Scientific Research Publishing

Pharmacology & Pharmacy, 2023, 14, 59-71
https://www.scirp.org/journal/pp
ISSN Online: 2157-9431
ISSN Print: 2157-9423

Epalrestat Enhances MTS Reduction Activity Independent of Cell Numbers in Bovine Aortic Endothelial Cells

Keisuke Sato[1], Shoya Endo[1], Natsuki Ota[1], Akira Takaguri[2], Kumi Satoh[2], Ryosuke Tatsunami[1]

[1]Department of Pharmacy, Hokkaido University of Science, Hokkaido, Japan
[2]Department of Pharmacology, Hokkaido University of Science, Hokkaido, Japan
Email: tatunami@hus.ac.jp

How to cite this paper: Sato, K., Endo, S., Ota, N., Takaguri, A., Satoh, K. and Tatsunami, R. (2023) Epalrestat Enhances MTS Reduction Activity Independent of Cell Numbers in Bovine Aortic Endothelial Cells. *Pharmacology & Pharmacy*, **14**, 59-71.
https://doi.org/10.4236/pp.2023.143005

Received: February 8, 2023
Accepted: March 13, 2023
Published: March 16, 2023

Copyright © 2023 by author(s) and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).
http://creativecommons.org/licenses/by/4.0/

Open Access

Abstract

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay is used as a major method to evaluate cell viability. However, in some cases, the results may reflect mitochondrial status regardless of viability. Epalrestat (EPS) is currently available for the treatment of diabetic neuropathy. In this study, we report that EPS at near-plasma concentrations increases MTS reduction activity independent of cell number in bovine aortic endothelial cells. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that plays a pivotal role in inducing the expression of genes encoding detoxifying and defensive proteins. Sulforaphane (an Nrf2 activator) also increased MTS-reducing activity, similar to EPS. Knockdown of Nrf2 by short interfering RNA suppressed EPS-induced MTS reduction. These results suggest that EPS increases MTS reduction activity via the Nrf2 pathway. Furthermore, the results that EPS increases ATP production and that electron transfer chain inhibitors suppress EPS-induced MTS reduction activity suggest that EPS may activate mitochondrial status. Because mitochondrial disorders cause numerous diseases, we suggest that EPS has new beneficial properties that may prevent the development and progression of disorders caused by mitochondrial dysfunction.

Keywords

EPS, MTS, Nrf2, Mitochondria, BAECs

1. Introduction

Various methods have been used to measure cell proliferation and toxicity,

including the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assays [1] [2] [3]. MTT is a water-soluble tetrazolium salt that is converted into a water-insoluble dark blue formazan by reductive cleavage [1]. The necessary step of removing the medium and the solubilization of the formazan prior to its quantification can be troublesome and lead to errors. XTT and MTS assays have been developed to shorten time and eliminate errors [2] [3]. These require an electron coupling reagent for optimal formazan yield. For this, phenazine methosulfate is normally used. The MTS assay is the most popular and is widely used to measure the number of viable cells because it provides results in a short time [4] [5]. MTS, a tetrazolium salt compound, was used as a substrate for dehydrogenase in the MTS assay [4]. After permeabilization of the cell membrane, MTS is reduced to a formazan dye by dehydrogenase in the mitochondria. It can be applied to most animal cells; by using a microplate, many samples can be processed simultaneously. Moreover, the amount of formazan produced corresponds to the number of viable cells. In contrast, the MTS assay reflects mitochondrial activity because it is based on mitochondrial dehydrogenase activity in viable cells [6]. As a result, it is conceivable that the MTS assay may only indicate mitochondrial status, independent of the number of viable cells.

Mitochondria are major intracellular organelles that consume more than 90% of cellular oxygen and produce ATP [7]. They have a dual nature in that they can oxidize metabolic substrates to generate cellular energy, but they also produce genotoxic reactive oxygen species (ROS) [8] [9]. Mitochondria-derived ROS directly cause cell injury to proteins, lipids, and nucleic acids, resulting in aging, atherosclerosis, diabetes mellitus, and mitochondria diseases [10] [11] [12] [13].

Epalrestat (5-[(1Z,2E)-2-methyl-3-phenylpropenylidene]-4-oxo-2-thioxo-3-thiazolidine acetic acid; EPS), approved in Japan in 1992, is currently used to treat diabetic neuropathy [14]. EPS is obtained by condensation of rhodanin-*N*-acetic acid and α-methylcinnamaldehyde in acetic acid in the presence of sodium acetate [15]. EPS is an inhibitor of aldose reductase, which is a rate-limiting enzyme in the polyol pathway [16]. Under hyperglycemic conditions, EPS reduces the accumulation of intracellular sorbitol, which is implicated in the development of diabetic complications [17]. Recently, we reported that EPS increased glutathione (GSH) levels by upregulating glutamate-cysteine ligase (GCL) via activation of nuclear factor erythroid 2–related factor 2 (Nrf2) in rat Schwann cells and bovine aortic endothelial cells (BAECs) [18] [19]. Although EPS exhibits antioxidant effects by regulating glutathione levels, the effect of EPS on mitochondrial status is not clear. The purpose of this study was to determine whether EPS can affect MTS-reducing activity and has the potential to influence mitochondrial activity in BAECs.

2. Materials and Methods

2.1. Cell Culture and Treatment with EPS

BAECs were purchased from Dainippon Sumitomo Pharma Co. Ltd. (Osaka, Japan). BAECs were grown to 80% - 90% confluence in DMEM containing 10% fetal bovine serum (FBS), L-glutamine (4 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were passaged by trypsinization. Before treating cells with EPS (Wako Pure Chemical Industries, Ltd., Osaka, Japan), the culture medium was replaced with DMEM containing 2% FBS. EPS (10, 50, and 100 μM) was then added to the medium.

2.2. Cell Viability

Cell viability was assessed by measuring acid phosphatase activity, which is an accurate indicator of the number of endothelial cells in culture. It was assayed using the method described by Connolly *et al.* [20]. After treatment of BAECs in 96-well plates with EPS, detached BAECs were removed by washing twice with phosphate-buffered saline (PBS) at pH 7.4, and the cells remaining in the 96-well plates were incubated with 100 mL of 0.1 M sodium acetate buffer (pH 5.5) containing 0.1% Triton X-100 and 10 mM *p*-nitrophenyl phosphate at 37˚C for 20 min. The reaction was stopped by adding 10 μL of 1 M NaOH. The *p*-nitrophenol produced was measured at 405 nm using a Bio-Rad Model 680 microplate reader (Hercules, CA, USA). Acid phosphatase activity was expressed as the ratio of surviving cells to the untreated control cells.

2.3. MTS Reduction Activity

MTS reduction activity was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS assay); Promega (Madison, WI, USA). Briefly, BAECs in 96-well plates were incubated with EPS for 24 h at 37˚C. After the medium was removed, the cells remaining on the 96-well plates were washed with FBS-free DMEM and incubated with fresh DMEM (100 μL) and MTS assay solution (10 μL) for 60 min at 37˚C. The amount of MTS formazan produced was measured at 490 nm using a Bio-Rad Model 680 microplate reader.

2.4. Knockdown of Nrf2 in BAECs with Small Interfering RNA (siRNA)

Oligonucleotides targeting bovine Nrf2 (Sigma-Aldrich Co., St. Louis, MO, USA) and control siRNA (Thermo Fisher Scientific, Waltham, MA, USA) were transfected into BAECs using Lipofectamine RNAiMAX (Thermo Fisher Scientific), according to the manufacturer's protocol. Briefly, both Nrf2 siRNA and control siRNA were diluted in Opti-MEM, and then diluted Lipofectamine RNAiMAX was added. The transfection mixture was then incubated at room temperature for 20 min. When the BAECs reached 30% - 50% confluency, the culture medium was replaced with FBS-free, and the transfection mixture was

added to each well. The final siRNA concentration was 20 nM.

2.5. Measurement of ATP

Intracellular ATP levels were measured using an Intracellular ATP assay kit v2 (TOYO B-Net Co., Ltd., Tokyo, Japan). Briefly, BAECs in 96-well plates were treated with EPS (10, 50, and 100 μM). After 24 h of incubation, aliquots of the cell extracts were sampled to determine the ATP concentration. The assay was performed according to the manufacturer's instructions.

2.6. Other Procedures

Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard.

3. Statistical Analysis of Data

All experiments were performed independently at least thrice. Data were combined and expressed as mean ± S.D. Statistical significance was determined using the Student's t-test after analysis of variance or two-way analysis of variance (ANOVA) with Tukey's *post-hoc* test. Statistical significance was set at $P < 0.05$.

4. Results

4.1. Effect of EPS on MTS Reduction Activity and Viability in BAECs

We first evaluated MTS reduction activity using an MTS assay with EPS-induced BAECs. The MTS assay was performed in cells treated and not treated with EPS (50 and 100 μM) and incubated for 24 h. Figure 1(a) shows that EPS increased MTS reduction activity by 2-fold at 50 and 100 μM. In contrast, EPS at all tested

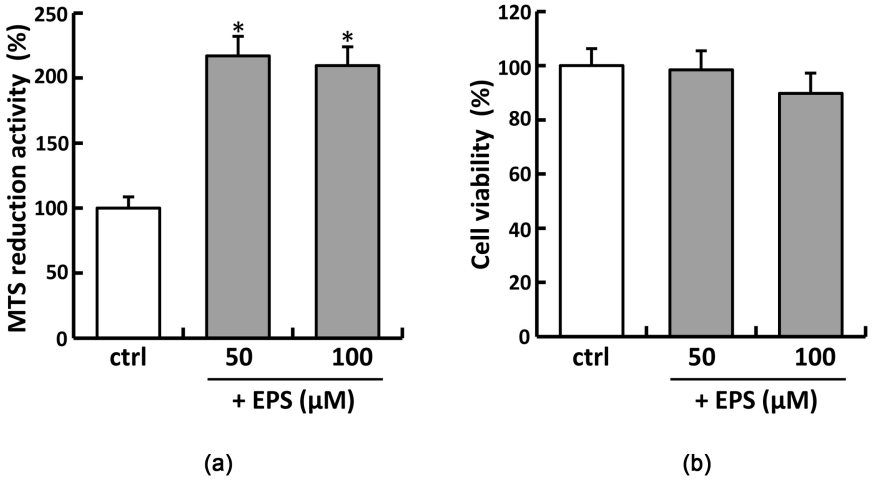

Figure 1. Effect of EPS on MTS reduction activity and viability in BAECs. BAECs were treated with EPS (50 and 100 μM) for 24 h. (a) MTS reduction activity was estimated using MTS assay. (b) Cell viability was assessed by measuring the acid phosphatase activity. Values represent the mean ± SD of six experiments. *Significant difference compared with the control value ($P < 0.05$).

conditions had no effect on acid phosphatase activity in the BAECs (Figure 1(b)). Acid phosphatase activity, an accurate indicator of the number of endothelial cells in culture, was assayed using the method described by Connolly *et al.* [20]. These results indicated that the increase in MTS reduction activity by EPS was not dependent on cell viability.

4.2. Effect of Aldose Reductase Inhibitor and Nrf2 Activator on EPS-Induced MTS Reduction Activity in BAECs

We next investigated the effect of an aldose reductase inhibitor on MTS reduction in BAECs. MTS reduction activity was measured using alrestatin and sorbinil, which have an aldose reductase inhibitory activity similar to that of EPS. Unlike the results with EPS, the same aldose reductase inhibitors, alrestatin (50 μM) and sorbinil (50 μM), had no effect on MTS reduction activity (Figure 2(a)). On the other hand, as shown in Figure 2(b), sulforaphane (5 μM) (which is known to have an Nrf2 activating effect) had increased MTS reduction activity, similar to that of EPS. These results suggest that the increase in MTS reduction activity by EPS is not due to aldose reductase inhibition, which is the pharmacological action of EPS, but rather due to the activating action of Nrf2.

4.3. Effect of Nrf2 on EPS-Induced MTS Reduction Activity in BAECs

Next, we examined whether Nrf2 could alter the increase in MTS reduction activity in BAECs treated with 50 μM EPS by Nrf2 knockdown. BAECs were transfected with either control siRNA (siControl) or Nrf2 siRNA (siNrf2). Nrf2 mRNA expression levels in cells transfected with Nrf2 siRNA were reduced by 80% compared to those in control siRNA-transfected cells (data not shown). As shown in Figure 3(a), the increase in MTS reduction activity by EPS treatment was inhibited by knockdown of Nrf2 expression using siRNA. These results

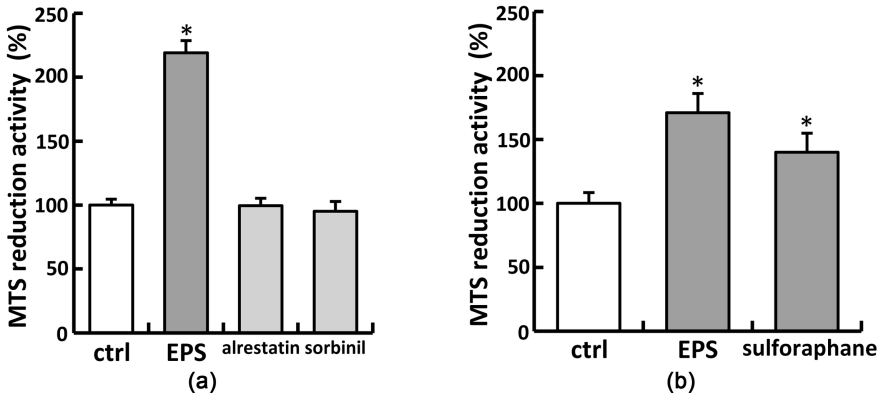

Figure 2. Effects of aldose reductase inhibitor and Nrf2 activator on EPS-induced MTS reduction activity in BAECs. BAECs were treated with EPS (50 μM) and aldose reductase inhibitors [(a); alrestatin (50 μM), sorbinil (50 μM)], and Nrf2 activator [(b); sulforaphane (5 μM)] for 24 h. MTS reduction activity was estimated using the MTS assay. Values represent the mean ± SD of six experiments. *Significant difference compared with the control value ($P < 0.05$).

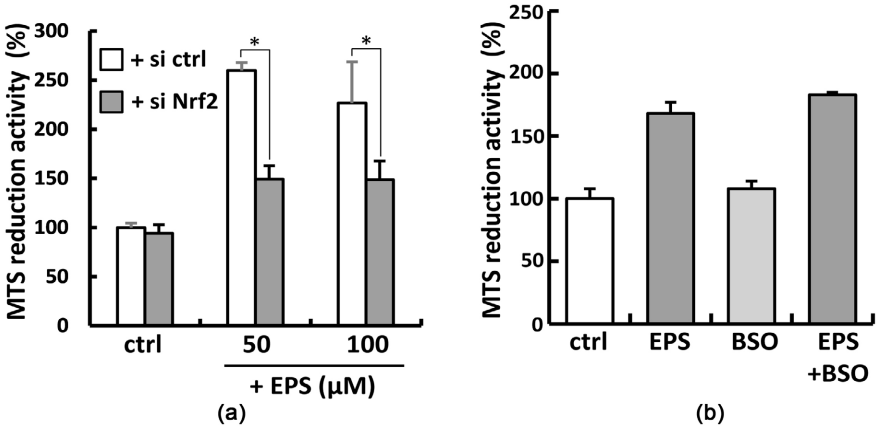

Figure 3. Effect of Nrf2 on EPS-induced MTS reduction activity in BAECs. (a) BAECs were transfected with control siRNA (si ctrl) or Nrf2 siRNA (si Nrf2) and were treated with EPS (50 and 100 μM) for 24 h. (b) BAECs were pretreated with BSO (1 mM) for 16 h. Subsequently, the cells were treated with EPS (50 μM) for 24 h. MTS reduction activity was estimated by MTS assay. Values are means ± SD of four experiments. *Significant difference ($P < 0.05$).

suggest that EPS increases MTS reduction activity via the activation of Nrf2 in BAECs.

Some studies have reported that Nrf2 plays a pivotal role in inducing the expression of genes encoding detoxifying and defensive proteins, including GCL, by binding to the antioxidant response element (ARE) [21] [22] [23]. We also reported that EPS increases GSH levels in BAECs in association with the Nrf2 pathway [19]. Although we expected that GSH would contribute to EPS-induced MTS reduction activity, L-buthionine-(S,R)-sulfoximine (BSO), a GSH-depleting agent, did not affect the EPS-induced MTS reduction activity (**Figure 3(b)**).

4.4. Effect of EPS on ATP Production in BAECs

The role of the Nrf2 pathway in the maintenance of mitochondrial function has recently been elucidated. We evaluated intracellular ATP levels in BAECs treated with EPS (10, 50, and 100 μM) and incubated for 24 h. **Figure 4** shows that EPS increased ATP levels by 1.3-fold at 50 μM.

4.5. Effect of EPS on Electron Transfer Chain in BAECs

The electron transfer chain contains transmembrane protein complexes (I-IV) and is located in the inner membrane. These complexes form the basis of ATP production via oxidative phosphorylation. We examined whether electron transfer chain inhibitors affected the EPS-induced MTS reduction activity in BAECs. As shown in **Figure 5(a)** and **Figure 5(b)**, rotenone (5 μM) and antimycin A (10 μM) suppressed EPS-induced MTS reduction activity.

4.6. Effect of EPS on MTS Reduction Activity in Schwann Cells and NRK-52E Cells

Finally, we examined whether the EPS-induced increase in MTS reduction activity

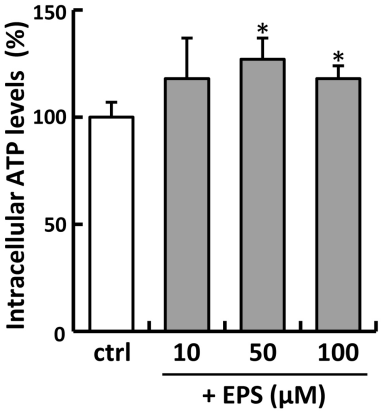

Figure 4. Effect of EPS on ATP production in BAECs. (a) BAECs were treated with EPS (10, 50, and 100 μM) for 24 h. ATP levels were measured by the Intracellular ATP assay kit. Values are means ± SD of three experiments. *Significant difference from control value ($P < 0.05$).

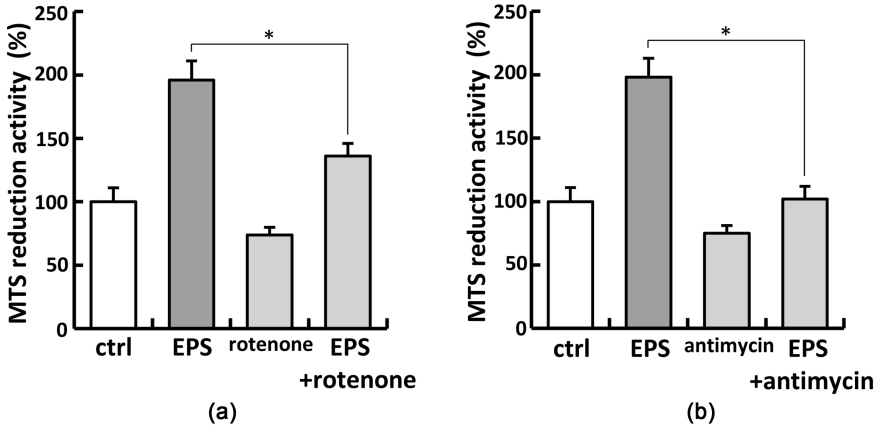

Figure 5. Effects of electron transport chain inhibitors on EPS-induced MTS reduction activity in BAECs. BAECs were treated with EPS (50 μM) and electron transfer chain inhibitors [(a); rotenone (5 μM), (b); antimycin A (10 μM)] for 24 h. MTS reduction activity was estimated using the MTS assay. Values represent the mean ± SD of six experiments. *Significant difference ($P < 0.05$).

is a phenomenon that also occurs in other tissue cell lines. The results showed that in Schwann cells and NRK-52E cells, the increase in MTS reduction activity by EPS was slight, unlike the results obtained using BAECs (**Figure 6(a)** and **Figure 6(b)**).

5. Discussion

The MTS assay is a widely used method for measuring the number of viable cells. However, in some cases, the results may reflect mitochondrial status without reflecting viability. In fact, in the MTT assay (which is similar to the MTS assay), the mitochondrial uncoupler dicumarol has been reported to disrupt MTT assay results [24]. The present study shows that EPS increases MTS reduction activity, which is the principal mechanism of the MTS assay. Furthermore,

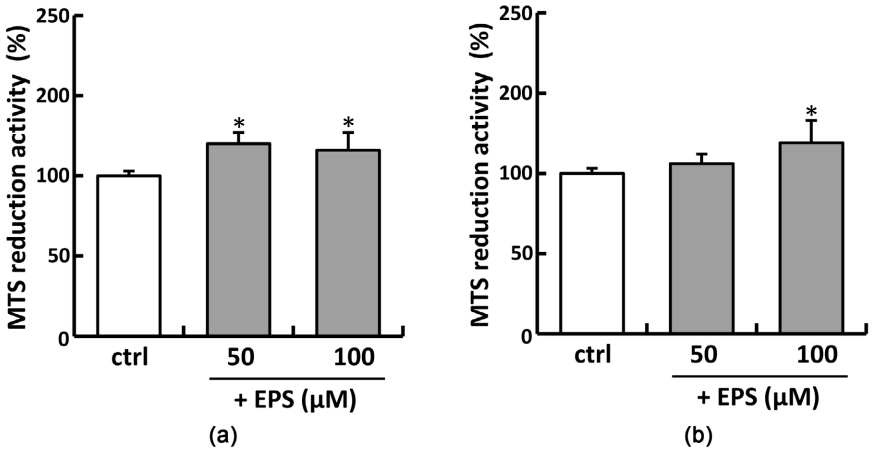

Figure 6. Effect of EPS on MTS reduction activity in Schwann cells and NRK-52E cells. Cells were treated with EPS (50 and 100 μM) for 24 h. MTS reduction activity was estimated by MTS assay in rat Schwann cells (a) and rat NRK-52E cells (b). Values represent the mean ± SD of six experiments. *Significant difference compared with the control value ($P < 0.05$).

the results that EPS increases ATP production and that electron transfer chain inhibitors suppress EPS-induced MTS reduction activity suggest that EPS may activate the mitochondrial status.

Mitochondria are the major intracellular organelles involved in ATP production as well as in the production of ROS that damage cells at a rate of 1% - 5% of the consumed oxygen [25]. Mitochondrial ROS directly causes cell injury, leading to aging, atherosclerosis, diabetes mellitus, and mitochondrial diseases. Mitochondrial diseases are a group of disorders that are caused by mitochondrial dysfunction. There have been few epidemiological studies on mitochondrial disease in the world. However, the disease may occur in approximately 1 in 5000 people regardless of age or form of inheritance. Since mitochondrial DNA mutations in all diabetes patients are reported to be approximately 1% [26] [27], the number of potential disease patients with mitochondrial disorders is probably larger than estimated. The pathology of mitochondrial disorders is extremely varied because the role of mitochondria in maintaining vital functions is closely related to energy metabolism, oxidative stress, apoptosis, and carcinogenesis [28] [29]. Particularly in cases where the disease occurs in childhood, the symptoms vary widely and there is a high risk of serious or fatal consequences. Currently, there is no effective treatment for this condition. Furthermore, mitochondrial dysfunction has been shown to cause not only mitochondrial diseases, but also neurodegenerative diseases, heart disease, kidney disease, diabetes, and other diseases. Our present study indicates the possibility that it may be used to inhibit the onset and progression of many diseases.

Recently, it was reported that 4-(2,4-difluorophenyl)-2-(1H-indole-3-yl)-4-oxobutanoic acid (MA-5) increased cellular ATP levels, rescued mitochondrial disease fibroblasts, and prolonged the life span of a disease mouse model [30]. Increased ATP levels by enhancing the production ability may be beneficial for

the treatment of mitochondrial diseases. Nrf2 is a key transcription factor that plays a central role in regulating the expression of proteins with cytoprotective functions [31]. There is an interest in the direct involvement of Nrf2 in modulating mitochondrial function [32] [33] [34]. Holmström *et al.* showed that ATP levels were decreased in cells and mitochondria isolated from Nrf2-knockout mice, while they were increased in Keap1-knockout and Keap1-knockdown mice [35]. These results mean that the activation of Nrf2 is involved in increasing ATP levels. Sulforaphane, a compound present in broccoli sprouts, is a potent Nrf2 activator [36]. In the present study, sulforaphane increased MTS reduction activity, similar to that of EPS. This indicates that Nrff2 may influence mitochondrial status. However, although we have reported that EPS activates Nrf2 and promotes GSH biosynthesis in Schwann cells [37], a sufficient increase in MTS reduction activity was not observed in neither Schwann nor renal cells (NRK-52E), the latter of which is known to express of Nrf2 [37]. These results indicate that there may be an unknown factor that attenuates the effect of Nrf2, which is involved in the increase in MTS inhibitory activity. In recent studies, sulforaphane prevented the decline in ATP production and decreased the activity of electron transfer chain components (complexes I, II, and IV) [38] [39]. Similarly, quercetin, another Nrf2 activator, also prevented the decrease in ATP production [40] [41]. However, unlike our present results, most of these reports have shown that Nrf2 activators prevent the decrease in ATP production. Additionally, few reports have shown that they increase ATP production under normal conditions.

The electron transfer chain contains transmembrane protein complexes (I-IV) and resides in the inner membrane. These complexes form the basis of ATP generation via oxidative phosphorylation [42]. In the present study, the increase in EPS-induced MTS reduction activity was inhibited by rotenone (an inhibitor of complex I) and antimycin A (an inhibitor of complex III). These findings suggest that EPS increases MTS reduction activity via its involvement in the electron transfer chain. However, the mRNA expression levels of complexes I-III were unaffected by EPS (data not shown). Further studies are needed to clarify the mechanism underlying EPS-induced MTS reduction.

Drug repurposing is a new strategy in drug discovery and development that has emerged as a new treatment for diseases [43]. This strategy involves a comprehensive molecular-level examination of the pharmacological effects of existing drugs that have already been clinically proven safe and pharmacokinetic and have been approved for use; the results are then employed in the development of new drugs. These results can also be applied to the development of existing drugs for the treatment of other diseases. EPS is currently the only aldose reductase inhibitor available for the treatment of diabetic neuropathy [44]. Under normal dosage, EPS is administered orally at a dose of 50 mg three times daily; a single oral dose of 50 mg results in a plasma EPS concentration of 3.9 mg/ml (12 mM) at 1 h (package insert). In this study, the effect of near plasma EPS concentrations on BAECs was demonstrated. Interestingly, other aldose reductase inhi-

bitors, alrestatin and sorbinil failed to increase MTS reduction activity. No significant dose-dependent effects on aldose reductase activity were observed at any of the tested EPS concentrations (data not shown). These results suggest that the inhibition of aldose reductase does not contribute to the ability of EPS to increase MTS reductive activity.

Our findings led us to propose that targeting the regulation of ATP production by EPS is a promising therapeutic approach for mitochondrial disorders.

6. Conclusion

This study could lead to breakthroughs in drug discovery and development. In this study, we demonstrated that EPS increased MTS reduction activity and ATP production, suggesting the beneficial effects of EPS. Therapeutic administration of EPS may be a useful new strategy to ameliorate not only diabetes but also diverse diseases related to mitochondrial disorders, such as neurodegenerative, heart, kidney, and other diseases.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Vistica, D.T., Skehan, P., Skudiero, D., Monks, A., Pittman, A. and Boyd, M. (1991) Tetrazolium-Based Assays for Cellular Viability: A Critical Examination of Selected Parameters Affecting Formazan Production. *Cancer Research*, **51**, 2515-2520.

[2] Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H. and Boyd, M.R. (1988) Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines. *Cancer Research*, **48**, 589-601.

[3] Goodwin, C.J., Holt, S.J., Downes, S. and Marshall, N.J. (1995) Microculture Tetrazolium Assays: A Comparison between Two New Tetrazolium Salts, XTT and MTS. *Journal of Immunol Methods*, **179**, 95-103. https://doi.org/10.1016/0022-1759(94)00277-4

[4] Berg, K., Zhai, L., Chen, M., Kharazmi, A. and Owen, T.C. (1994) The Use of a Water Soluble Formazan Complex to Quantitate the Cell Number and Mitochondrial Function of Leishmania Major Promastigotes. *Parasitology Research*, **80**, 235-239. https://doi.org/10.1007/bf00932680

[5] Tominaga, H., Ishiyama, M., Ohseto, F., Sasamoto, K., Hamamoto, T., Suzuki, K. and Watanabe, M. (1999) A Water-Soluble Tetrazolium Salt Useful for Colorimetric Cell Viability Assay. *Analytical Communications*, **36**, 47-50. https://doi.org/10.1039/A809656B

[6] Rai, Y., Pathak, R., Kumari, N., Sah, D.K., Pandey, S., Kalra, N., Soni, R., Dwarakanath, B.S. and Bhatt, A.N. (2018) Mitochondrial Biogenesis and Metabolic Hyperactivation Limits the Application of MTT Assay in the Estimation of Radiation Induced Growth Inhibition. *Scientific Reports*, **8**, Article No. 1531. https://doi.org/10.1038/s41598-018-19930-w

[7] Javadov, S., Kozlov, A.V. and Camara, A.K.S. (2020) Mitochondria in Health and

Diseases. *Cells*, **9**, Article 1177. https://doi.org/10.3390/cells9051177

[8] Sullivan, L.B. and Chandel, N.S. (2014) Mitochondrial Reactive Oxygen Species and Cancer. *Cancer & Metabolism*, **2**, Article No. 17. https://doi.org/10.1186/2049-3002-2-17

[9] Mori, M.P., Penjweini, R., Knutson, J.R., Wang, P.Y. and Hwang, P.M. (2022) Mitochondria and Oxygen Homeostasis. *FEBS Journal*, **289**, 6959-6968. https://doi.org/10.1111/febs.16115

[10] Li, X., Fang, P., Mai, J., *et al.* (2013) Targeting Mitochondrial Reactive Oxygen Species as Novel Therapy for Inflammatory Diseases and Cancers. *Journal of Hematology & Oncology*, **6**, Article No. 19. https://doi.org/10.1186/1756-8722-6-19

[11] Mikhed, Y., Daiber, A. and Steven, S. (2015) Mitochondrial Oxidative Stress, Mitochondrial DNA Damage and Their Role in Age-Related Vascular Dysfunction. *International Journal of Molecular Sciences*, **16**, 15918-15953. https://doi.org/10.3390/ijms160715918

[12] Nowak, W.N., Deng, J., Ruan, X.Z. and Xu, Q. (2017) Reactive Oxygen Species Generation and Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **7**, e41-e52. https://doi.org/10.1161/atvbaha.117.309228

[13] Hayashi, G. and Cortopassi, G. (2015) Oxidative Stress in Inherited Mitochondrial Diseases. *Free Radical Biology and Medicine*, **88**, 10-17. https://doi.org/10.1016/j.freeradbiomed.2015.05.039

[14] Ramirez, M.A. and Borja, N.L. (2008) Epalrestat: An Aldose Reductase Inhibitor for the Treatment of Diabetic Neuropathy. *Pharmacotherapy*, **28**, 646-655. https://doi.org/10.1592/phco.28.5.646

[15] Ishida, T., In, Y., Inoue, M., Ueno, Y., Tanaka, C. and Hamanaka, N. (1989) Structural Elucidation of Epalrestat(ONO-2235), a Potent Aldose Reductase Inhibitor, and Isomerization of Its Double Bonds. *Tetrahedron Letters*, **30**, 959-962. https://doi.org/10.1016/S0040-4039(00)95290-0

[16] Yang, B.B., Hong, Z.W., Zhang, Z., Yu, W., Song, T., Zhu, L.L., Jiang, H.S., Chen, G.T., Chen, Y. and Dai, Y.T. (2019) Epalrestat, an Aldose Reductase Inhibitor, Restores Erectile Function in Streptozocin-Induced Diabetic Rats. *International Journal of Impotence Research*, **31**, 97-104. https://doi.org/10.1089/jop.2016.0103

[17] Steele, J.W., Faulds, D. and Goa, K.L. (1993) Epalrestat. A Review of Its Pharmacology, and Therapeutic Potential in Late-Onset Complications of Diabetes Mellitus. *Drugs & Aging*, **3**, 532-555. https://doi.org/10.2165/00002512-199303060-00007

[18] Sato, K., Yama, K., Murao, Y., Tatsunami, R. and Tampo, Y. (2013) Epalrestat Increases Intracellular Glutathione Levels in Schwann Cells through Transcription Regulation. *Redox Biology*, **2**, 15-21. https://doi.org/10.1016/j.redox.2013.11.003

[19] Yama, K., Sato, K., Abe, N., Murao, Y., Tatsunami, R. and Tampo, Y. (2015) Epalrestat Increases Glutathione, Thioredoxin, and Heme Oxygenase-1 by Stimulating Nrf2 Pathway in Endothelial Cells. *Redox Biology*, **4**, 87-96. https://doi.org/10.1016/j.redox.2014.12.002

[20] Connolly, D.T., Knight, M.B., Harakas, N.K., Wittwer, A.J. and Feder, J. (1986) Determination of the Number of Endothelial Cells in Culture Using an Acid Phosphatase Assay. *Analytical Biochemistry*, **152**, 136-140. https://doi.org/10.1016/0003-2697(86)90131-4

[21] Kensler, T.W., Wakabayashi, N. and Biswal, S. (2007) Cell Survival Responses to Environmental Stresses via the Keap1-Nrf2-ARE Pathway. *Annual Review of Pharmacology and Toxicology*, **47**, 89-116. https://doi.org/10.1146/annurev.pharmtox.46.120604.141046

[22] Kalyanaraman, B. (2013) Teaching the Basics of Redox Biology to Medical and Graduate Students: Oxidants, Antioxidants and Disease Mechanisms. *Redox Biology*, **1**, 244-257. https://doi.org/10.1016/j.redox.2013.01.014

[23] Kansanen, E., Kuosmanen, M., Leinonen, H. and Levonen, A.L. (2013) The Keap1-Nrf2 Pathway: Mechanisms of Activation and Dysregulation in Cancer. *Redox Biology*, **1**, 45-49. https://doi.org/10.1016/j.redox.2012.10.001

[24] Collier, A.C. and Pritsos, C.A. (2003) The Mitochondrial Uncoupler Dicumarol Disrupts the MTT Assay. *Biochemical Pharmacology*, **66**, 281-287. https://doi.org/10.1016/S0006-2952(03)00240-5

[25] Das, K. and Roychoudhury, A. (2014) Reactive Oxygen Species (ROS) and Response of Antioxidants as ROS-Scavengers during Environmental Stress in Plants. *Frontiers in Environmental Science*, **2**, Article 53. https://doi.org/10.3389/fenvs.2014.00053

[26] Kadowaki, T., Kadowaki, H., Mori, Y., *et al.* (1994) A Subtype of Diabetes Mellitus Associated with a Mutation of Mitochondrial DNA. *The New England Journal of Medicine*, **330**, 962-968. https://doi.org/10.1056/nejm199404073301403

[27] Murphy, R., Turnbull, D.M., Walker, M. and Hattersleyet, A.T. (2008) Clinical Features, Diagnosis and Management of Maternally Inherited Diabetes and Deafness (MIDD) Associated with the 3243A>G Mitochondrial Point Mutation. *Diabetic Medicine*, **25**, 383-399. https://doi.org/10.1111/j.1464-5491.2008.02359.x

[28] Birch-Machin, M.A. (2006) The Role of Mitochondria in Ageing and Carcinogenesis. *Clinical and Experimental Dermatology*, **31**, 548-552. https://doi.org/10.1111/j.1365-2230.2006.02161.x

[29] Seo, A.Y., Joseph, A.M., Dutta, D., Hwang, J.C., Aris, J.P. and Leeuwenburgh, C. (2010) New Insights into the Role of Mitochondria in Aging: Mitochondrial Dynamics and More. *Journal of Cell Science*, **123**, 2533-2542. https://doi.org/10.1242/jcs.070490

[30] Matsuhashi, T., Sato, T., Kanno, S.I., *et al.* (2017) Mitochonic Acid 5 (MA-5) Facilitates ATP Synthase Oligomerization and Cell Survival in Various Mitochondrial Diseases. *EBioMedicine*, **20**, 27-38. https://doi.org/10.1016/j.ebiom.2017.05.016

[31] Tonelli, C., Chio, I.I.C. and Tuveson, D.A. (2018) Transcriptional Regulation by Nrf2. *Antioxidant & Redox Signaling*, **29**, 1727-1745. https://doi.org/10.1089/ars.2017.7342

[32] Dinkova-Kostova, A.T. and Abramov, A.Y. (2015) The Emerging Role of Nrf2 in Mitochondrial Function. *Free Radical Biology and Medicine*, **88**, 179-188. https://doi.org/10.1016/j.freeradbiomed.2015.04.036

[33] Esteras, N., Dinkova-Kostova, A.T. and Abramov, A.Y. (2016) Nrf2 Activation in the Treatment of Neurodegenerative Diseases: A Focus on Its Role in Mitochondrial Bioenergetics and Function. *Biological Chemistry*, **397**, 383-400. https://doi.org/10.1515/hsz-2015-0295

[34] Ludtmann, M.H., Angelova, P.R., Zhang, Y., Abramov, A.Y. and Dinkova-Kostova, A.T. (2014) Nrf2 Affects the Efficiency of Mitochondrial Fatty Acid Oxidation. *Biochemical Journal*, **457**, 415-424. https://doi.org/10.1042/bj20130863

[35] Holmström, K.M., Kostov, R.V. and Dinkova-Kostova, A.T. (2016) The Multifaceted Role of Nrf2 in Mitochondrial Function. *Current Opinion Toxicology*, **1**, 80-91. https://doi.org/10.1016/j.cotox.2016.10.002

[36] Dinkova-Kostova, A.T., Fahey, J.W., Kostov, R.V. and Kensler, T.W. (2017) KEAP1 and Done? Targeting the NRF2 Pathway with Sulforaphane. *Trends in Food Science & Technology*, **69**, 257-269. https://doi.org/10.1016/j.tifs.2017.02.002

[37] Pan, J., Shen, F., Tian, K., Wang, M., Xi, Y., Li, J. and Huang, Z. (2019) Triptolide Induces Oxidative Damage in NRK-52E Cells through Facilitating Nrf2 Degradation by Ubiquitination via the GSK-3β/Fyn Pathway. *Toxicology in Vitro*. **58**, 187-194. https://doi.org/10.1016/j.tiv.2019.03.032

[38] Jardim, F.R., Almeida, F.J.S., Luckachaki, M.D. and Oliveira, M.R. (2020) Effects of Sulforaphane on Brain Mitochondria: Mechanistic View and Future Directions. *Journal of Zhejiang University Science B*, **21**, 263-279. https://doi.org/10.1631/jzus.b1900614

[39] Carrasco-Pozo, C., Tan, K.N., Gotteland, M. and Borges, K. (2017) Sulforaphane Protects against High Cholesterol-Induced Mitochondrial Bioenergetics Impairments, Inflammation, and Oxidative Stress and Preserves Pancreatic β-Cells Function. *Oxidative Medicine and Cell Longevity*, **2017**, Article ID: 3839756. https://doi.org/10.1155/2017/3839756

[40] Qiu, L., Luo, Y. and Chen, X. (2018) Quercetin Attenuates Mitochondrial Dysfunction and Biogenesis via Upregulated AMPK/SIRT1 Signaling Pathway in OA Rats. *Biomedicine & Pharmacotherapy*, **103**, 1585-1591. https://doi.org/10.1016/j.biopha.2018.05.003

[41] Li, X., Wang, H., Gao, Y., Li, L., Tang, C., Wen, G., Zhou, Y., Zhou, M., Mao, L. and Fan, Y. (2016) Protective Effects of Quercetin on Mitochondrial Biogenesis in Experimental Traumatic Brain Injury via the Nrf2 Signaling Pathway. *PLOS ONE*, **11**, e0164237. https://doi.org/10.1371/journal.pone.0164237

[42] Zhao, R.Z., Jiang, S., Zhang, L. and Yu, Z.B. (2019) Mitochondrial Electron Transport Chain, ROS Generation and Uncoupling (Review). *International Journal of Molecular Medicine*, **44**, 3-15. https://doi.org/10.3892/ijmm.2019.4188

[43] Sahoo, B.M., Ravi, Kumar, B.V.V., Sruti, J., Mahapatra, M.K., Banik, B.K. and Borah, P. (2021) Drug Repurposing Strategy (DRS): Emerging Approach to Identify Potential Therapeutics for Treatment of Novel Coronavirus Infection. *Frontiers Molecular Biosciences*, **8**, Article 628144. https://doi.org/10.3389/fmolb.2021.628144

[44] Hotta, N., Akanuma, Y., Kawamori, R., Matsuoka, K., Oka, Y., Shichiri, M., Toyota, T., Nakashima, M., Yoshimura, I., Sakamoto, N. and Shigeta, Y. (2006) Long-Term Clinical Effects of Epalrestat, an Aldose Reductase Inhibitor, on Diabetic Peripheral Neuropathy: The 3-Year, Multicenter, Comparative Aldose Reductase Inhibitor-Diabetes Complications Trial. *Diabetes Care*, **29**, 1538-1544. https://doi.org/10.2337/dc05-2370