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Effect of Exosomal Protein Expression and Electrochemical Nano Interface in Morphine Analgesia Tolerance

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ABSTRACT

The current understanding of neurons cannot well explain the phenomenon of morphine tolerance, and even if some neuronal drugs are used in combination with morphine, they cannot achieve good results. In recent years, exosomal proteins have been the role of morphine tolerance and morphine-induced hyperalgesia is becoming more and more important. In clinical application, a larger dose is needed to achieve the desired analgesic effect, and large doses of morphine will aggravate the adverse drug reactions. The purpose of this experiment is to explore the role and mechanism of exosomal protein expression and electrochemical nanointerface in morphine analgesic tolerance and provide a theoretical basis for the application of exosomal protein in the clinical prevention and treatment of morphine tolerance. In this paper, adult male rats were randomly divided into groups and analyzed from four aspects: enhancing the analgesic effect of high-dose morphine, preventing morphine tolerance, the effect of morphine-induced hyperalgesia, and changes in mRNA levels of related genes. Experiments have shown that the mRNA levels of exosomal protein-related genes in the spinal cord of morphine-tolerant rats are significantly increased, and the rats given exosome protein inhibitors can prolong morphine analgesia and relieve morphine tolerance and hyperalgesia in behavior. It can inhibit the increase of exosomal protein-related genes. It shows that low-dose long-term morphine treatment can cause spinal endoplasmic reticulum stress, and the exosomal protein inhibitor 4-PBA or TUDCA can enhance the analgesic effect of morphine in neuropathic pain.

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Introduction

In recent years, the role of glial cells in morphine tolerance and morphine-induced hyperalgesia has become increasingly important. Glial cells are a type of non-neuronal cells that regulate the homeostasis of the nervous system (1). Morphine tolerance is defined as reducing the effectiveness of opioids to control pain. This means that the dose to achieve the same analgesic effect must be increased over time (2). Hyperalgesia and tolerance have some basic mechanisms in common (3). However, the exact underlying mechanism is still not very clear and seems to involve several complex pathways (4). The current understanding of neurons cannot well explain the phenomenon of morphine tolerance, and even if some neuronal drugs are used in combination with morphine in clinical practice, good results cannot be achieved. Opioid-induced inflammatory factors play an important role in morphine tolerance and hyperalgesia (5). Blocking the activation of glial cells or blocking the production of pro-inflammatory factors will alleviate these two conditions (1).

There have been many research results in foreign exosomal protein expression and electrochemical nano interface (6). Morphine analgesic tolerance can activate immune cells and secrete a series of cytokines by identifying pathogen-related molecular patterns and endogenous danger signals. Thereby promoting the process of neuroinflammatory reaction. Xingliang assembles Raman molecules and sulfhydryl DNA complementary to the target sequence on silver nanoparticles (7). When the target DNA appears and hybridizes, the silver nanoparticles will aggregate, and the color of the solution will change from yellow to blue-green, its SERS signal will be enhanced many times (8). Heidi S used glial cell modulator valprofylline to verify that the production of morphine analgesic tolerance requires the activation of spinal

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cord glial cells (9). Powers B uses plasma coupling and monitors DNA hybridization. Its signal-to-noise ratio is significantly better than that of traditional molecular beacons. It is found that a single nucleotide mismatch is lower than its melting temperature, so it can distinguish single-base mismatches (10).

In recent years, there have been more and more researches on exosomal protein expression and electrochemical nanointerface in China. With the improvement of science and medical technology, there are more and more researches on exosomal protein expression and electrochemical nanointerface. Mature, it is gradually used in the clinical evaluation of morphine analgesia and morphine tolerance (11, 12). Theodoraki MN proposed that LCN2 deficiency also inhibits formalin-induced activation of microglia in the spinal cord stratum corneum, which is a prerequisite for central sensitization and is important for the development of noxious behaviors and the inflammation required for pain hypersensitivity (13). Song Y proposed that chronic morphine-induced tolerance of potassium conduction activation in mouse locus coeruleus neurons was similar to that previously reported in wild-type neurons and was eliminated in b-arrestin2 knockout (14). Rugivan found that the degree of endocytosis of locus coeruleus neurons in animals chronically treated with morphine was no different from that induced by ME, but the degree of receptor reinsertion into the plasma membrane was reduced (15).

This study mainly discusses the application status and influencing factors of exosomal protein expression and electrochemical nanointerface in morphine analgesic tolerance. The advantages and disadvantages of previous studies were analyzed, and then a model of morphine analgesic tolerance was proposed based on the exosomal protein inhibitor 4-PBA or TUDCA. The systematic and objective evaluation of the clinical significance of the exosomal protein inhibitor 4-PBA or TUDCA in morphine analgesic tolerance indicates that intrathecal administration of the exosomal protein inhibitor 4-PBA or TUDCA can enhance the analgesic effect of morphine. Prevent the development of morphine tolerance and relieve morphine-induced hyperalgesia.

Materials and methods

Test Subject

In this experiment, healthy adult male rats, 2.5-3 months old and weighing 220-250g, were raised in the experimental animal center. During the animal feeding process, free eating, drinking, natural light and dark cycles, and maintaining room temperature (22±1°C)). Adult male rats were randomly divided into the normal saline group, morphine tolerance group (10μg morphine), morphine+4-PBA (5, 15 and 50μg) group (prepared and diluted with normal saline) and morphine+TUDCA (50, 100 and 50μg). 200μg) group (prepared and diluted with physiological saline). The analysis was conducted from four aspects: enhancing the analgesic effect of high-dose morphine, preventing morphine tolerance, the effect of morphine-induced hyperalgesia, and changes in the mRNA levels of related genes.

Experimental Method

Intrathecal administration (the rat is in a sitting position under isoflurane anesthesia, the L5-6 spinous process space is used as the puncture point, and the vertical distance between the puncture point and the highest point on the back of the rat is >3cm. Then the experimenter's left-hand Hip nodules, the horizontal position is the rat’s L5-6 spinous process space. Then the left thumb and middle finger are placed on both sides of the rat’s L5-6 spinous process space, and the skin is tightened outward. Take the food at the designated position and hold the insulin syringe in the right hand slowly insert the needle vertically from the gap. When the tail-flick occurs, the syringe draws back the cerebrospinal fluid and then injects 20 μL of the medicine, and the tail-flick occurs frequently after the injection), twice a day for seven consecutive days, every half an hour on the first day of administration the thermal pain threshold was measured by tail flicking in a 52-degree water bath, and the maximum time of tail flicking was 10 seconds until the thermal pain threshold was close to the normal value. After half an hour of administration every day, the thermal pain threshold was measured by a 52-degree water bath tail flick. On the seventh day, the mechanical pain threshold of each group was measured with von Frey filaments.

Western Blotting Detection

Western blotting was used to detect the expression levels of exosomal protein, TLR4, p-p38MAPK and p38MAPK protein in the spinal cord tissue. The remaining spinal cord tissue was extracted, and total protein was extracted according to the steps of the KeyGEN protein extraction kit. BCA quantitative kit was used for quantitative analysis. The loading amount is 25μg/well, the protein is separated by 10% SDS-PAGE electrophoresis, and then transferred to PVDF membrane, blocked with TBST solution containing 5% BSA for 1 hour at room temperature, and the primary antibody exosomal protein (1:1000), TLR4 (1:1000), p-p38MAPK(1:1000), p38MAPK(1:1000), β-actin(1:5000), incubate overnight, wash the membrane 3 times with TBST, and mark specificity with horseradish peroxidase at room temperature incubate with the secondary antibody (1:5000) for 1 h, use ECL chemiluminescent solution in fluorescence and visible light gel imaging system to develop and perform grayscale analysis, calculate the ratio of the gray value of the target protein band to the gray value of the internal reference protein band, Semi-quantitative analysis of the expression level of the target protein.

Statistical Analysis

ImageJ 2x software was used to collect the images of Western blotting results, and GraphPad Prism 4 software was used for mapping and analysis. The experimental results are all expressed as mean±standard deviation, and SPSS13.0 was used for statistical analysis. $P<0.05$ indicates that the difference is statistically significant.

Results and discussion

Evaluation Index System Based on Index Reliability Testing

It is generally believed that the α coefficient above 0.8 indicates that the effect of the index set is very good, and above 0.7 is also acceptable. Here we analyze the reliability of each type of object, and the reliability index we choose for each type of object is slightly different. The results are shown in Table 1.

It can be seen from Figure 1 that the data obtained by enhancing the analgesic effect of high-dose morphine, preventing the effect of morphine tolerance and morphine-induced hyperalgesia have a very good effect on this experiment (α>0.8), the data obtained from changes in the mRNA level of related genes have an acceptable impact on this experiment (α>0.7), indicating that the four indicators selected in this article based on morphine analgesic tolerance are reasonable, which is a follow-up continue the experiment to provide a basis.

Table 1. Data Sheet of Evaluation Index System for Index Reliability Testing; Enhance the Analgesic Effect of High-dose Morphine (A), Prevent Morphine Tolerance (B), Morphine Induced Hyperalgesia (C), Changes in mRNA Levels of Related Genes (D)

	Very Clear	Clear	General	Not Clear	Chaotic	Alpha
A	0.207	0.392	0.183	0.122	0.096	0.9273
B	0.249	0.307	0.176	0.182	0.086	0.8335
C	0.207	0.251	0.297	0.167	0.078	0.8174
D	0.112	0.276	0.311	0.182	0.119	0.7617

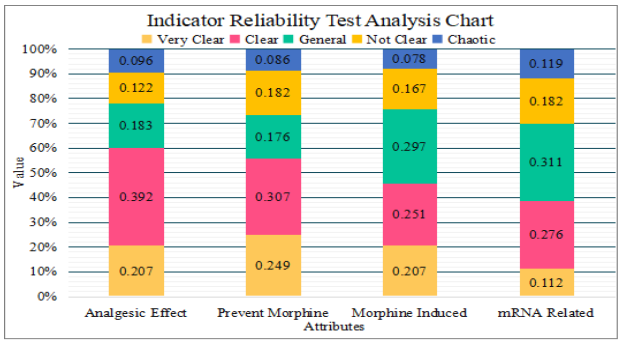

Figure 1. Indicator reliability test analysis chart

Morphine Analgesia Tolerance and Hyperalgesia

In order to investigate the effect of exosomal protein inhibitors on the analgesic effect of morphine, we administered 20μl intrathecal morphine (2μg, 5 μg and 10μg) (diluted with saline) and 4-PBA (50μg) or TUDCA (100μg) in different doses. (Prepare and dilute with physiological saline) The mixture is administered once on the same day, and the tail-flick is measured every half an hour after the administration. The maximum time of each tail flick is 10s to prevent damage. The exosomal protein inhibitor is detected by the tail-flick method influence on the analgesic effect of morphine. The results are shown in Table 2.

Table 2. Data sheet of exosomal protein inhibitors for analgesia of different doses of morphine

Time (Min)	Morphine (2μg) + Saline	Morphine (5μg) + Saline	Morphine (2μg) + 4-PBA (50μg)	Morphine (2μg) + 4-PBA (50μg)	Morphine (2μg) + TUDCA (100μg)
0	2.32	3.37	2.67	3.01	3.11
30	7.98	7.89	9.13	9.06	8.83
60	8.36	8.32	8.42	9.28	8.39
90	7.74	7.86	8.29	7.96	8.57
120	7.25	7.48	7.59	7.67	7.63
150	6.89	7.03	6.98	7.14	7.42

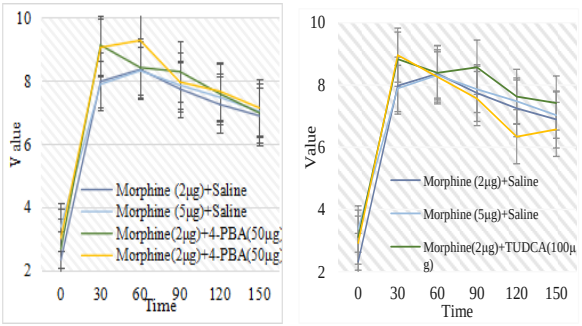

Figure 2. Analgesic effects of exosomal protein inhibitors on different doses of morphine

It can be seen from Figure 2 that the exosomal protein inhibitor TUDCA or 4-PBA can only prolong the analgesic effect of morphine at a dose of 10 μg of morphine, but has no effect on the analgesic effect of low-dose morphine. Through the tail-flick experiment, we found that different doses of morphine 4-PBA or TUDCA enhance the analgesic effect of morphine with a dose-effect.

Morphine tolerance is mainly manifested by weakened analgesic effect, shortened duration, increased pain and hyperalgesia after stopping the drug. In clinical application, a larger dose is required to achieve the ideal analgesic effect. After 0.5h of administration, it is thrown in a 52-degree water bath. The effect of the endoplasmic reticulum stress inhibitor on the development of morphine tolerance was tested by the tail. Each tail flick was done for a long time for 10 seconds. The results are shown in Table 3.

It can be seen from Figure 3 that intrathecal administration of exosomal protein inhibitors TUDCA and 4-PBA can prevent the right shift of the morphine tolerance dose-response curve. In order to further verify that exosomal protein inhibitors can alleviate morphine tolerance, we are in morphine after the tolerance model was established, a 52-degree water bath was used to test whether the exosomal protein inhibitor can reverse the morphine tolerance. Morphine tolerance rats can still alleviate the morphine tolerance after intrathecal administration of the exosomal protein inhibitor.

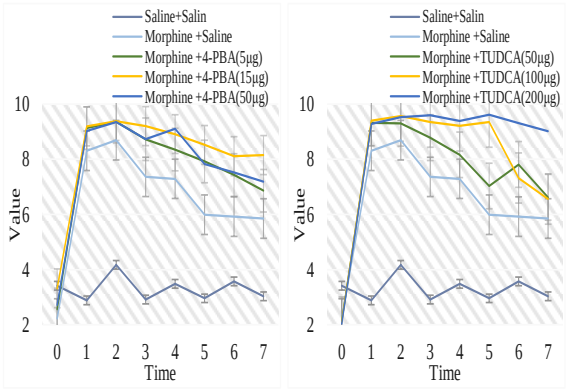

Figure 3. Effects of 4-PBA or TUDCA on the development of morphine tolerance

In order to explore the role of exosomal protein in morphine-induced hyperalgesia, we administered a mixture of morphine and exosomal protein intrathecally twice a day for seven consecutive days. On the seventh day, the mechanical pain threshold was measured by using VonFrey fiber filament, and the thermal pain threshold was measured by a thermal radiance meter. The effects of exosomal protein inhibitors on morphine-induced hyperalgesia were discussed. The results are shown in Table 4.

It can be seen from Figure 4 that intrathecal administration of morphine (10μg) and endoplasmic reticulum stress inhibitor 4-PBA (15μg and 50μg) or TUDCA (100μg and 200μg) alleviated the mechanical hyperalgesia induced by morphine. And the endoplasmic reticulum stress inhibitor 4-PBA (5, 15 and 50μg) or TUDCA (50, 100 and 200μg) mixture alleviates morphine-induced thermal tingling hypersensitivity.

From our animal behavior experiments, we know that intrathecal administration of exosomal protein inhibitors and morphine mixture can enhance the analgesic effect of morphine and relieve morphine tolerance and hyperalgesia. However, whether long-term administration of morphine will cause extra spinal cord exosomal protein has yet to be verified, so we use immunofluorescence staining to detect

whether morphine induces spinal cord exosomal protein expression, as shown in Figure 5.

Table 3. 4-PBA or TUDCA tolerance to morphine data sheet; A) Time (Day), B) Saline + Saline, C) Morphine + Saline, D) Morphine + 4-PBA (5μg), E) Morphine + 4-PBA (15μg), F) Morphine + 4-PBA (50μg), G) Morphine + TUDCA (50μg), H) Morphine + TUDCA (100μg), I) Morphine + TUDCA (200μg)

A	B	C	D	E	F	G	H	I
0	3.42	2.23	2.56	3.32	2.72	2.13	2.10	2.04
1	2.89	8.31	9.13	9.19	9.02	9.32	9.40	9.28
2	4.17	8.69	9.36	9.38	9.35	9.30	9.56	9.52
3	2.92	7.36	8.72	9.20	8.73	8.78	9.35	9.59
4	3.49	7.29	8.35	8.91	9.10	8.16	9.21	9.39
5	2.96	5.99	7.92	8.52	7.83	7.03	9.35	9.61
6	3.57	5.92	7.43	8.11	7.52	7.81	7.32	9.31
7	3.03	5.85	6.86	8.15	7.19	6.62	6.56	9.01

Table 4. Data sheet of exosomal protein inhibitors on morphine-induced hyperalgesia; (A) Time (Day), (B) Morphine + Saline, (C) Morphine + 4-PBA (5μg), (D) Morphine + 4-PBA (15μg), (E) Morphine + 4-PBA (50μg), (F) Morphine + TUDCA (50μg), (G) Morphine + TUDCA (100μg), (H) Morphine + TUDCA (200μg)

A	B	C	D	E	F	G	H
0	23.62	23.14	25.67	17.25	22.98	21.73	24.25
7	7.85	7.15	10.35	8.75	8.15	11.25	18.50

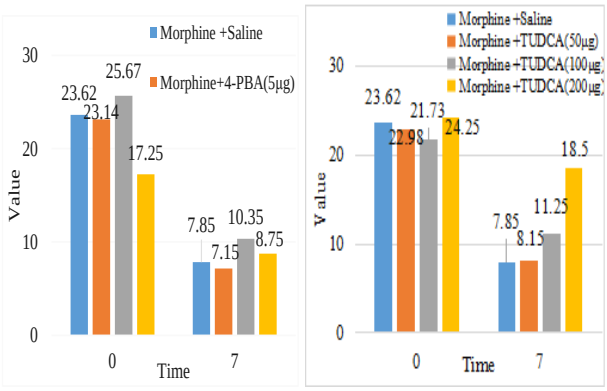

Figure 4. Effects of 4-PBA or TUDCA on the development of morphine tolerance

It can be seen from Figure 5 that intrathecal administration of morphine twice a day for seven consecutive days can cause the accumulation of unfolded protein in the spinal cord, which in turn causes the expression of the exosomal protein. It is proved that it can selectively bind protein aggregates, especially β-sheet protein, and can be used as a tool for detecting protein aggregates as a tool for measuring the stress level of the endoplasmic reticulum in living cells.

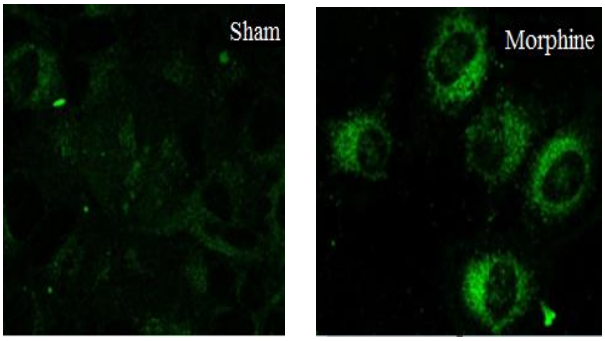

Figure 5. Enhancement of ThT fluorescence in the spinal cord of morphine-tolerant rats *(Picture source: https://baike.baidu.com/item/)*

In order to further explore the prolonged treatment of intrathecal morphine which can cause exosomal proteins, we detected the mRNA expression levels of exosomal protein-related genes (ATF4, GRP78, CHOP and XBIP1s) by fluorescence quantitative PCR. The experiment was divided into four groups: Sham group, morphine group, morphine+TUDCA group and morphine+4-PBA group. The intrathecal administration was administered twice a day for seven consecutive days. On the seventh day, most of the spinal lumbar expansion was taken for the Q-PCR experiment. The results are shown in Table 5.

It can be seen from Figure 6 that the mRNA of spinal cord exosomal protein-related genes in morphine-tolerant rats was significantly increased, and the rats given exosome protein inhibitors could prolong morphine analgesia and relieve morphine tolerance and hyperalgesia. , mRNA level can inhibit the increase of exosomal protein-related genes.

Exosomal proteins are often overexpressed or ectopically expressed under stress and are involved in the process of signal transduction. Among them, HSP60 is involved in a variety of clinical-pathological processes, as well as immune-related diseases such as transplant rejection (16).

Table 5. Morphine causes exosomal protein mRNA level changes data table

mRNA Types	Sham	Morphine + Saline	Morphine + TUDCA (100μg)	Morphine + 4-PBA (50μg)
ATF4	1.12	1.78	1.16	0.98
GRP78	1.21	2.11	1.36	1.05
CHOP	1.17	0.93	0.81	1.14
XBIP1s	1.46	1.72	1.25	1.11

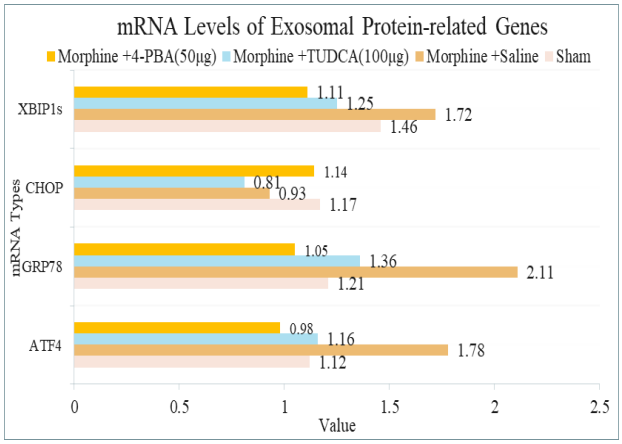

Figure 6. Morphine causes changes in mRNA levels of exosomal protein-related genes

At present, the use of single tumor markers for diagnosis often cannot accurately predict the occurrence, development and postoperative evaluation of cancer (12). With the development of genome, proteomics and metabolomics, studies have found that the occurrence of complex diseases such as cancer is accompanied by changes in many types of molecules. Compared with a single type of marker, the use of multiple molecular changes to predict the occurrence and development of diseases can provide more accurate diagnostic information. Therefore, the development of a platform that can be used for multi-level detection is extremely critical (17, 18). Many current biological analysis methods are relatively simple, and their applications are quite limited because they are difficult to transform into other types of molecular detection. However, general recognition probes are usually limited to nucleic acids or aptamers. Therefore, it is extremely important to develop a platform compatible with various types of molecules including nucleic acids, proteins, and small molecules (19, 20).

Exosomes contain a variety of substances, of which the most widely studied is miRNA. Since this region can participate in the regulation of subcellular localization, nuclear transport and stabilization of transcripts, miRNA molecules can regulate many target genes. It has been found that about >60% of all Human genes are regulated by miRNA molecules (21, 22). They are abnormally expressed in many human cancer types. Among all miRNA molecules, their proportion in exosomes is higher than that of the source cells, which indicates that the components in exosomes are actively regulated by cells rather than random packaging.

Endoplasmic reticulum stress is involved in regulating the activation of NLRP3 and the release of LCN2. The endoplasmic reticulum is the main organelle for the synthesis and folding of secreted proteins and transmembrane proteins. These activities depend to a large extent on quality control mechanisms to ensure that newly synthesized proteins are correctly folded and accurately allocated to the appropriate secretory pathway. Different stress signals, including oxidative stress or the imbalance of calcium homeostasis in the endoplasmic reticulum, can lead to the accumulation of unfolded protein (23, 24). This will trigger a series of biochemical and transcriptional responses aimed at rebuilding the homeostasis of the endoplasmic reticulum, and at the same time reduce the overall protein synthesis, the unfolded protein response. The endoplasmic reticulum stress signaling pathway is shown in Figure 7. Endoplasmic reticulum stress activates LCN2 through the PERK-ATF4 signaling pathway, and inhibiting the activation of LCN2 can alleviate the promotion of inflammation and apoptosis caused by endoplasmic reticulum stress, and the endoplasmic reticulum stress inducer TG induces endoplasmic reticulum stress At the same time, it can promote the increase of LCN2 expression. Therefore, in the process of morphine tolerance, whether endoplasmic reticulum stress-induced NLRP3 inflammasome and LCN2 play a role is the focus of our research (25, 26).

In this study, continuous intrathecal injection of morphine was used to establish a morphine analgesia tolerance model. Western blotting results confirmed that the expression of the exosomal protein in the morphine group was significantly increased, and exosomal protein can significantly improve morphine tolerance. Exosomal proteins can reduce morphine tolerance by inhibiting the activation of microglia and the release of inflammatory factors. The TLR4-p38MAPK signaling pathway participates in this

process, providing exosomal proteins for clinical prevention and treatment of morphine tolerance.

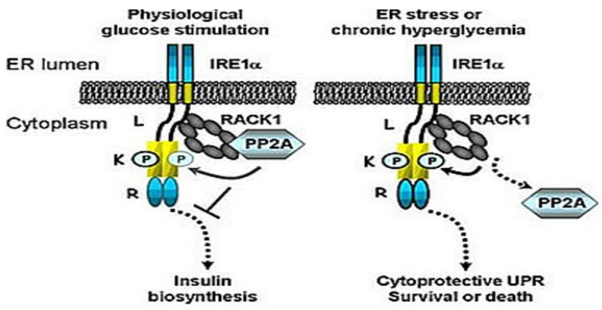

Figure 7. Endoplasmic reticulum stress signaling pathway *(Picture source: https://baike.baidu.com/item/)*

The endoplasmic reticulum is the main organelle for the synthesis and folding of secreted proteins and transmembrane proteins. These activities depend to a large extent on quality control mechanisms to ensure that newly synthesized proteins are correctly folded and accurately allocated to the appropriate secretory pathway. In this paper, Western Blot was used to verify the changes in the protein levels of the three signaling pathways of spinal cord exosomal proteins in morphine-tolerant rats. Western Blotting results showed that all three signaling pathways of exosomal proteins in the spinal cord of morphine-tolerant rats were activated and could be blocked by exosomal protein inhibitors. The experiment further confirmed that exosomal proteins are involved in morphine tolerance and hyperalgesia. development of.

Glutamate transporters play a vital role in the glutamatergic regulation of physiological glutamate homeostasis, neurotoxicity and opioid tolerance. Intrathecal administration of the exosomal protein inhibitor TUDCA or 4-PBA at the protein level can prevent the down-regulation of the glutamate transporter GLT-1 caused by morphine. Chronic morphine exposure does not cause changes in the glutamate transporter at the mRNA level. In in vitro experiments, chronic morphine exposure to protein and mRNA levels can cause the down-regulation of GLT-1, and the exosomal protein inhibitor TUDCA or 4-PBA can prevent this change.

Acknowledgements

None.

Interest conflict

None declared.

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