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Protective effect of dihydropteridine reductase against oxidative stress is abolished with A278C mutation*

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Abstract: Objective: To evaluate the antioxidation of dihydrobiopterin reductase and to explore the effect of A278C mutation of the quinoid dihydropteridine reductase (QDPR) gene on its antioxidant activity. Methods: First, plasmids with different genes (wild and mutant QDPR) were constructed. After gene sequencing, they were transfected into human kidney cells (HEK293T). Then, the intracellular production of reactive oxygen species (ROS) and tetrahydrobiopterin (BH4) was detected after cells were harvested. Activations of nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4), glutathione peroxidase 3 (GPX3), and superoxide dismutase 1 (SOD1) were analyzed to observe the oxidative stress after transfection. The expression of the neuronal nitric oxide synthase (nNOS) gene was analyzed by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). We also detected the activation of transforming growth factor β1 (TGF-β1) by enzyme-linked immunosorbent assay (ELISA) to observe the connection of TGF-β1 and oxidative stress. Results: The exogenous wild-type QDPR significantly decreased the expression of nNOS, NOX4, and TGF-β1 and induced the expression of SOD1 and GPX3, but the mutated QDPR lost this function and resulted in excessive ROS production. Our data also suggested that the influence on the level of BH4 had no significant difference between mutated and the wild-type QDPR transfection. Conclusions: Wild-type QDPR played an important role in protecting against oxidative stress, but mutant QDPR failed to have these beneficial effects.

Key words: Dihydropteridine reductase; Transforming growth factor β1 (TGF-β1); Nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4); Superoxide dismutase 1 (SOD1); Glutathione peroxidase 3 (GPX3); Oxidative stress

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1 Introduction

In terms of diabetic nephropathy (DN), several mechanisms have been postulated that may combine to produce pathologic correlates of DN, such as advanced glycation end products (AGEs)-induced increased oxidative stress, activated production of cytokines, hyperglycemia-induced renal hyper filtration, chemokines, and different inflammatory signals related to renal injury (Bhattacharjee *et al*., 2016). Among various factors, oxidative stress has been considered to play an important role in the underlying onset and propagation of DN.

Oxidative stress is a condition in which the prooxidant-antioxidant balance in the cell is disturbed and is characterized by excessive production of reactive oxygen species (ROS) (Brownlee, 2001; Pan *et al.*, 2010). ROS is a term for a collection of intermediates formed during oxidative metabolism, including superoxide anion (O_2^-), hydroxyl radicals, oxygen

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radicals, non-radical reactive oxygen derivatives, and hydrogen peroxide. Under certain pathological conditions, increased generation of ROS or antioxidant system depletion will generate enhanced ROS activity and oxidative stress, which finally result in tissue damage. A variety of systemic diseases, such as metabolic syndrome, diabetes mellitus, infections, and hypertension, can induce oxidative stress in kidney tissue. Therefore, ROS has been proposed as a potential pathogenic factor of DN, neuronal damage, and cardiac dysfunction (Dobashi *et al.*, 1991). Scavenging of ROS appears to protect tissues against oxygen radical toxicity in certain disease processes (Horie *et al.*, 1997; Schnackenberg and Wilcox, 2001; Shah *et al.*, 2013).

Pan *et al.* (2010) found that increased oxidative stress induces up-regulation of transforming growth factor β1 (TGF-β1), leading to injuries of the vasculature, glomeruli, and tubular interstitial tissue. Accumulating evidence has shown that malondialdehyde (MDA) and dihydronicotinamide adenine dinuclectide phosphate (NADPH) oxidase are increased in the DN state (Wang *et al.*, 2012). Antioxidant enzymes, such as glutathione peroxidase 3 (GPX3) and superoxide dismutase 1 (SOD1), which are the primary antioxidant enzymes involved in scavenging of ROS (Kim *et al.*, 2006), exhibit a relatively low expression in the glomerular microvasculature and reduce the bioactivity of NO, resulting in the progress of DN (Zeng *et al.*, 2000; Sharma *et al.*, 2012).

The enzyme quinoid dihydropteridine reductase (QDPR), which possesses an essential thiol group at the active site, maintains and salvages the intracellular pool of tetrahydrobiopterin (BH4) (Thöny *et al.*, 2000). BH4, which is considered as an essential cofactor for the three nitric oxide synthase (NOS) isoforms, can increase nitric oxide (NO) synthesis and scavenge superoxide and peroxynitrite. Our previous research demonstrated that QDPR might mediate the process of DN through regulating the expression of TGF-β1 and NADPH oxidases (Gu *et al.*, 2013). In addition, sequencing of the QDPR gene from Otsuka Long-Evans Tokushima Fatty (OLETF) rats revealed a single substitution (A278C) in the sense strand, resulting in an amino acid change (lysine to threonine) in the corresponding gene product. However, whether QDPR regulates oxidative stress and the point mutation's function has not been determined. In this study, we constructed wild-type and mutant recombinant plasmids to investigate the effect of QDPR on oxidative stress. We also assessed the involvement of point mutation of QDPR in HEK293T cells.

2 Materials and methods

Our group investigated the effect of QDPR on autophagy and oxidative stress from different pathways. It was found that QDPR affected the occurrence of autophagy (Si *et al*., 2017). In this study, we investigated the effect of QDPR on oxidative stress using the similar methods described by Si *et al*. (2017).

2.1 Construction of wild and mutant strains

The wild and mutant complementary DNAs (cDNAs) were extracted from the renal cortex of a normal rat or an OLETF diabetic rat, respectively. QDPR fragments were amplified using forward primer with *Eco*RV enzyme and reverse prime with *Xba*I enzyme. All polymerase chain reactions (PCRs) described here used polymerase following the published protocol (Invitrogen, CA, USA). After being ligated with the pCR2.1-T vector, the fusion fragment containing the wild or mutant QDPR was subcloned into DH5α (Zeng *et al.*, 2000). Therefore, strains with correct QDPR fragments were screened by sequence analysis and then ligated with the vector of pcDNA3.1/V5-His-A to form wild-type recombinant plasmid of QDPR (rQDPR(wt)) and mutant recombinant plasmid of QDPR (rQDPR(mut)).

2.2 Transfection of plasmids

To observe the effect of QDPR on oxidative stress, 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies/GIBCO, Grand Island, NY, USA) at 37 °C, which contained 100 U/ml penicillin and 100 μg/ml streptomycin. The concentration of cells was 2.5×10^5 293T cells per well. Plasmid of rQDPR(wt) or rQDPR(mut) was transfected into the cells following the instructions as described in protocol (Promega, WI, USA). We measured the transfection efficiency using epifluorescence inverted microscopy at 24, 48, and 72 h. After 72 h, 80% of cells could express green fluorescent protein (GFP). rQDPR(wt), rQDPR(mut) and control vector were transfected into the 293T cells to observe the expression of oxidative stress.

2.3 RT-PCR experiment

At the indicated time points, cells were harvested for the extraction of RNA (TRIzol, Invitrogen). Then 2 μg of total RNA was reversely transcribed into cDNA according to the manufacturer's instructions (Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, Fermentas). To measure mRNA expression of neuronal NOS (nNOS) and TGF-β1, the following primers were used for nNOS: forward TTGCGACGTCTCCCTCCCCA, reverse GAGAGC AGGAGCCGGGGTGA, and for TGF-β1: forward GAAGTGGATCCACGAGCCCAAG, reverse GCT GCACTTGCAGGAGCGCAC. Both experienced the cycle: initial denaturation at 93 °C for 10 min, denaturation at 94 °C for 30 s, annealing at 36 and 37 °C respectively for 30 s, and elongation at 72 °C for 1 min. PCR fragments were separated on a 1.2% (0.012 g/ml) agarose gel and stained with GoldView. The bands were quantitated by densitometry and analyzed using the ImageJ analysis software. Meanwhile, cyclophilin B was employed as the internal reference.

2.4 ROS measurement

The ROS level was examined first by flow cytometry using an oxidation-sensitive probe as described by Si *et al.* (2017). The cells were cultured as shown in Section 2.2, and 2.5×10^5 cells were incubated in a medium containing 1 mmol/L 2',7'-dichlorofluorescein diacetate (DCFH-DA; Applygen Technologies, Beijing, China) at 37 °C for 30 min for cellular incorporation. Then, we washed the cells twice with phosphate-buffered saline (PBS). Finally, we used an inverted fluorescent microscope (Olympus, Japan) to observe the level of intracellular ROS using 10000 cells per sample.

2.5 Western blot

Protein of transfected cells was extracted with Tris-HCl buffer following the published protocol (Ugolino *et al.*, 2016), and 60 μg protein was subjected to electrophoresis with 12% (0.12 g/ml) sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nylon membrane. The antibodies of rabbit anti-SOD1 (Epitomics, USA), rabbit anti-GPX3 (Epitomics, USA), rabbit anti-nicotinamide adenine dinucleotide phosphate oxidase (NOX4; Epitomics, USA), and mouse anti-V5 (Invitrogen, USA) were used to incubate nylon membranes at 4 °C overnight. After incubation with goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody conjugated with horseradish peroxidase for 1.5 h, membranes were washed by PBS and developed. After reaction with enhanced chemiluminescent (ECL) reagent (PerkinElmer, Waltham, MA, USA) as substrate, the bands were quantitated by densitometry and the signals were detected and analyzed using the ImageJ analysis software and β-actin was used for normalization.

2.6 ELISA assay

Enzyme-linked immunosorbent assay (ELISA) assay was used to detect the production of BH4 and TGF-β1. After 72 h of transfection, cells were collected to detect the degree of BH4 generation, according to the manufacturer's instructions (Antibodies-Online, USA). Simultaneously, the expression of TGF-β1 from culture media was also detected by the ELISA kit (US Reagent Center, Delaware, USA).

2.7 Statistical analysis

For quantitative analysis, data are expressed as mean±standard error of the mean (SEM), and each experiment was performed three times. The mean data were analyzed using Student's *t*-test and a *P* value of <0.05 was considered statistically significant. Statistical analysis used SPSS 16.0 software (IBM Company, Stanford, USA).

3 Results

3.1 Detection of recombinant QDPR in cells

After transfection into HEK293T cells, QDPR protein had a significant elevation in the rQDPR(wt) or rQDPR(mut) group. The empty vector (control) caused no exogenous fusion protein to be detected using V5 antibody (Fig. 1a).

3.2 BH4 generation activated by QDPR

BH4 levels of the groups transfected with control vector, rQDPR(wt), or rQDPR(mut) were examined using an ELISA kit. As expected, BH4 levels in both rQDPR(wt) and rQDPR(mut) groups were significantly up-regulated compared with the control group, suggesting that BH4 production was up-regulated in response to the overexpressed wild-type or mutated QDPR ($P<0.05$; Fig. 1b).

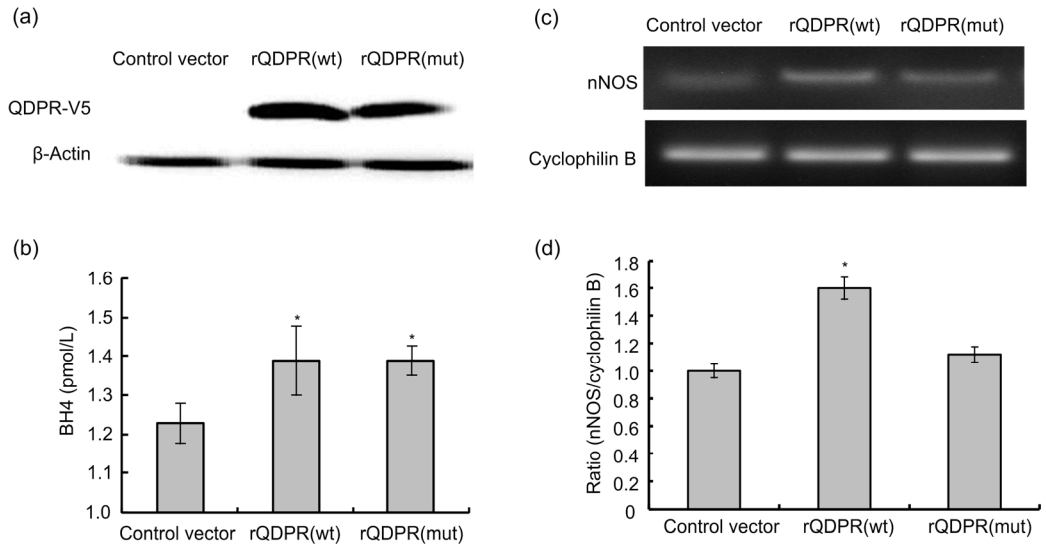

Fig. 1 Fusion protein, BH4, and nNOS levels detected by QDPR overexpression

(a) Bands display expression of fusion protein by Western blotting using V5 antibody; (b) BH4 level in three groups; (c) Expression of nNOS mRNA in cells with overexpressed wild-type and mutated QDPR; (d) Densitometric quantification of nNOS with respect to control. Data are expressed as mean±SEM (n=3). * P<0.05, compared with control vector

3.3 Gene expression of nNOS mediated by overexpression of QDPR

To illustrate the functional importance of BH4 on nNOS, we detected the mRNA level of nNOS in the cells after 72 h of transfection. The expression of nNOS appeared to be higher in the wild-type QDPR group (P<0.05) than in the control, but was reduced in the mutated QDPR group (Figs. 1c and 1d).

3.4 Analysis of ROS generation affected by overexpressed QDPR

We have shown that wild QDPR could regulate the level of nNOS. Next, the production of intracellular ROS was detected in 293T cells after exogenous QDPR was overexpressed in cells and our results suggested that ROS levels were significantly increased in the group transfected with mutated QDPR compared with the control. There was no difference between the wild QDPR group and the control vector group (P>0.05; Fig. 2).

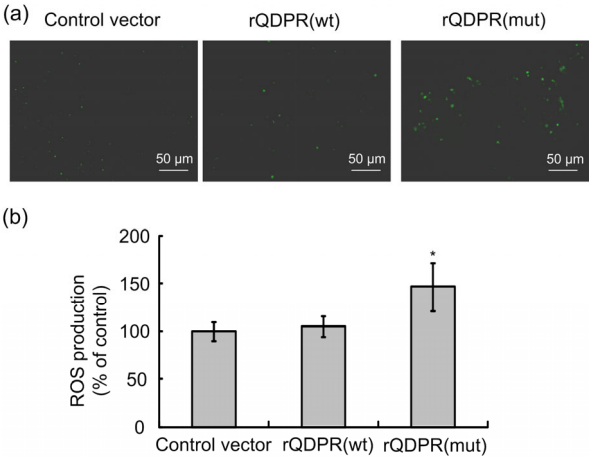

Fig. 2 Effect of QDPR gene on ROS production

(a) ROS generation in 293T cells when concentrations of QDPR were increased for 72 h. (b) Bar graph shows the effects of QDPR on ROS production. Data are expressed as mean±SEM (n=3). * P<0.05, significant difference as compared with control vector

3.5 Expression of NOX4, GPX3, and SOD1 after transfection

Expression of NOX4 in the cells transfected with rQDPR(wt), rQDPR(mut), or the control vector was investigated. The expression of NOX4 in rQDPR(wt) group was significantly decreased compared with that of the control vector group, whereas GPX3 and SOD1 in the rQDPR(wt) group were increased (P<0.05; Fig. 3). Accordingly, the expression of NOX4, GPX3, and SOD1 showed no difference in the rQDPR(mut) group compared with the control vector group (P>0.05).

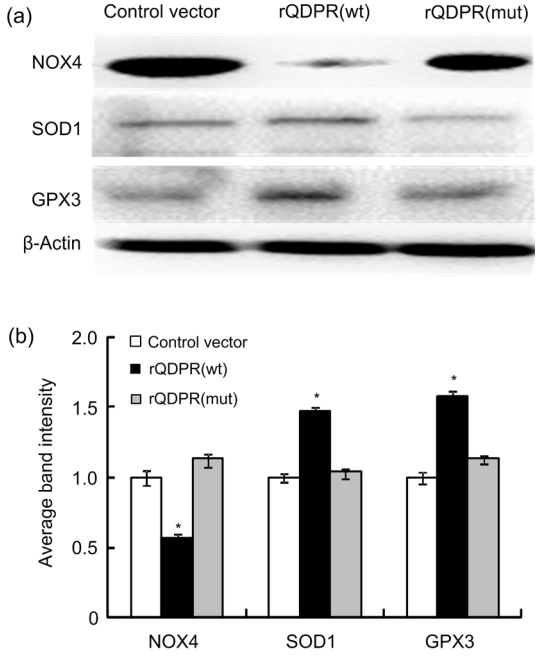

Fig. 3 NOX4, SOD1, and GPX3 levels regulated by QDPR overexpression

(a) NOX4, SOD1, and GPX3 protein levels with QDPR overexpression; (b) Densitometric quantification of Western blotting. Data are expressed as mean±SEM (n=3). $^{*}P$<0.05, significant difference as compared with control vector

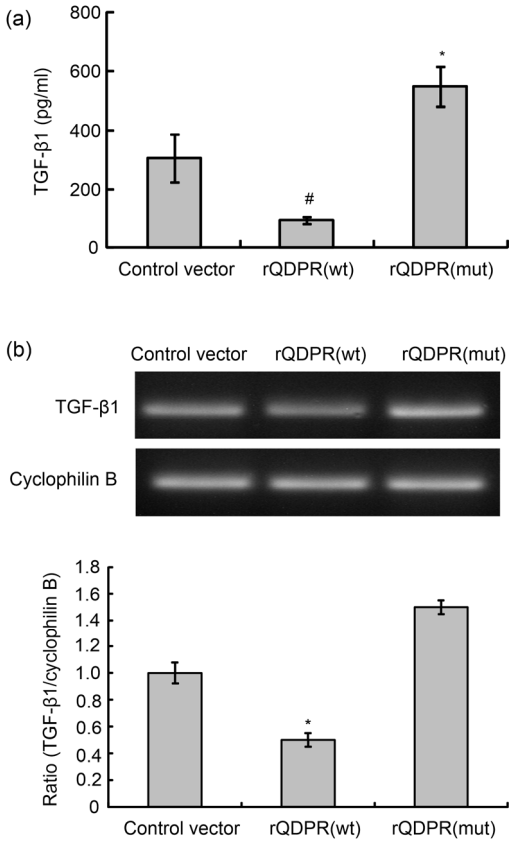

Fig. 4 Effect of wild and mutated QDPR gene on TGF-β1 level

(a) Protein level of TGF-β1 was tested by ELISA assay in the control vector, rQDPR(wt), and rQDPR(mut) groups. (b) Quantitative assessment of TGF-β1 gene level. Data are expressed as mean±SEM (n=3). $^{\#}P$<0.01, $^{*}P$<0.05, compared with control vector

3.6 TGF-β1 expression mediated by QDPR

So far we have shown that ROS could be regulated by overexpressed QDPR. Here we explored the connection of TGF-β1 and QDPR (Fig. 4). As expected, there was decreased expression of TGF-β1 (P<0.01) in the wild-type QDPR group. The data are in accord with our previous results (Gu *et al*., 2013). Moreover, the level of TGF-β1 was significantly up-regulated in the mutated QDPR group compared with the control vector group (P<0.05).

4 Discussion

The results showed that QDPR regulates the levels of BH4, nNOS, ROS and the enzymes associated with oxidative stress. Many studies have proven that oxidative stress plays a major role in most pathogenic pathways of DN. Excessive ROS in kidney tissue can cause renal inflammation, fibrosis, apoptosis, and a series of reactions through mediating a multiple signaling pathways cascade reaction, such as TGF-β1/Smad, p38 mitogen-activated protein kinase (p38MAPK), c-Jun N-terminal kinases (JNK), and nuclear factor κB (NF-κB) signaling, which ultimately leads to DN. Our study has found that overexpression of QDPR reduced the expression of TGF-β1, which regulated many biological processes such as fibrosis, extracellular matrix production, migration, cell proliferation and differentiation of a variety of cell types. However, the relationship between QDPR and oxidative stress is still unclear. Here, we discuss the mechanism by which QDPR might suppress the process of oxidative stress leading to the increase of TGF-β1 expression.

Accumulation of ROS and oxidative damage have been linked to multiple salient features including

cell signaling, proliferation, and cell migration. Levels of oxidative stress are concerned with the balance of ROS generation and their elimination caused by endogenous antioxidants. Therefore, in addition to the increase in ROS production, a large amount of antioxidant consumption and inactivation are also involved in renal tissue oxidative damage, such as SOD1, GPX3, catalase (CAT), and glutathione peroxidase (GSH-Px). We found a marked increase in SOD1 and GPX3 expression and a decrease in NOX4 in HEK293T cells transfected with wild QDPR. This study appears to verify that QDPR exerts a renal protective effect via decreasing oxidative stress. We reason that the inhibition of pro-oxidation by the augmented QDPR gene level promotes the dissipation of ROS and limits oxidative stress. However, there was no significant increase of antioxidant enzymes when QDPR had the A278C mutation. Thus this mutation appears to suppress its antioxidant capability.

Previously, our study has shown that NADPH oxidases (NOX), especially NOX4, increased in OLETF rats with increased TGF-β1 expression (Gu *et al.*, 2013). The isoform of NOX4, which is an important mediator in inducing ROS generation, may contribute to an important function in mesangial cells and is markedly expressed when stimulated by a high glucose level (Oliveira *et al.*, 2003; Uchizono *et al.*, 2006; Newsholme *et al.*, 2012). Additionally, GPX3 and SOD1 represent the most important intracellular antioxidant enzymes, with NADPH oxidases being inhibited by genetic overexpression of GPX3 and SOD1 (Shah *et al.*, 2013). In the high glucose state, activity and expression of antioxidant enzymes are low (Feng *et al.*, 2013), but the mechanism of down-regulation of GPX3 and SOD1 by high glucose is not clear (Yu *et al*., 2015). We have not only found that ROS generation and NOX4 expression were decreased, but also that GPX3 and SOD1 levels were increased after transfection of the wild-type QDPR. Moreover, if QDPR had mutated, these functions were inhibited. It is suggested that there is a relationship between QDPR and oxidative stress.

Nevertheless, QDPR may regulate oxidative stress also in the following manner. QDPR mediates the recycling of BH4 and this is important for three NOS isoforms. If QDPR is absent, the BH4 level may be attenuated. It will result in NOS uncoupling, which generates superoxide production (Ong *et al.*, 2011). BH4 is a cofactor of NOS and it has been reported that nNOS expression is significantly increased in diabetic patients' kidneys (Möllsten *et al.*, 2009). Deficiency in BH4 may allow NOS enzymes to function as a peroxynitrite synthase generating superoxide (Satoh *et al.*, 2008). Furthermore, uncoupling of the NOS enzymes will inhibit NO production (Delgado-Esteban *et al.*, 2002). Overall, ROS can promote BH4 oxidation as inactive metabolites and the uncoupling of endothelial NOS (eNOS), which will generate O_2^- in turn. Moreover, O_2^- can react with NO to further reduce the expression of eNOS through activation of RhoA. Our study indicates that wild-type QDPR appears to reduce oxidative stress by up-regulating BH4 and nNOS involved in the formation of ROS in HEK293 cells. We also found that the mutated QDPR gene significantly increased generation of BH4. Surprisingly, a relatively low level of nNOS was observed in the mutated group. We speculated that mutant QDPR with A278C destroyed the effect of BH4 on nNOS expression, resulting in increased oxidative stress.

5 Conclusions

This study suggests that QDPR, via increasing the expression of nNOS, may promote the production of NO. Therefore, QDPR may have a beneficial effect as a free radical scavenger through regulating protein expression related to intracellular oxidative stress and attenuating ROS production in DN. Therefore, the A278C mutation of the QDPR gene, which results in increased levels of ROS and antioxidant enzymes or decreased products of NADPH oxidases, may play a role in the oxidative burden of the diabetic kidney. We thus deduce that this point mutation can affect modulation of oxidative stress, the mechanism of which needs further exploration. This study has some limitations. For example, we only measured protein levels to assess oxidative stress without measuring their activity.

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Compliance with ethics guidelines

Yan-ting GU, Yan-chun WANG, Hao-jun ZHANG, Ting-ting ZHAO, Si-fan SUN, Hua WANG, Bin ZHU, and Ping LI declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：A278C 位点突变减弱了二氢生物蝶呤还原酶的抗氧化作用

目　的：评估二氢生物蝶呤还原酶（QDPR）的抗氧化作用，并初步探讨 QDPR 基因 A278C 位点突变对其抗氧化作用的影响。

创新点：首次在体外实验中发现 QDPR 有抗氧化作用，且此作用在 A278C 位点突变后减弱。

方　法：我们构建了野生型和突变型 QDPR 质粒，且分别转染至人胚肾 293 细胞中（HEK293T）。实验可分为以下三组：空白质粒对照组、野生型 QDPR 组和突变型 QDPR 组。三天后收集细胞观察活性氧（ROS）和四氢生物蝶呤（BH4）的表达量，使用免疫印迹的方法检测烟酰胺腺嘌呤二核苷酸磷酸氧化酶 4（NOX4）、谷胱甘肽过氧化物酶 3（GPX3）和超氧化物歧化酶 1（SOD1）的蛋白表达水平。用半定量逆转录-聚合酶链反应（RT-PCR）方法分析神经型一氧化氮合成酶（nNOS）基因的表达。用酶联免疫吸附测定（ELISA）试剂盒检测转化生长因子-β1（TGF-β1）的活性。

结　论：本实验中野生型 QDPR 可以显著降低 nNOS、NOX4 和 TGF-β1 的水平，同时提高 SOD1 和 GPX3 表达。但当 QDPR 发生位点突变后没有观察到上述现象，并且突变型会导致 ROS 过量产生。我们的数据还表明，野生型和突变型 QDPR 对 BH4 含量的影响无显著差异。综上所述，QDPR 有抗氧化作用，但 A278C 位点突变后会影响 QDPR 的抗氧化功能。

关键词：二氢生物蝶呤还原酶（QDPR）；转化生长因子-β1（TGF-β1）；烟酰胺腺嘌呤二核苷酸磷酸氧化酶 4（NOX4）；超氧化物歧化酶 1（SOD1）；谷胱甘肽过氧化物酶 3（GPX3）；氧化应激