Interactive Responses of Enkephalin, δ-opioid Receptor and Dopamine Receptor D1/D2 to Hypoxia and/or MPP+ Stress in PC12 Cells

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Research Article

Keywords: Hypoxia, MPP+, δ-opioid receptor, Enkephalin, Prohormone convertases 1 and 2, Dopamine receptor D1 and D2, Parkinson's disease

Posted Date: December 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1167672/v1

Abstract

Hypoxic/ischemic brain injury is a potential etiology of Parkinson's disease (PD). There is evidence suggesting that the up-regulation of enkephalin, an endogenous opioid, in the midbrain may have a compensatory effect against Parkinson's disease (PD) related motor symptoms. To explore the potential mechanism underlying this action, we investigated the effects of hypoxia and MPP^+, a pathological inducer PD, on enkephalin, δ-opioid receptor (DOR, an enkephalin receptor), and prohormone convertases 1 and 2 (PC1/PC2) on in- vitro PD model of PC12 cells. We found that (1) short-term hypoxia could inducing cell protection by up-regulating the level of enkephalin, accompanied by the synergistic up-regulation of δ-opioid receptor (DOR) ; (2) a longer period of hypoxia or MPP^+ insult accelerated the proteolysis of proenkephalin by up-regulating PC1/PC2 which might produce more active enkephalin and thus activating DOR for cell protection; (3) The levels of enkephalin and DOR decreased significantly after a prolonged hypoxia or MPP^+ insult; and (4) a certain degree of hypoxia improved cell viability and enhance the transcription of dopamine D1/D2 receptorby increasing their mRNA level. Our findings suggest that hypoxia may induce an interactive reaction of enkephalin, DOR and dopamine receptor D1/D2, which is potentially beneficial for cell surviving to severe/prolonged hypoxia and PD condition.

Introduction

Parkinson's disease (PD) is one of the most common progressive neurodegenerative disease with the classic quadriad of severe motor symptoms including resting tremor, bradykinesia, rigidity, and impaired postural reflexes. The pathobiology of PD is characterized by the accumulation of eosinophilic cytoplasmic inclusions called Lewy-bodies in neurons, and the loss of dopaminergic neurons in the substantianigra of the midbrain [1, 2]. PD is a multifactorial illness with likely genetic and environmental determinants [3–7]. Indeed, the susceptibility of midbrain dopaminergic neurons to oxidative stress and environmental neurotoxins such as MPP^+ has long been suggested as potential causes of PD [8–10]. Furthermore, there is ample evidence to show that an insufficient blood or oxygen supply to brain, could attenuate neurons' resistance to environmental damage, and even trigger cell death [11–13]. Therefore, hypoxia/ischemia stress is also likely an apathogenic factor that contribute to the development of PD [14–16].

In recent years, our studies and those of others have well documented the neuroprotective effect ofδ-opioid receptor (DOR) [15–23]. There is also evidence suggesting that the up-regulation of DOR and its endogenous ligand, enkephalin, in the midbrain may have a compensatory effect against PD-related motor symptoms [15, 16, 24, 25]. Indeed, an exogenous addition of DOR agonist could effectively attenuates hypoxia and/or MPP^+ induced injury [15, 16]. It seems that cellular DOR system might be impaired by hypoxia and/or MPP^+, while an increased DOR activity is neuroprotective against Parkinsonian injury. However, there is no information available regarding the regulation of DOR and its endogenous ligands in the condition of hypoxia and/or MPP^+ insult. Clarifying this fundamental issue is

important for understanding the mechanisms of DOR protection against Parkinsonian injury and for developing a novel therapy for PD treatment in future.

Enkephalin is a kind of opioid neuropeptides, two forms of enkephalin were revealed in 1975, i.e., the opioid pentapeptides methionine-enkephalin (Met-enkephalin) and leucine-enkephalin (Leu-enkephalin) [26, 27]. They are highly expressed in human nervous system. By immunostaining techniques, the most intense enkephalin staining was found in the globuspallidus, substantianigra, caudate, and putamen [28, 29], suggesting their role in the striatal control of motor and behavioral function [30, 31]. Moreover, recent studies found an up-regulation of endogenous opioid enkephalin in the midbrain as a compensatory mechanism to alleviate PD-related motor symptoms [32–34].

To learn the regulation of DOR system in response to simulative Parkinsonian insults, we asked, in this study, several critical questions: 1) Isthe cellular level of enkephalin changed by hypoxia and/or MPP^+ insult and do the changes vary with the duration of the stress? 2) Is cellular DOR density changed by the same stress? 3) Are their changes parallel with each other or not? and 4)Is there any relevance between the changes in enkephalin and/or DOR and cell viability? In addition, we also detected the changes in dopamine receptor D1/D2 simultaneously since their regulation matters the pathophysiology of PD. Our outcome data show that a mild or short-term stress could up-regulate DOR system, which may reflect a compensative response to the stress, while a severe or prolonged stress significantly decreased the levels of both enkephalin and DOR, accompanying with a major injury to cell viability and the expression of dopamine receptor.

Material And Methods

Chemicals and reagents

Deulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) for cell culture medium, protease inhibitors for Western blot, andMPP^+(1-methyl-4-phenylpyridinium) for simulative Parkinsonian insult were all purchased from Sigma Chemical Co. (St Louis, MO, USA). Anti-opioid receptor antibody was purchased from Chemicon Inc. (Temecula, CA, USA). PC-12 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Anti-enkephalin antibody, Primers of the prohormone convertases 1 and 2 (PC1/PC2) and D1and D2 dopamine receptor (D1/D2) were purchased from Abcam (Cambridge, MA, USA). Cytoscan-LDH cytotoxicity assay kit was purchased from G-Biosciences Inc. (St Louis, MO, USA). TaqMan® Reverse Transcription Kit was purchased from Biosystems Inc. (Foster City, CA). RIPA buffer was from Life Science Research Pierce Biotechnology Inc. (Rockford, IL, USA). Transfer buffer and running buffer for Western blotting were purchased from Boston Bioproducts Inc. (Ashland, MA, USA).

Cell culture and treatments

The PC-12 cells were cultured in DMEM [15], containing 10% FBS. For normoxic control, the cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C. For hypoxic treatment, the cells were cultured

in a hypoxic chamber (Galaxy 48r, New Brunswick, Edison, NJ, USA) at 37°C, the O_2 level was strictly maintained at 0.5% or 1% by continuous nitrogen flushing. To mimic Parkinsonian insult, the cells were exposed to 0.5 mM of MPP^+. For protein and gene expression experiments, the cells were incubated in 60-mm dishes, and for cell viability, the PC-12cells were plated on 96-well plates at a density of 2×10^5 cells/mL.

Cell injury assessment

Cytotoxicity was assessed quantitatively by measuring the lactate dehydrogenase (LDH) activity in the medium using the cytoscan-LDH cytotoxicity assay kit (G-Biosciences Inc., St Louis, Mo, USA) [15, 29, 30]. The cells were treated with the specified compounds and cultured for the required time (including no treatment control). Fifty µl supernatant was transferred to each well of a flat-bottom 96-well enzymatic assay plate and mixed with 50 µl of recombinant substrate mixture, which were then cultured at room temperature and kept out of light for 30 minutes. Then, 50 µl of stop solution was added to terminate the reaction. Finally, LDH assay was determined with a micro flat reader (Tecan Sunrise, Switzerland), and the absorption wavelength was 490 nm.

Western Blotting

The PC12 cells Cultures were washed twice with ice-cold Dulbecco's modified PBS (DPBS) then Cells were ultrasonically homogenized in ice-cold RIPA buffer (25 mM Tris·HCl pH 7.6, 1% SDS, 1% NP-40,150 mM NaCl and 1% sodium deoxycholate) containing a cocktail of proteinase inhibitors (100 mg/mL Phenyl methane sulfonyl fluoride, 100 µg/mL Benzamidine, 1 mg/mL Pepstatin A, 1 mg/mL Leupeptin, 1 mg/mL Aprotinin, 8 mg/mL Calpain I and II and 0.1 mM PMSF). The homogenates were centrifuged at 12,000 g for 15 min at 4°C, and the supernatants were used for electrophoresis. Fifty micrograms of total protein samples were applied onto 10% SDS-PAGE gels (standard Laemmli formulation). Following electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (PVDF, Amersham Biosciences, USA). The membranes were probed overnight at 4°C with a 1:5,000 dilution of anti-DOR and anti-enkephalin antibody. Then the membrane was incubated for 1 hr at room temperature with goat anti-rabbit IgG (Chemicon Inc. Temecula, CA, USA) secondary antibody (1:5,000 final dilution) and immunoreactive bands were visualized under Piercer® ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA).

Quantitative Real-time Pcr

The total RNA was isolated from the collected cell homogenate using TRIzol Reagent (Thermo Fisher, USA) according to the manufacturer's instructions. Total cDNA was produced by reverse transcription using theTaqMan Reverse Transcription Kit (Biosystems, Foster City, CA) of 0.2 µl of purified RNA, 3 µl 10× RT buffer, 2.0 µl $MgCl_2$, 1.6 µl of 2.5 mM dNTPs, 1.5 µl random hexamers, 0.6 µl RNAse inhibitor, and 0.75 µl Multiscribe RT. The samples were incubated for 10 min at 25°C, 30 min at 48°C, and 5 min at

95°C. Quantitative real-time PCR was performed in the Light Cycler Instrument (Roche, Mannheim, Germany). The PCR reactions were carried out in a total volume of 20 μl, which included SYBR Green I with a Taq DNA polymerase reaction buffer, deoxynucleotide triphosphate mixture, 10 mM $MgCl_2$, PCR-grade water, 2 μl of template DNA, and primers. The reaction conditions were as follows: preincubation at 95°C for 5 min, followed by 45 cycles (amplification) of 95°C for 15s, 55°C for 5s, and 72°C for 10s. Specificity of amplicons was confirmed by melting curve analysis, evidence of a single band upon gel electrophoresis, authenticity of the DNA sequence of the band isolated from the gel, and resolution by BLAST analysis that the sequences of the amplicons were unique to D1 and D2 dopamine receptor (D1/D2) and the prohormone convertases 1 and 2 (PC1/PC2). Real-time PCR data was analyzed using the comparative Ct method where the relative amount of gene copies was extrapolated using beta-actin as a normalizer as a calibrator.

Statistical analysis

All values are expressed as mean ± S.E. One way analysis of variance (ANOVA) followed by Bonferroni test was used for multiple pairwise tests to determine the significance. P values less than 0.05 were considered statistically significant.

Results

Effects Of Hypoxia And/or Mppon Pc12 Cell Viability

We detailly examined the effects of MPP^+ and/or hypoxia at different oxygen levels for different durations on PC12 cell viability with LDH leakage assay. As shown in Figure 1A, hypoxic exposure at 1% O_2 for 24 hrs did not increase but rather decreased the LDH leakage (-16.2%) (Fig. 1A). However, more severe and longer duration of hypoxia markedly increased the leakage. LDH leakage significantly increased with hypoxiaat 1% O_2 for 48 hrs, while at 0.5% O_2 gradually increased the leakage from 4 to 48 hrs. In contrast, only 1-hr MPP^+ exposure in normoxia caused a large increase in LDH leakage (+32.9%) when compared to the control group (p□0.05; Fig. 1A). These results suggest that both MPP^+ insult and severe hypoxia caused cell injury and the injury increased with the severity of hypoxia and the duration of hypoxia or MPP^+ insult. And interestingly, hypoxia, either 1% or 0.5% O_2, rendered the MPP^+-induced injury to appear later than that of MPP^+ insult alone (Fig. 1A,1B).

Changes in cellular enkephalin in hypoxia and/or MPP^+ insult

Since DOR is determinant in neuronal survival/injury [15–18] and enkephalin is its endogenous ligand [37], we used Western blot assays to detect changes in the level of enkephalin in the PC12 cells exposed to hypoxia and/or MPP^+.

The results revealed that the level of enkephalin had a slight increaseat the 8th hour of 1% of O_2 and then came back to the control level from 12th to 24th hour of hypoxia. However, it was largely decreased after 48-hr hypoxic exposure ($P < 0.05$ vs. the control; Fig. 2).

At a lower level of oxygen (0.5% O_2), the level of enkephalin increased after 1-hr hypoxia and then decreased after 2-4 hours of hypoxia. After 8 hours of hypoxic exposure, the level of enkephalin returned to approximately normal levels. It decreased with the extension of hypoxia with a major reduction after 48-hr hypoxia ($P < 0.05$ vs. the control; Fig. 2). In the condition of MPP^+, the level of enkephalin were significantly decreased after 2-hr exposure and then had a continuous decline until the end of hypoxic exposure without any significant increase during the whole period of hypoxia, although there was a slight upturn in the middle of the injury ($P < 0.05$ vs. the control; Fig. 2). With the existence of MPP^+, hypoxiaat either 0.5% or 1% O_2 caused a decline in all the time points, from 1 to 48 hrs after the initiation of hypoxia plus MPP^+ insult, although there was a slight upturn in the middle of the injury ($P < 0.05$ vs. the control; Fig. 3).

These data suggest that the level of enkephalin was significantly changed by hypoxia or/and MPP^+ insults with a major decrease after severe and/or prolonged stress.

Changes in DOR density under hypoxia and/or MPP^+ insults

Furthermore, we observed the change in DOR density under hypoxic and/or MPP^+ insults. At 1% of O_2, the level of DOR had an increase from 8th to 24th hours after hypoxia and then decreased after 48-hr hypoxia ($P < 0.05$ vs. the control; Fig. 4A, 4B). Even with the existence of MPP^+, hypoxia at 1% O_2 still increased the level of DOR after 1-hr exposure ($P < 0.05$ vs. the control; Fig. 5A, 5B). At a lower level of oxygen (0.5% O_2), the level of DOR increased after 1-hr hypoxic exposure and then decreased ($P < 0.05$ vs. the control; Fig. 4A, 4B). With the existence of MPP^+, severe hypoxia (0.5% O_2) caused a major decline in all the times, from 1 hr to 48 hrs after the initiation of hypoxia plus MPP^+ insult ($P < 0.05$ vs. the control; Fig. 5A, 5B).

Interactive changes in enkephalin, DOR and cell viability in hypoxia and/or MPP^+

In the first 24 hours of hypoxia at 1% O_2, enkephalin and DOR levels were maintained at the same or even higher levels with a reduced LDH leakage. After 48 hours of exposure, the levels of enkephalin and DOR decreased, while the leakage of LDH significantly increased (Fig. 6A), suggesting that mild hypoxia might protect the cells by the up-regulation of enkephalin and DOR.

In the early stage of hypoxia at 0.5% O_2 (e.g., 1 hr after hypoxia), the levels of enkephalin and DOR were still increased without appreciable change in the leakage of LDH, indicating the protective effect of transient hypoxia(Fig. 6B).

Under the exposure conditions of hypoxia at 0.5% O_2, MPP^+ plus hypoxia at 1% O_2 or MPP^+ plus hypoxia at 0.5% O_2, the level of enkephalin increased briefly in the middle stage of hypoxia or injury (e.g., 8hrs or

12 hrs after hypoxia), during which the increase of LDH leakage slowed down, but the level of DOR changed little. With the extension of exposure time, the expression of enkephalin and DOR decreased, and LDH leakage increased significantly (Fig. 6B, 6C, 6D).

These results suggest that short-term or mild hypoxia stress can up-regulate the expression of DOR, thus protecting neurons from severe injury, which is consistent with our previous study that δ - opioid receptor (DOR) has neuroprotective effect on hypoxia/ischemia injury [15–23]. At the same time, in this study, we found that short-term or mild hypoxia stress also increased the expression of enkephalin, which showed that enkephalin and DOR were involved in the neuroprotective effect of hypoxia in the early stage.

Effects of hypoxia and/or MPP^+ insult on PC1/PC2 mRNAs

PC1 and PC2 are key enzymes involved in the proteolytic processing of proenkephalin and their up-regulated is capable of generating active opioid units from proenkephalin [38–41]. In Figure 6B, 6C and 6D, why did enkephalin levels increase briefly in the middle stage of hypoxia or injury (e.g., 8 hrs or 12 hrs)? Is PC1 / PC2 activated to promote the decomposition of proenkephalin and produce more active enkephalin? We therefore investigated their changes in response to hypoxia and/or MPP^+ insult by measuring the levels of PC1 and PC2 mRNAs with quantitative real-time PCR analysis. The results depicted in Figure 7 showed that in response to hypoxia alone, both PC1 and PC2 mRNAs increased after 8-24 hrs exposure and decreased after 48-hr exposure. In the condition of MPP^+ alone or MPP^+ plus hypoxia, both PC1 and PC2 mRNAs increased after 8-12 hrs exposure and largely decreased after the exposure for 24-48 hours (Fig. 7A, 7B, 7C, 7D).

Effects of hypoxia and/or MPP^+ insult on D1 and D2 mRNAs

Since hypoxia and/or MPP^+ insults are underlying the pathophysiological causes of PD [15], we wondered whether D1 and D2 dopamine receptors mRNA levels are modulated by hypoxiain PC12 cell models and the role of hypoxia intensity and exposure duration. As shown in Figure 8, both D1 and D2 mRNAs increased after hypoxiaat 1% O_2 (e.g., 24 hrs after hypoxia) and at 0.5% O_2 (e.g., 12 hrs after hypoxia) with a major decrease after 48-hr exposure, while MPP^+ or hypoxia plus MPP^+ significantly decreased the levels of both D1 and D2 mRNAs after 24-48 hrs of the exposure, especially in the case of hypoxia plus MPP^+ exposure (Fig. 8A, 8B, 8C, 8D). These results suggest that hypoxia is involved in the occurrence and development of Parkinson's disease by affecting the transcriptional levels of dopamine D1 and D2 receptors, which may be related to the time and intensity of exposure.

Discussion

We made several interesting findings in this work. Slight and short-term hypoxia showed a protective effect on PC12 cell with short-term reactive up-regulation of not only enkephalin but also DOR levels. At a longer time, hypoxia or MPP^+ injury accelerated the proteolysis of proenkephalin by up-regulating PC1/PC2, which might produce more active enkephalin, so as to activate DOR to protect cells. All our results suggest a important role of DOR system in the protection against PD injury.

The opioid receptors consist of three major subclasses, namely μ, δ and κ (MOR, DOR, and KOR respectively) [42, 43]. They are involved in the regulation of striatal dopamine function [44–47]. Enhanced delta opioid receptor transmission may represent an endogenous compensatory mechanism in parkinsonism to reduce the activity of the indirect striatopallidal pathway following dopamine depletion [48]. Opioid neuropeptides, enkephalin, mainly activating DOR which is expressed in abundance in the basal ganglia and cortical regions. Studies have shown that the function of dopaminergic neurons in PD is associated with the regulation of the opioidergic neuropeptides enkephalin and DOR in the striatum [49]. Increased opioid transmissions are thought to play a compensatory role in altered functions of the basal ganglia at an early stage of PD and can alleviate PD-related motor symptoms [46, 48].

Since Hugh et al discovered enkephalin in 1975, the research on the action mechanism of endogenous enkephalin in the pathophysiological process has been deepened with the development of neuromolecular biology and pathophysiology [50–53]. Similar to typical peptide hormones, proenkephalin have no biological activity and can produce active molecules by enzymatic hydrolysis and transformation. This study found that a series of changes occurred in the level of enkephalin during the whole process of hypoxia and/or MPP^+ stress. In the early stage, the levels of enkephalin and DOR increased synchronously. It is speculated that stress response may promote the release of enkephalin in synaptic vesicles, which DOR was involved in the automatic regulation of enkephalin release from axon terminals in this region. Interestingly, we observed a transient rise in enkephalin levels in the metaphase of PC12 cell injury. What mechanism is involved in the re-elevation of enkephalin in the middle term of PC12 cell injury? It is well recognized that enkephalin biosynthesisis from proenkephalin processing in secretory vesicles is achieved by the prohormone convertases 1 and 2 (PC1/PC2) [38–41]. Our data show that the alteration in enkephalin levels was probably due to changes in prohormone convertases 1 and 2 (PC1/PC2) mRNA in both MPP^+ and hypoxia conditions. Consistent with our prediction, the changes of PC1/PC2 in the injured PC12 cell were nearly paralleled with enkephalin, suggesting that the up-regulation of enkephalin resulted from the activation of PC1/PC2, which seemed to be an adaptive or compensatory response. Unfortunately, we did not find an increase in DOR expression in the middle of cell injury.

Taken together, we speculate that in normal enkephalin neurons, the majority of mature peptides are present in mature granules near the axon terminals, ready for release to the circulation. In the early stage of hypoxic treatment, mild or transient hypoxia can increase the expression of DOR, thus inducing cell protection through cAMP-response element binding protein (CREB)pathway [15, 16, 54]. In hypoxia and other stress conditions, the enkephalin neurons may release more enkephalin to further enhanced the protective effect by activated DOR [55]. In the middle period of hypoxia or MPP^+ injury, enkephalin neurons produce more enkephalin by enhancing the hydrolysis of enkephalin precursor protein through PC1/PC2, which the re-elevation of enkephalin may play a DOR-mediated protection to resist external stress. When facing further aggravation of cell injury, the levels of DOR, enkephalin and PC1/PC2 decreased, the apoptosis of cells finally occurred (Fig. 9).

The physiological effects of dopamine, a major striatal neurotransmitter, are mediated by several distinct receptor subtypes [56–58]. On the basis of structural, pharmacological, functional, and distributional

similarities, these subtypes fall into one of two groups, the D1-like receptor family and the D2-like receptor family [59–61]. Hypoxia in the brain has been detected in several neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and Huntington's disease [62]. Several studies have examined the effect of hypoxia on ligand binding properties of the striatal dopaminergic receptors, the authors suggested that hypoxia leads to the changes of D1 and/or D2 receptors and the long-term disorder of DA signal, which plays an important role in the pathogenesis of PD [63–66].

Under the condition of hypoxia alone, the transcription levels of D1 and D2 mRNA increased in varying degrees within a certain time. With the extension of exposure time, the levels of D1 and D2 mRNA decreased, while MPP^+ and/or hypoxia plus MPP^+ significantly reduced the levels of D1 and D2 mRNA, especially under the condition of hypoxia and MPP^+ co-exposure. It is suggested that a certain degree of hypoxia may improve the tolerance of cells to hypoxia, and have anti-Parkinson effects by increasing the transcription level of dopamine receptor. Long-term or severe hypoxia may lead to disorder of DA signaling pathway and increase the susceptibility to PD by reducing the level of dopamine receptor. In our previous studies, hypoxia was involved in the occurrence and development of as an environmental factor [15, 16, 67, 68]. These data suggest that dopamine D1 and D2 receptors are also involved.

Our results demonstrate that hypoxia has a regulatory effect on endogenous enkephalin. This may, at least partially, happen to obstructthe development of Parkinson's disease. This speculation is partially supported by our previous research results [10, 15, 16, 67, 68]. Short-term or mild hypoxia can up-regulate the level of enkephalin, which is coupled with coordinated up-regulation of DOR. DOR is considered to be the key to the cytoprotective effect of mild or transient hypoxia (Fig. 9). We hypothesized that this regulation will eventually lead to more efficient processing of mature peptides, stimulate the release of endogenous enkephalin from enkephalin neurons, and protect neurons from damage by activating DOR. The transcriptional regulation of PC1 and PC2 gene expression by hypoxia is another stage of regulating energy homeostasis. A little longer period of hypoxia or injury, the processing of proenkephalin increased, and then active enkephalin stimulated endogenous DOR, which constituted an additional key checkpoint. At this stage, hypoxia accelerates the proteolysis of proenkephalin by activating PC1/PC2 to produce more active enkephalin. In addition, a certain degree of hypoxia may improve the tolerance of cells to hypoxia, and play an anti-Parkinson role by increasing the transcription level of dopamine D1/D2 receptor (Fig. 9). In general, the relationship between the regulation of hypoxia on enkephalin, DOR and dopamine receptor D1/D2 and the effect of PC1/PC2 on proenkephalin, will substantially improve our understanding of how hypoxia protects cells in a coordinated manner. Through the recognition of these coordination mechanisms, we can finally provide novel therapeutic strategies for the treatment of Parkinson's disease and other diseases related hypoxia.

Abbreviations

ANOVA	One way analysis of variance
CREB	cAMP-response element binding protein
D1/D2	D1and D2 dopamine receptor
DMEM	Deulbecco's Modified Eagle Medium
DOR	delta opioid receptor
FBS	fetal bovine serum
H	hypoxia
H+M	hypoxia plus MPP^+
LDH	Lactic Dehydrogenase
M	MPP^+
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PC1/PC2	Prohormone convertases 1 and 2
PD	Parkinson’s disease
PVDF	Polyvinylidene difluoride membrane

Declarations

Author contributions

YX and TC initiated the project, designed the experiments and interpreted the data. TC performed the experiments, analyzed the data and wrote the manuscript. QW and DC performed experiments and analyzed data. YD, YL, BC, JZ and GW participated in data processing and interpretation. YX provided research reagents and revised the manuscript. All authors have read and approved the manuscript.

Funding information

This work was supported by National Natural Science Foundation of China (81873361;81060096;81560202), Key Educational Reform Projects of Hainan Province (Hnjg2019ZD-15), High Level Talents of Hainan Natural Science Foundation (820RC759), Key R & D Plan Project of Hainan Province (ZDYF2021SHFZ091), Construction Project of Hainan Province Clinical Medical Centerand Science, The innovation platform for Academinicians of Hainan Province, Science and Technology Commission of Shanghai Municipality (18401970100).

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Yes.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Availability of data and materials

Not applicable.

Acknowledgements

Not applicable.

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Figures

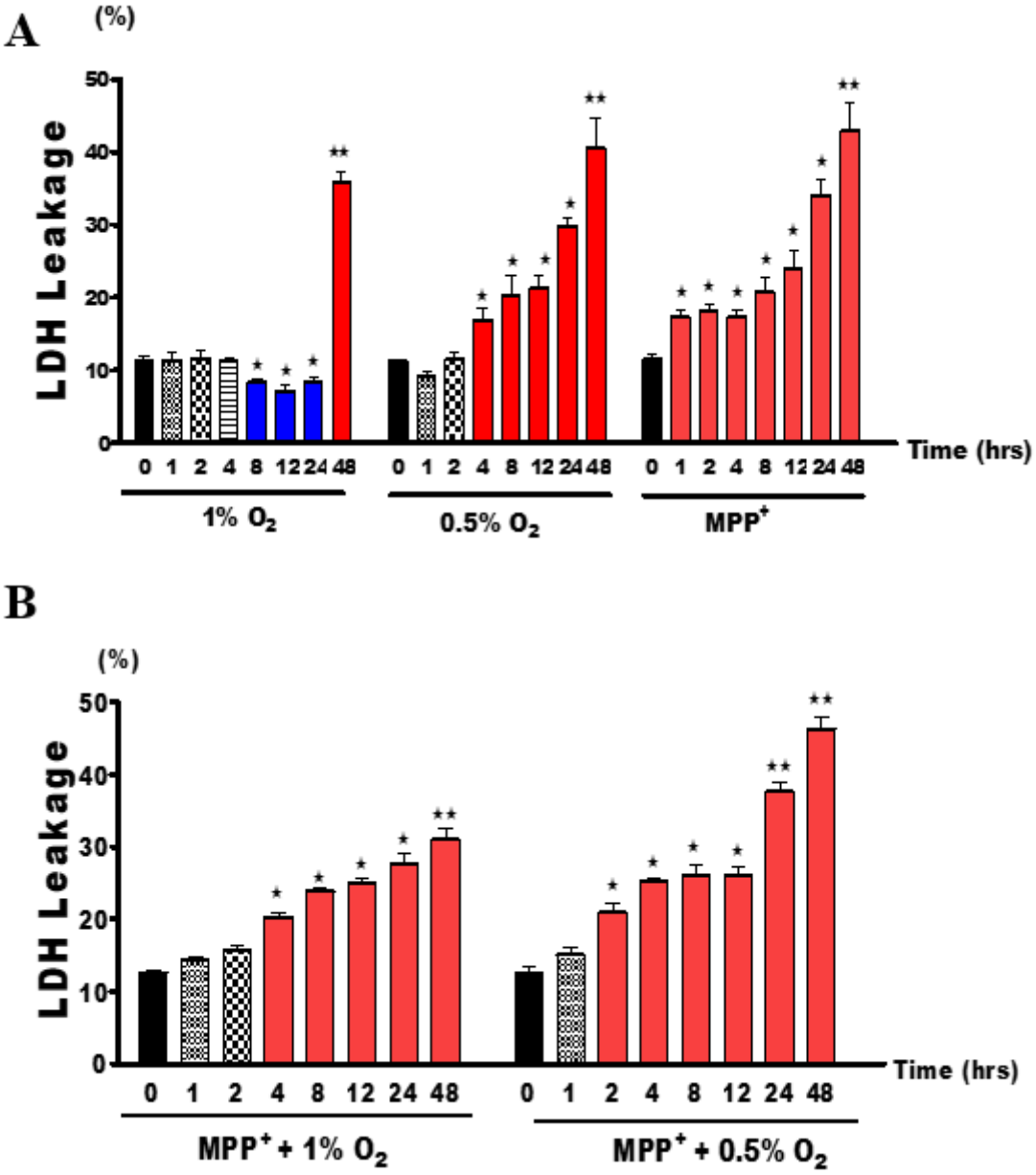

Figure 1

Hypoxia and/or MPP^+ induced injury in the PC12 cells. LDH leakage was measured as an index of cellular injury. **(A)** Cell viability in hypoxic stress (1% O_2 or 0.5% O_2) or MPP^+ insult. **(B)** Cell viability under the condition of hypoxia plus MPP^+ insult. N = 3 for each group. $^{\star}p<0.05$ and $^{\star\star}p<0.01$ vs. the control (0hr). Note that both hypoxic stress and MPP^+ insult caused cell injury and the injury increased with the severity of hypoxia and the duration of hypoxia or MPP^+ insult. Also note that hypoxia, either at 1% or 0.5% O_2, rendered the MPP^+-induced injury to appear later than that of MPP^+ insult alone.

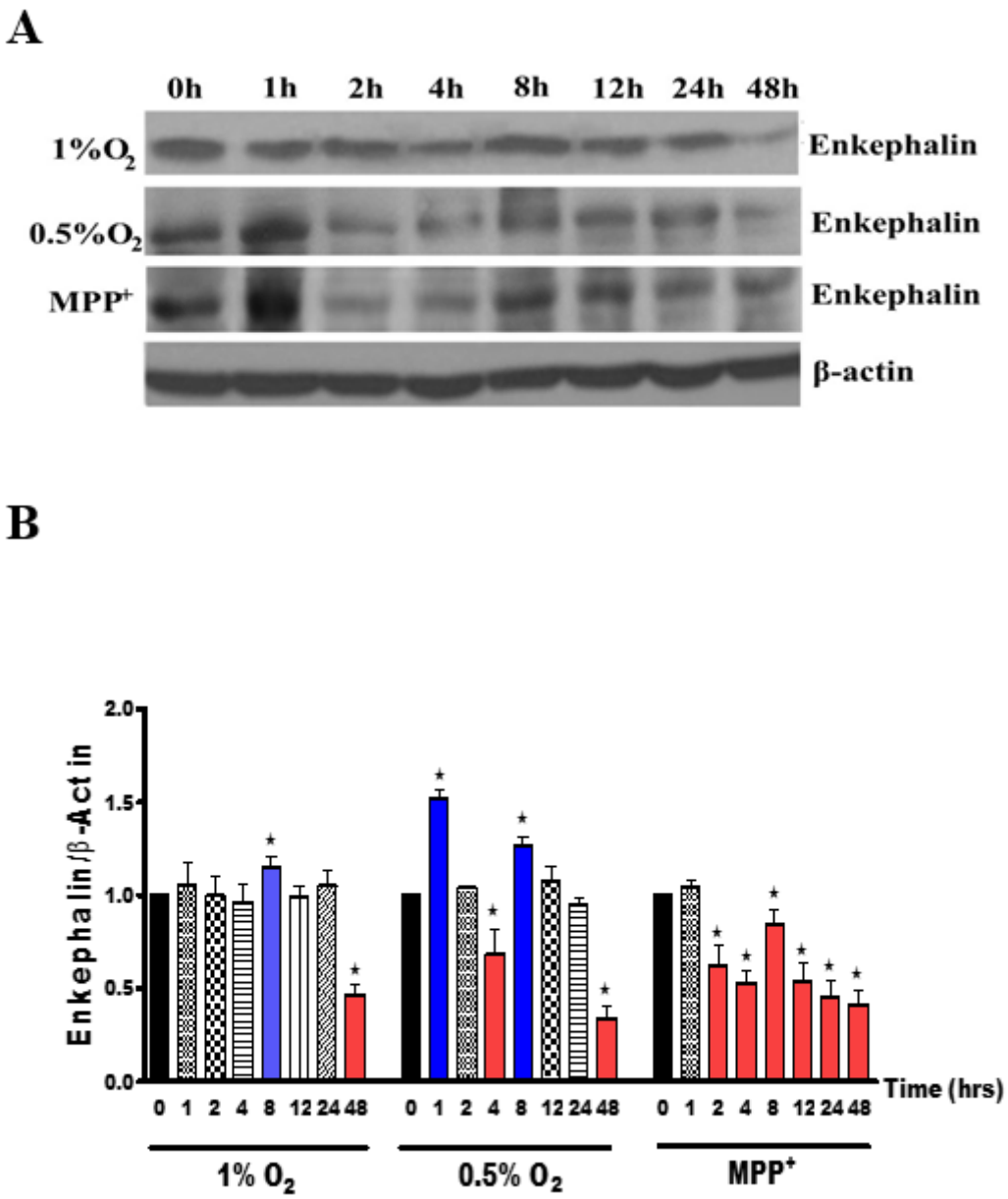

Figure 2

The levels of enkephalin in the PC12 cells exposed to hypoxia or MPP^+ insult. (A) Representative western blots of enkephalin in the PC12 cells in hypoxia (0.5 or 1% O_2) or MPP^+ (0.5 mM) insult. **(B)** Quantitative summary of western blot analysis in terms of cellular enkephalin level in hypoxia or MPP^+ insult. N = 3 for each group. $^{\star}p<0.05$ and $^{\star\star}p<0.01$ vs. the control (0 hr). Note that the level of enkephalin had a slight increase at the 8^{th} hour of hypoxia at 1% O_2 and then came back to the control level from 12^{th} to 24^{th} hour of hypoxia. However, it was largely decreased after 48-hr hypoxic exposure. At a lower level of oxygen (0.5 % O_2), the level of enkephalin increased after 1-hr hypoxia and then decreased after 2-4 hours of hypoxia. After 8 hours of hypoxic exposure, the level of enkephalin returned to approximately normal levels, and then decreased with the extension of hypoxia with a major reduction after 48-hr hypoxia. In the MPP^+ condition, the level of enkephalin was significantly decreased after 2-hr exposure and then had a

continuous decline until the end of observation (48 hrs) without any significant increase during the whole period of MPP^+ exposure, although there was a slight upturn in the middle of the injury.

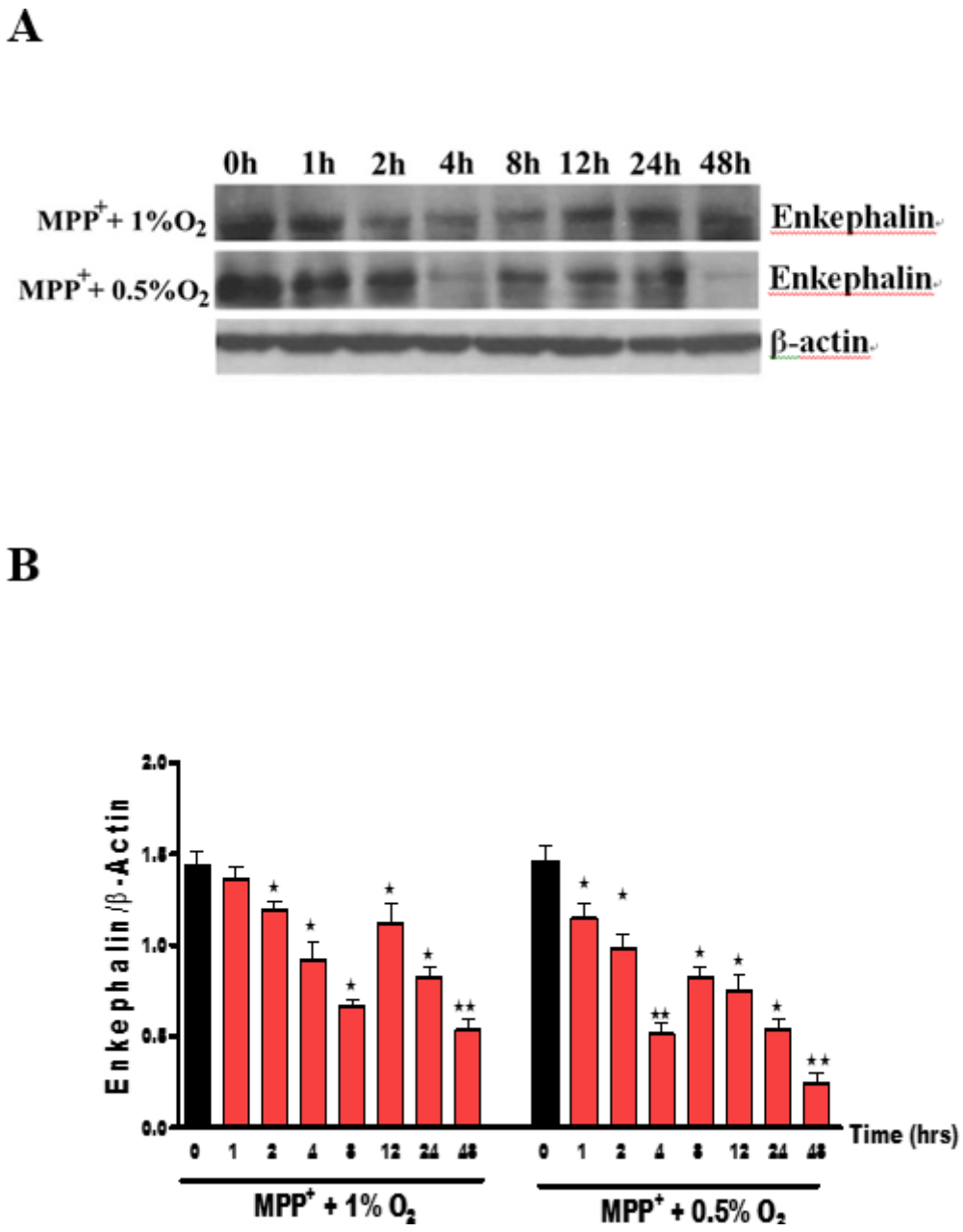

Figure 3

The levels of enkephalin in the PC12 cells under hypoxia plus MPP^+ insult. (A) Representative western blots of cellular encephalin. **(B)** Quantitative summary of western blot analysis of cellular enkephalin. N = 3 for each group. $^{*}p<0.05$, $^{**}p<0.01$ vs. the control (0 hrs). Note that with the existence of MPP^+, hypoxia at either 0.5% or 1% O_2 caused a decline at all the time points, from 1 to 48 hrs after the initiation of hypoxia plus MPP^+ insult, although there was a slight upturn in the middle of the injury.

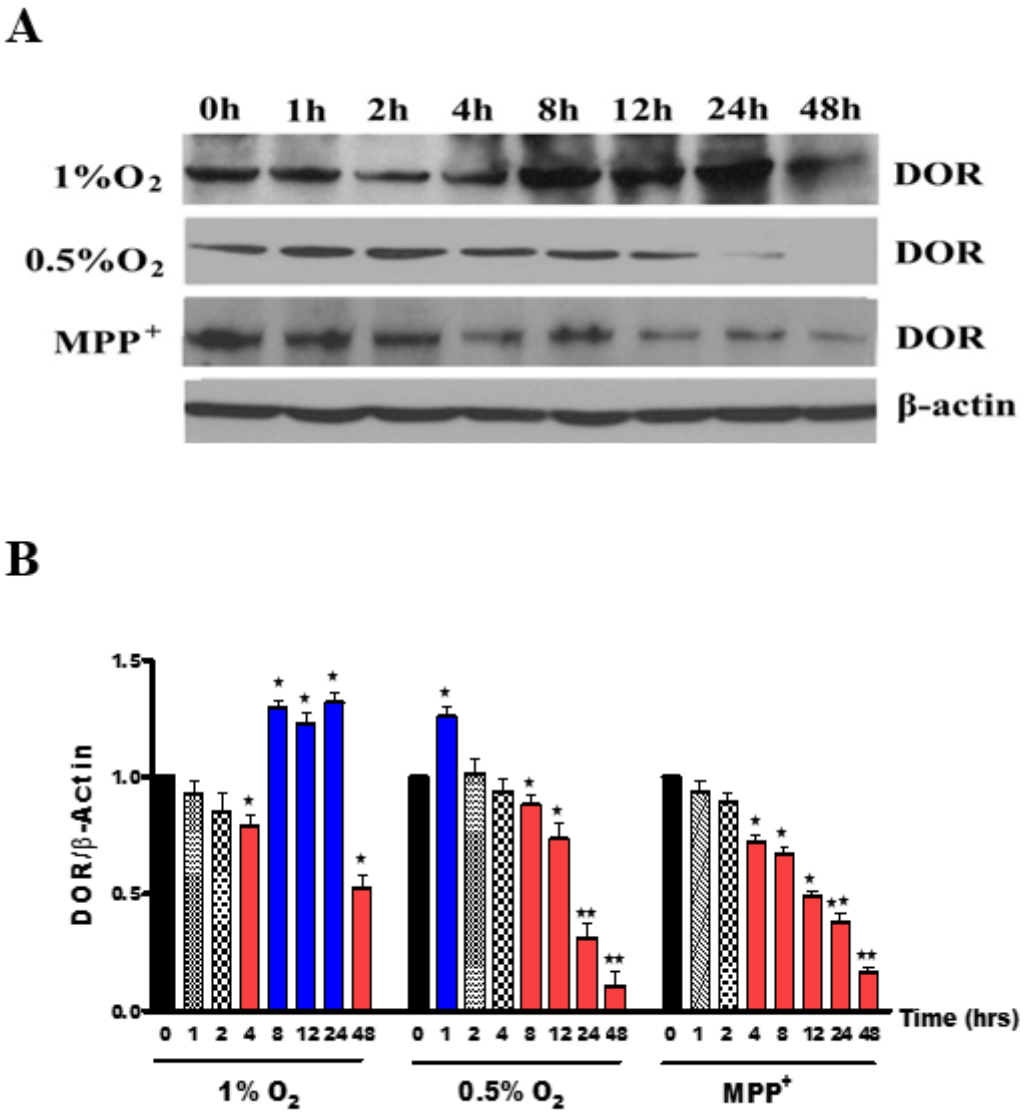

Figure 4

The levels of DOR in the PC12 cells under hypoxia or MPP+ insult. (A) Representative western blots of DOR in the PC12 cells in hypoxia (0.5 and 1% O_2) or MPP^+ (0.5 mM) insult. **(B)** Quantitative summary of western blot analysis in terms of DOR levels in the cells exposed to hypoxia or MPP^+. N = 3 for each group. *p<0.05 and **p<0.01 vs. the control (0 hr). Note that at 1% of O_2, the level of DOR had an increase from 8th to 24th hour after hypoxia and then decreased after 48-hr hypoxia. At a lower level of oxygen (0.5% O_2), the level of DOR increased after 1-hr exposure and then decreased. In exposure to MPP^+, the level of DOR gradually decreased after 4-hr exposure without any increase during the whole period of 48-hr exposure.

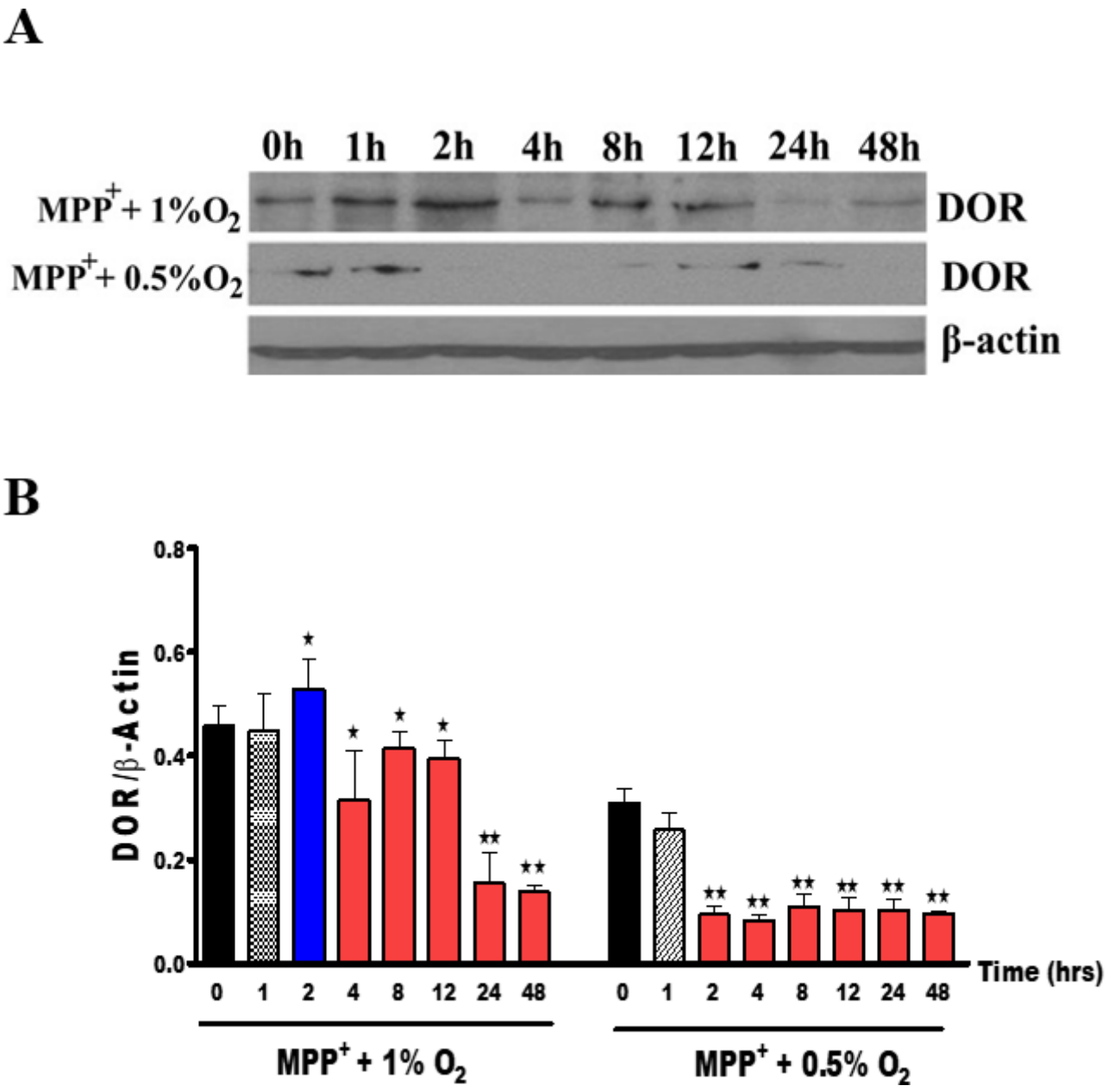

Figure 5

The levels of DOR in the PC12 cells exposed to both hypoxia and MPP^+ insult. (A) Representative western blots of cellular DOR. **(B)** Quantitative summary of western blot analysis of cellular DOR. N = 3 for each group. $^{\star}p<0.05$ and $^{\star\star}p<0.01$ vs. the control (0 hr). Note that with the existence of MPP^+, hypoxia (1% O_2) slightly increased the level of DOR after 1-hr exposure, but not afterwards, while severe hypoxia (0.5% O_2) caused a major decline in all the times after the initiation of hypoxia plus MPP^+ insult.

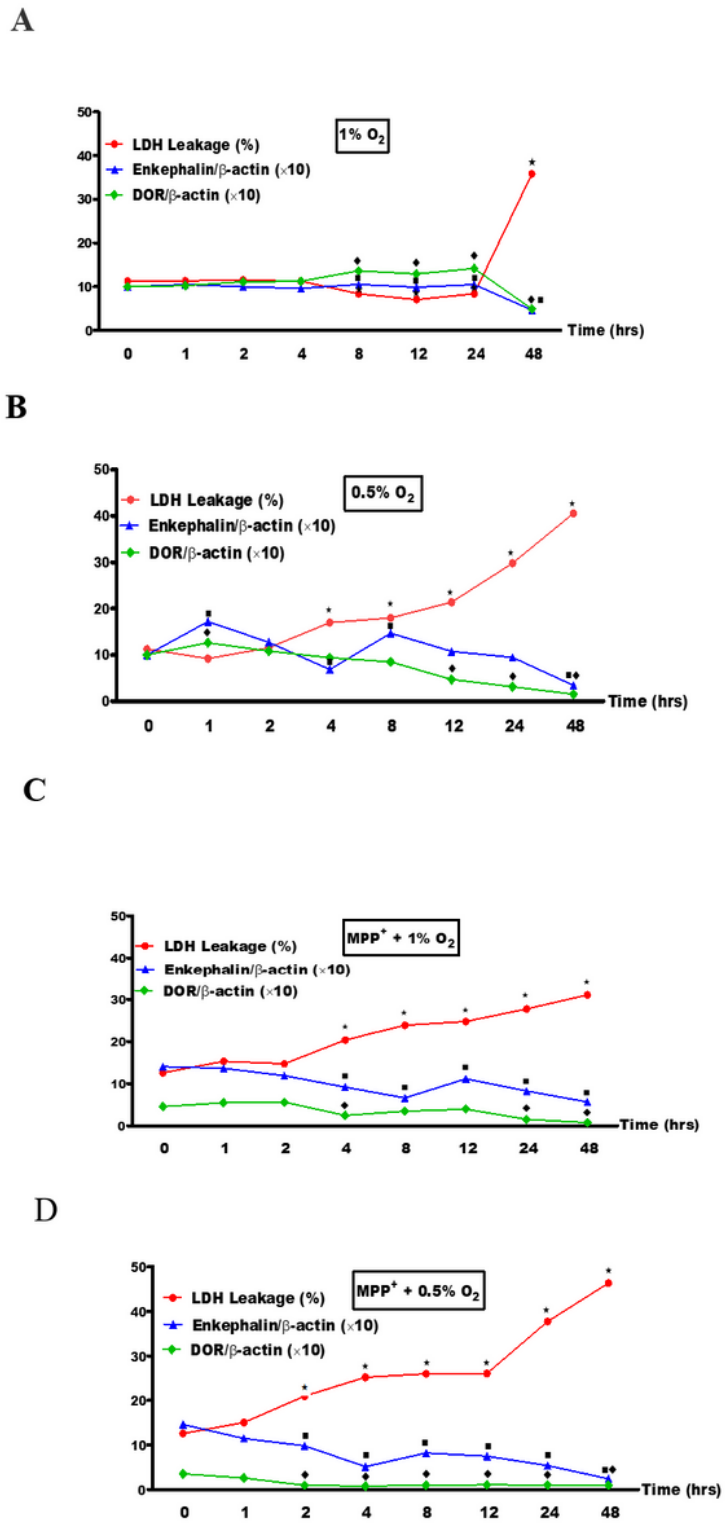

Figure 6

Interactive changes in enkephalin, DOR and cell viability in hypoxia and/or MPP^+. (A) Hypoxia at 1% O_2. **(B)** Hypoxia at 0.5% O_2. **(C)** Hypoxia at 1% O_2 plus MPP^+ insults. **(D)** Hypoxia at 0.5% O_2 plus MPP^+ insult. N = 3 for each group. □p<0.05 vs. the control of LDH. ■p<0.05 vs. the control of enkephalin. □p<0.05 vs. the control of DOR. Note that in the first 24 hours at 1% O_2 (6A), enkephalin and DOR levels were maintained

at the same or higher levels, while LDH leakage was not increased or even decreased. After 48 hours of exposure, the levels of enkephalin and DOR decreased with a major increase in LDH. Under the exposure conditions of hypoxia at 0.5% O_2, MPP^+ plus hypoxia at 1% O_2 or MPP^+ plus hypoxia at 0.5% O_2, the level of enkephalin increased briefly in the middle stage of hypoxia or injury (e.g., 8 hrs or 12 hrs after hypoxia), during which the increase of LDH leakage slowed down, but the level of DOR changed little. With the extension of exposure time, the levels of enkephalin and DOR decreased, and LDH leakage increased significantly (Fig. 6B, 6C, 6D).

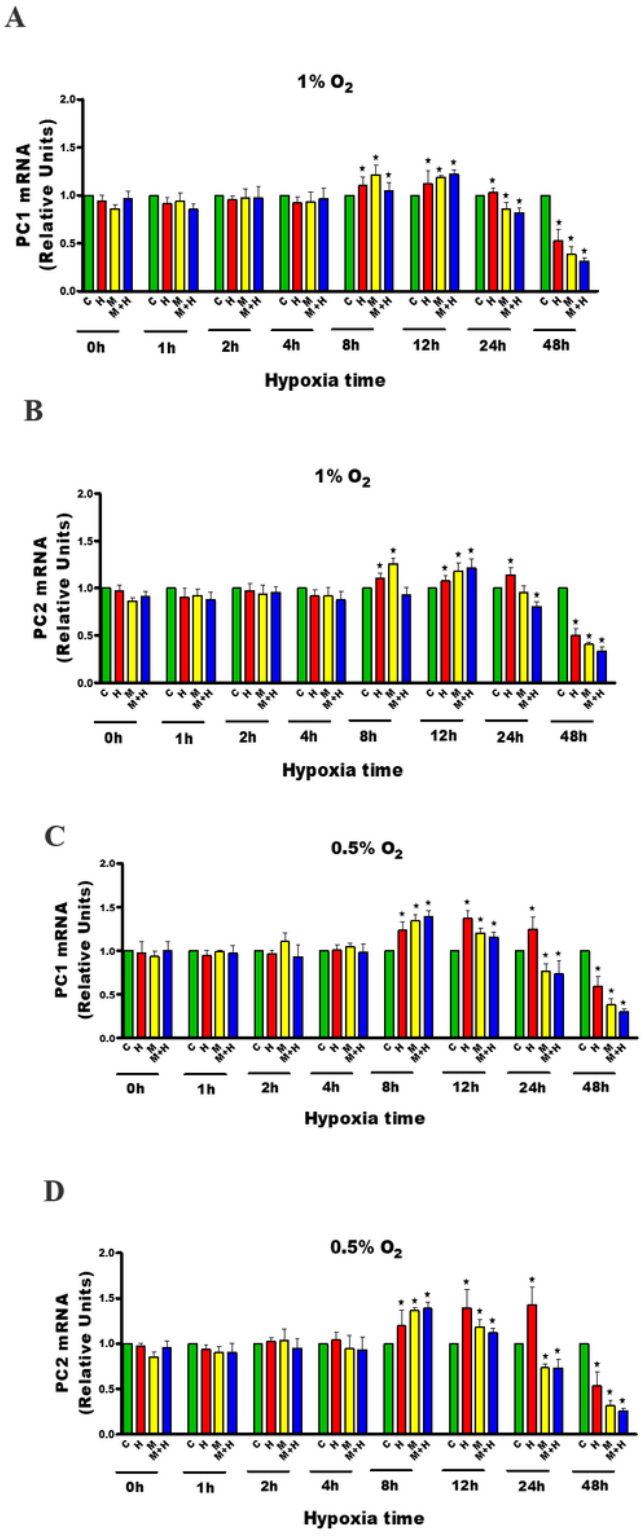

Figure 7

Effects of hypoxia and/or MPP^+ insult on the mRNAs of prohormone convertases 1 and 2 (PC1/PC2) in the PC12 cells. (A) Effect of hypoxia at 1% O_2 and/or MPP^+ insult on PC1 mRNA expression. **(B)** Effect of hypoxia at 1% O_2 and/or MPP^+ insult on PC2 mRNA expression. **(C)** Effect of hypoxia at 0.5% O_2 and/or MPP^+ insult on PC1 mRNA expression. **(D)** Effect of hypoxia at 0.5% O_2 and/or MPP^+ insult on PC2 mRNA

expression. C, normoxic control. H, hypoxia. M, MPP^+. H+M, hypoxia plus MPP^+. N = 3 for each group. *p<0.05 vs. the control. Note that in response to hypoxia alone, both PC1 and PC2 mRNAs increased after 8-24 hrs exposure and then decreased after 48-hr exposure. In exposure to MPP^+ alone or MPP^+ plus hypoxia, PC1 and PC2 mRNAs increased after 8-12 hrs exposure and largely decreased after the exposure for 24-48 hours.

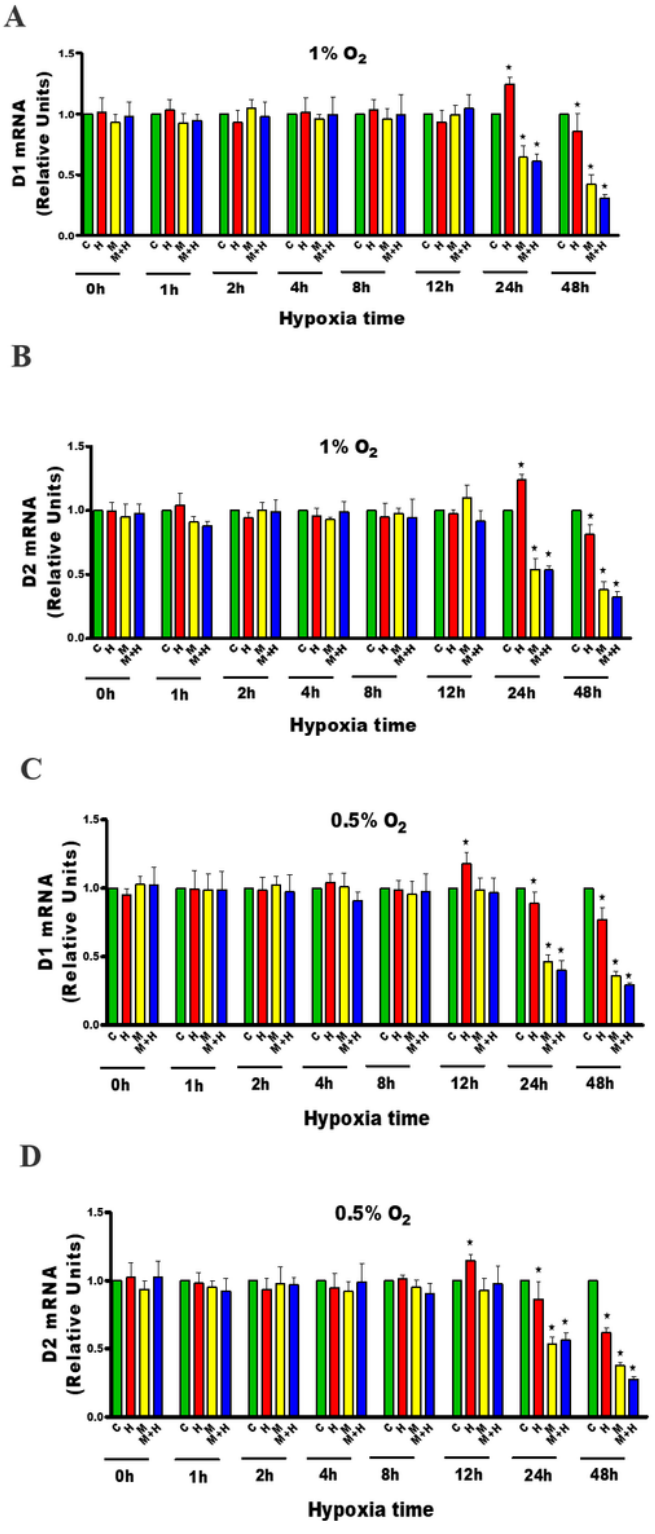

Figure 8

Effects of hypoxia and/or MPP^+ insult on cellular dopamine receptor D1/ D2 mRNAs in the PC12 cells. **(A)** Effect of hypoxia at 1% O_2 and/or MPP^+ insult on D1 mRNA expression. **(B)** Effect of hypoxia at 1% O_2 and/or MPP^+ insult on D2 mRNA expression. **(C)** Effect of hypoxia at 0.5% O_2 and/or MPP^+ insult on D2 mRNA expression. **(D)** Effect of hypoxia at 0.5% O_2 and/or MPP^+ insult on D2 mRNA expression. C, normoxic control. H, hypoxia. M, MPP^+. H+M, Combined exposure to hypoxia and MPP^+. N = 3 for each group. $^{\square}p<0.05$ vs. the control. Note that in response to hypoxia alone, both D1 and D2 mRNAs increased briefly after 24-hr (1% O_2) or 12-hr (0.5% O_2) hypoxic exposure and then decreased, whereas MPP^+ alone or MPP^+ plus hypoxia significantly decreased both D1 and D2 mRNAs after 24-48 hrs of the exposure, especially in the case of hypoxia plus MPP^+ exposure.

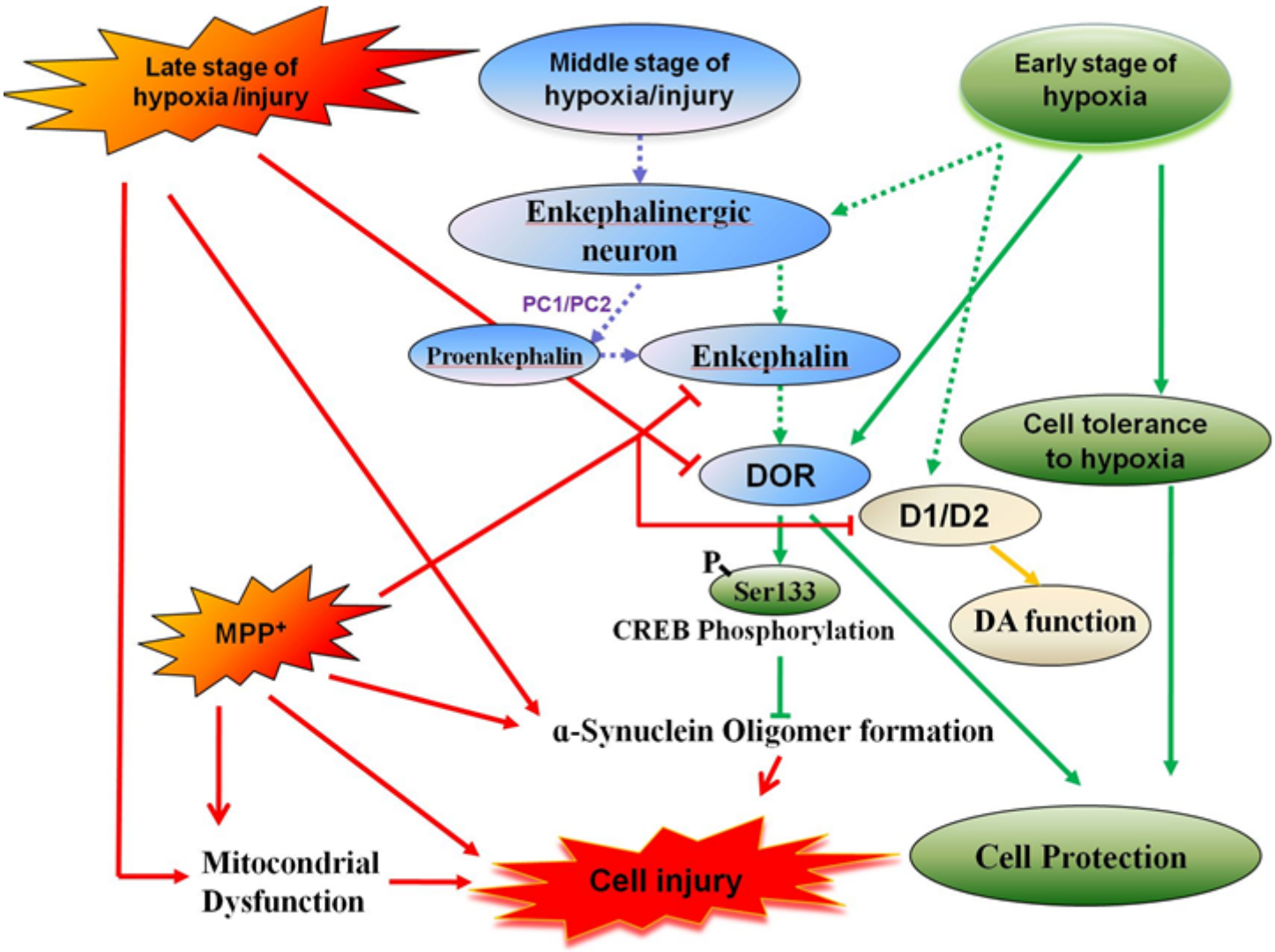

Figure 9

Potential changes in enkephalin, DOR and cell viability in response to hypoxia and MPP^+.