. However, to the best of our knowledge, our study exclusively offers a novel dimension to the neuroprotective role of vanillic acid. We present vanillic acid not only as an anticonvulsant but also as a potential antiepileptic since it reduced the seizure score, neuronal loss, astrocytosis, and prolonged seizure latency.

Astrocytosis has a crucial role in the process of epileptogenesis intertwined with oxidative stress which is a key hallmark in epilepsy. Puttachary et al. [33] mentioned that reactive astrocytosis is accompanied by hyperexcitability due to the down-regulation of glutamine synthase enzyme resulting in high levels of un-metabolized glutamate. Moreover, this hyperexcitability results in the overproduction of free radicals overwhelming the antioxidant capacity and causing oxidative stress. This is well consistent with our findings since PTZ exhibited a high GFAP level, a marker for astrocytosis, and high oxidative stress markers. In the current study, vanillic acid reduced the GFAP expression and the oxidative stress markers. The neuroprotective effect of vanillic acid demonstrated in our study seems to be owing to its antioxidant properties and slowing down astrocytosis. This is in harmony with Amin et al. [24] who mentioned that 30 mg/kg vanillic acid administration for 3 weeks after Aβ1–42 injection improved the oxidative state and attenuated astrocytosis in a mice model of Alzheimer’s disease.

Nrf2 is a transcription factor that promotes the production of many antioxidant enzymes including glutathione peroxidases and HO-1 [13]. In the current study, a 50 mg/kg alternate day PTZ injection for 14 days showed an insignificant change in the expression of Nrf2 and HO-1 between the PTZ and the controls. This is consistent with Wang et al. [14] who used repeated administration of sub-convulsive electric stimulation for a 15 days amygdala kindling rat model. On the other hand, Li et al. [34] displayed a lowered expression of Nrf2 and HO-1 with a daily 37 mg/kg PTZ for 40 days in mice. The difference might emerge from the fact that Nrf2 is activated in epilepsy after oxidative stress as an endogenous adaptive mechanism to protect against the neuronal oxidant insult [14]. Vanillic acid upregulated the Nrf2/HO-1 expression which is in line with its antioxidant effect. The up-regulation of Nrf2/HO-1 expression has been a well-investigated notion as a potential therapeutic target for epilepsy [13]. Chen et al. [15] displayed amelioration of glutamate-induced oxidative damage by activating Nrf2/HO-1 Signaling Pathway in HT22 Cells. Moreover, Amin et al. [24] showed a neuroprotective role of vanillic acid through Nrf2/HO-1 up-regulation in a mice model of Alzheimer’s disease. So, up to the best knowledge, our study exclu-
sively shows a neuroprotective role for vanillic acid in a PTZ-induced epilepsy model through up-regulation of Nr2/HO-1. However, we did not measure the expression of Nr2/HO-1 at the level of protein by western blotting which is considered as a limitation of the current study to be considered in further studies.

IGF-1 enhances neuronal differentiation, maturation, survival, and axon growth [16]. This is well depicted in our findings since vanillic acid elevated the PTZ-induced lowered levels of IGF-1. Also, there was a positive correlation between IGF-1 and Nr2 and a negative one between IGF1 and astrocytosis, neuronal loss, seizures score, and oxidative stress markers. Our hypothesis of the cross-talk between the two pathways of IGF-1 and Nr2/HO-1 has been postulated in various studies. Mahran [35] reported an improvement of cisplatin-induced nephrotoxicity through growth hormone administration that increased IGF-1 expression along with up-regulation of Nr2 promoting IGF-1 as a mediator for Nr2/HO-1 which eventually opposes the oxidative damage in renal cells induced by cisplatin. Bailey-Downs et al. [36] showed that IGF-1 deficiency impaired the vascular antioxidant responses by impairing Nr2 expression and its target genes. Also, Wang et al. [37] showed that IGF-1 treatment promoted the nuclear translocation of Nr2 and up-regulated the expression of its downstream gene (HO-1) in an Alzheimer’s disease model with improvement in the oxidative state. Moreover, PI3-K inhibition abolished the protective effect of IGF-1 on β-amyloid-induced ROS generation. This suggests that IGF-1 protects SH-SY5Y cells against β-amyloid induced cell injury by scavenging ROS via the PI3K/Akt-Nr2 signaling pathway. Furthermore, Amin, et al. [24] showed that daily 30 mg/kg vanillic acid for 3 weeks improved oxidative stress markers, Nr2, HO-1, Akt, and Gsk levels in mice brain homogenates after β-amyloid-induced oxidative stress. Although, the strongly suggested neuroprotective role of vanillic acid in PTZ-induced epilepsy in our study, a further clarification is needed concerning a more specific approach towards the involved signaling pathways such as PI3K/Akt pathway. Also, our study addressed a correlation between IGF-1, oxidative stress, Nr2 and astrocytosis which could open new perspectives in the study of the process of epileptogenesis. These potential novel perspectives require further studies.

5. Conclusions

In conclusion, vanillic acid demonstrated a neuroprotective and an anti-epileptic effect against PTZ-induced epilepsy. This outcome might be due to suppression of PTZ-induced astrocytosis, and oxidative stress. Vanillic acid seems to act through up regulation of IGF-1 and Nr2. Moreover, the potential cross-talk between IGF-1 and Nr2 could share in the neuroprotective and the anti-epileptic effect of vanillic acid in a PTZ-kindled rat model.

Author contributions

MAE, AY, AMH, EME and MAA conceived and designed the study. MAE, SS, WO, AMH performed all behavioral experiments and conducted biochemical and molecular experiments. AMH, SS, WO, EE, MAA, and WAA collected the data and performed statistical analysis. MAE, AY, EE, WO, AY, SS and AMH wrote the manuscript. All authors reviewed and approved the final draft.

Ethics approval and consent to participate

The IRB-committee approved all experimental procedures (code #R.20.11.1098.r).

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data will be available on reasonable request.

References


