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Original Research Article

Studies on *in vitro* binding of parecoxib to p38MAPK using spectroscopic methods

Xuejie Li[1], Kun Huang[1], Shan Zhong[2]*
[1]Department of Anesthesiology, West China Hospital, Sichuan University and The Research Units of West China (2018RU012), Chinese Academy of Medical Sciences, [2]Department of Anesthesiology, Children's Hospital of Nanjing Medical University, Nanjing 210004, China

For correspondence:* ***Email:* *tintin0211@163.com;* ***Tel:*** *+86-13002199949*

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Abstract

Purpose: *To investigate the interaction between parecoxib and p38MAPK under simulated physiological conditions.*
Methods: *The interaction between parecoxib and p38MAPK was studied under simulated physiological conditions using spectroscopy-based methods. The effect of parecoxib on the microenvironment and conformation of p38MAPK chromophore was studied by synchronous fluorescence spectroscopy, three-dimensional fluorescence, time-resolved fluorescence spectroscopy, circular dichroism spectroscopy, and ultraviolet-visible absorption spectroscopy.*
Results: *Synchronous fluorescence spectroscopy showed that addition of parecoxib changed the structure of p38MAPK and destroyed the original stable structure. Three-dimensional fluorescence spectroscopy showed that the hydrophilicity of the microenvironment in which the fluorescent chromophore is located was enhanced, and the polarity increased such that the serum protein macromolecules tend to be unfolded, and the alpha-helix content reduced. Time-resolved fluorescence spectroscopy showed that the presence of parecoxib hardly affected the fluorescence quenching of p38MAPK, and the combination of parecoxib and p38MAPK forms a stable complex (static quenching). Circular dichroism spectroscopy revealed the combined parecoxib change, the secondary structure of p38MAPK and reduced the alpha-helix content. Ultraviolet-visible absorption spectroscopy revealed changes in the microenvironment of the three amino acid residues as well as the tertiary structure of the protein.*
Conclusion: *The results shows that parecoxib has a significant effect on the structure of p38MAPK. In addition to explicitly inhibiting COX-2 and blocking arachidonic acid synthesis of prostaglandins, it inhibits the pathway involved in p38MAPK.*

Keywords: *Parecoxib, P38MAPK, Alpha-helix content, Fluorescence quenching, Spectroscopic study*

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INTRODUCTION

Mitogen-activated protein kinase (MAPK) is a relatively common serine/threonine-protein kinase. It is a recombinant protein expressed in *Escherichia coli*. It is now a promising area of research in signal transduction. In the 1990s, p38MAPK was reported by Brewster *et al* [1], in

studying the role played by fungi in hypertonic environments. This substance was then found in mammalian cells. Analysis of the substance revealed that it belongs to a subtype of MAPKs. Such substances have the same function as JNK and are protein kinases with stress-activated properties. Mitogen-activated protein kinase, p38MAPK play a crucial role in many cell responses, such as stress and inflammation mediation, and is closely related to cell development, growth, and differentiation [2].

Spinal dorsal horn microglia express a variety of neurotransmitter receptors, and the activation of p38MAPK in spinal microglia is associated with a variety of pain-related mediators (such as TNF-α, IL-1β, COX-2, brain-derived neurotrophic factor, etc.). These mediators have a direct regulatory relationship and participate in neuropathic pain caused by nerve damage [3]. At the same time, some studies have shown that p-p38MAPK, the activator of p38MAPK, is positively correlated with COX-2 protein expression and inhibition of p38MAPK phosphorylation leads to significant inhibition of COX-2 protein expression. Parecoxib has been shown to reduce the expression of COX-2 protein by inhibiting the pathway involved in p38MAPK [4]. In addition to explicitly inhibiting COX-2 and blocking arachidonic acid synthesis of prostaglandins, it also partially inhibits the pathway involved in p38MAPK. This study was therefore aimed at investigating the interactions between parecoxib and p38MAPK under simulated physiological conditions.

EXPERIMENTAL

Reagents and preparations

p38MAPK solution

Protein was quantified using bicinchoninic acid method and concentration of protein mother liquor was determined. The considered protein solution was added to the protein mother liquor and ultrapure water was added to a volumetric flask with a capacity of 100 mL. After shaking properly, a concentration of 1×10^{-5} mol/L p38MAPK stock solution was obtained. The stock solution was stored in a refrigerator at a temperature of 0 – 4 °C and then diluted according to experimental requirements. The phosphate buffer was prepared by dissolving 1.56 g of sodium dihydrogen phosphate and 3.58 g of disodium hydrogen phosphate in ultra-pure water to produce a concentration of 0.1 mol/L. The pH of the solution was adjusted to 7.4 using a pH meter (S400-K, Seven Compact, Swiss). A concentration of 0.1 mol/L phosphate buffer solution was used throughout the experiment. Parecoxib was dissolved in ultra-pure water (1×10^{-3} M) as a stock solution before use.

Fluorescence measurement

Phosphate buffer (1 mL, pH 7.4) was added in series to 10 mL colorimetric tubes. Thereafter, 1 mL 1.0×10^{-5} mol/L p38MAPK and varying concentrations of parecoxib solution were added. The sample was analyzed with an excitation wavelength of 278 nm using fluorescence spectroscopy (Hitachi F-7000, Hitachi, Japan) under specific conditions (excitation light and emission light wavelength range maintained at 200 - 500 nm, fluorescence excitation and emission slit width was 5.0 nm, photomultiplier tube voltage was set at 700 V, and the measurement speed was 12000 nm/min). Experimental conditions include the following: p38MAPK 1.0×10^{-6} mol/L; parecoxib 1 - 4: 0, 1, 2, 3×10^{-4} M; pH 7.4, T = 298 K. In three-dimensional fluorescence spectroscopy, p38MAPK was used at 1.5×10^{-6} mol/L; the concentration of parecoxib in the parecoxib-p38MAPK system (A - B) 0, 5×10^{-4} mol/L. Furthermore, in the time-resolved fluorescent measurement, the conditions were set at p38 MAPK 1.0×10^{-6} mol/L; parecoxib 0, 5×10^{-4} M; pH 7.4; T = 298 K.

Ultraviolet-visible absorption spectrometry

Phosphate buffer (1 mL pH 7.4), 1 mL of 1.0×10^{-5} mol/L p38MAPK solution, and 1×10^{-3} mol/L parecoxib stock solution were placed in a 10 mL colorimetric tube and centrifuged in series of concentration protein-drug reaction systems. The spectra was obtained using a UV-visible spectrophotometer (UV-2500 Shimadzu, Tokyo, Japan) at wavelength within 200 - 450 nm, width of cuvette and instrument slit was kept at 10 mm and 2.0 nm, respectively.

Circular dichroism determination

The phosphate buffer (1 mL) was mixed with 0.1 ml of 1.0×10^{-5} mol/L p38MAPK solution and graded concentration of parecoxib solution (1.00×10^{-3} mol/L). A dichroic spectrometer (J-815 JASCO, Japan Spar Corporation) was used to analyze the sample at specified conditions (scanning speed at 200 nm/min, and wavelength interval of 1.0 nm). The scans were done in triplicate and average taken. High-purity nitrogen (10 L/min) was used to protect the deuterium lamp of the instrument during measurement. Circular dichroism spectrum was carried out at a concentration of 2×10^{-7} mol/L p38MAPK, 0, 1, 2×10^{-5} mol/L parecoxib in parecoxib-p38MAPK system, and the reference pool contains the

same concentration of parecoxib. Absorption spectrum was carried out at a concentration of 1 × 10^{-6} mol/L p38MAPK, 0, 1.5 × 10^{-4} mol/L parecoxib in parecoxib-p38MAPK system while the reference pool contains the same concentration of parecoxib.

RESULTS

Fluorescence spectrometry

The result revealed that in the environment of pH 7.4, the resonance light scattering intensity signal of p38MAPK was low, and after mixing, the light scattering signal became stronger. The maximum scattering peak can be observed at 325 nm, and the light scattering signal was found to be very high, stable and strong. As the concentration of the drug increases, the intensity increases.

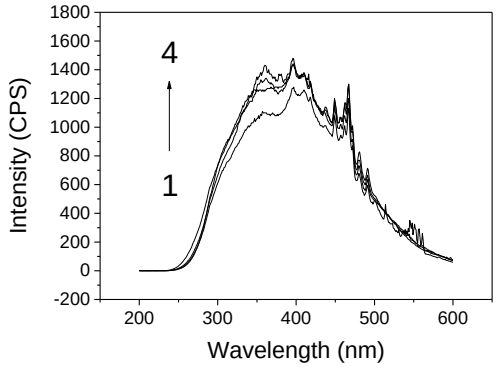

Figure 1: The effect of parecoxib on resonance light scattering of p38MAPK

Three-dimensional fluorescence spectroscopy

Three-dimensional fluorescence spectra of the p38MAPK and p38MAPK-parecoxib systems are shown in Figure 2 A and B respectively. The p38MAPK solution produced three peaks: peak A was the Rayleigh scattering peak of water, peak B was the fluorescence peak of the protein, and peak C was the backbone structure peak of the protein. When concentration of p38MAPK was 1.5 × 10^{-6} mol/L, the relative fluorescence intensity at the top of peak B and the peak top coordinates (F, λem/λex) are (7538, 280.0/339.5). When parecoxib was added, the relative fluorescence intensity and peak top coordinates (F, λem/λex) of the peak top of p38MAPK are (4210, 280.0/338), and the fluorescence intensity was reduced by 44.2 %. The relative fluorescence intensity and peak top coordinates (F, λem/λex) of peak C are (8742, 220.0/347.5). When parecoxib was added, the ratio of its concentration to protein concentration was 333:1, the peak top of p38MAPK. The relative fluorescence intensity and peak-top coordinates (F, λem/λex) are (5893, 220.0/348.5), and the fluorescence intensity was reduced by 32.6 %.

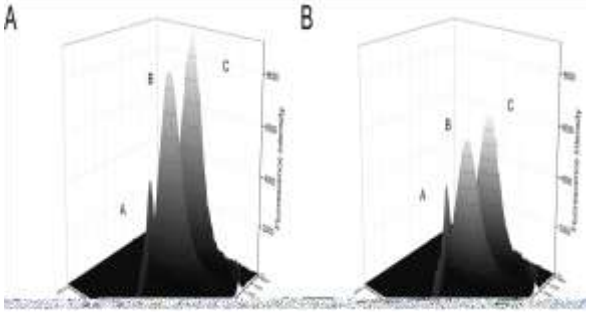

Figure 2: Resonance scattering spectra of p38MAPK in the presence of different concentrations of parecoxib

Time-resolved fluorescence measurement

Time-resolved fluorescent measurement revealed that the presence of parecoxib hardly affected the fluorescent quenching of p38MAPK, with the protein lifetime varying from 7.96 to 7.90 nS (Figure 3).

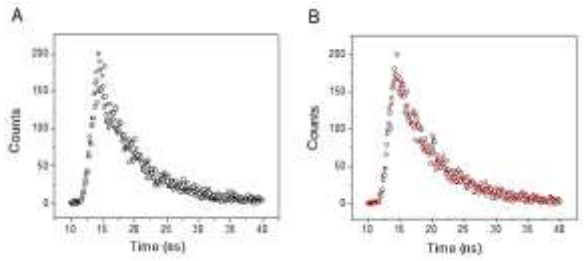

Figure 3: Effect of parecoxib on the fluorescence lifetime of p38MAPK

The effect of parecoxib and p38MAPK binding on protein structure

To investigate the effect of the combination of parecoxib and p38MAPK on the secondary structure of p38MAPK, the circular dichroism of parecoxib and p38MAPK present at various concentrations was analyzed (Figure 4 A). The circular dichroism chromatogram obtained by determination of p38MAPK has a total of two negative peaks, each of which is located at a distance of 208 and 222 nm, and accurately expresses the secondary structure of the protein α helix. When a certain concentration of parecoxib was added to p38MAPK system, the α helix of p38MAPK decreased from 56.3 % to 54.2 and 50.9 %, respectively (Table 1).

Table 1: Protein secondary structure content of parecoxib-p38MAPK system

Sample	Structural elements			
	α-Helix (±3%)	***β-Sheet (±1%)***	***β-Turn (±1%)***	***Random coil (±3%)***
1	56.3	8.2	11.2	25.4
2	54.2	9.2	11.4	26.0
3	50.9	10.5	12.5	26.9

When a certain concentration of parecoxib was added to the p38MAPK system, the α helix of p38MAPK decreased from 56.3 to 54.2 % and 50.9 %, respectively

Ultraviolet–visible absorption spectra

To study the effect of parecoxib on the structure of p38MAPK, the absorption spectra of p38MAPK in the presence of different concentrations of parecoxib were measured (Figure 4), and the result revealed that p38MAPK has two absorption peaks.

At 0 concentration of parecoxib, only some skeleton structures on the surface were exposed to water. At this time, the π→π* electron cloud transition energy of C=O in the skeleton structure of p38MAPK was lower. After parecoxib enters the protein molecule, the protein structure became loosed, and there were more spaces to contact water molecules. The overall environment of the skeleton structure became hydrophilic.

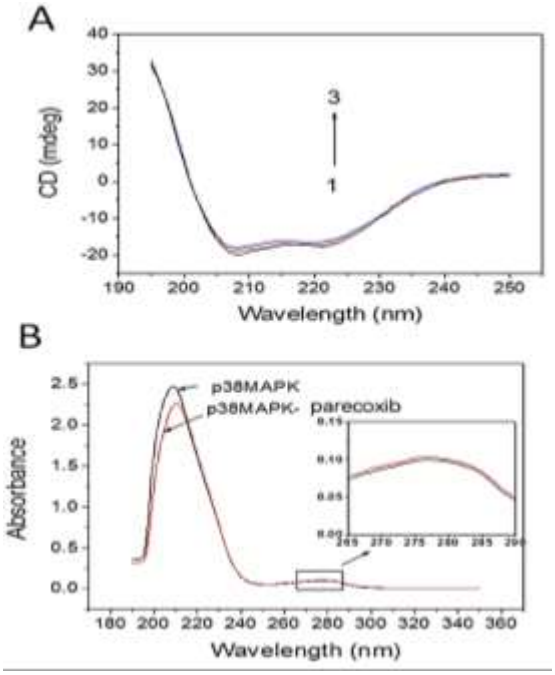

Figure 4: The circular dichroism spectrum (A) and absorption spectrum (B) of p38MAPK in the presence of different concentrations of parecoxib

DISCUSSION

The mitogen-activated protein kinase (p38MAPK) is a relatively common serine/threonine-protein kinase, and it is also an essential component of mitogen-activated protein kinases. Recent studies have shown that changes in expression and activity of p38MAPK effectively regulate critical signaling pathways and effectively control cell apoptosis [5,6]. Parecoxib inhibits COX-2 pathway, p38MAPK participates in the regulation of COX-2 transcription and synthesis of prostaglandins. When the p38MAPK pathway is inhibited, the expression of COX-2 in the dorsal horn of the spinal cord is significantly reduced, blocking the synthesis of prostaglandins and alleviating neuropathic pain. Fluorescence spectroscopy technology is an important method for studying the combination of body proteins and drugs and their functional mechanism. Some molecules of substances fluoresce after absorbing energy, The wavelength difference effectively distinguishes the unique fluorescent chromophores in protein molecules. Subsequent changes in protein conformation are effectively recorded due to specific changes in wavelength of the emission spectrum. The difference between three-dimensional fluorescence spectroscopy and general fluorescence analysis is that it obtains information on fluorescence intensity as emission wavelength and excitation wavelength changes. In this study, addition of parecoxib changed the structure of p38MAPK and destroyed the original stable structure. Thus, p38MAPK changed from the original structure to the activated structure.

In three-dimensional fluorescence spectrum, the luminescence level changes with the emission wavelength, and the excitation wavelength is measured. The maximum fluorescence emission wavelength of amino acid residues in proteins usually depends on the polarity of their environment. Studies show that enhancing the hydrophilicity of the microenvironment in which the fluorescent chromophore is located, serum protein macromolecules tend to be unfolded, and the alpha-helix content is reduced. In this study, parecoxib may be distributed in the hydrophobic area of the p38MAPK cavity, resulting in the formation of a new disordered structure of p38MAPK [7,8]. Time-resolved fluorescence spectroscopy technology has been applied in various research fields such as materials, chemistry, and medicine. If the corresponding excitation is performed by a pulsed laser light source, the time scan of the fluorescence intensity of the fluorescent substance is completed after the excitation is stopped so that the fluorescence decay curve of the molecule

over time is depicted [9-11]. Molecules of different substances have different fluorescence lifetimes. If the fluorescence lifetime is very long, it reaches the microsecond level, while the shorter lifetime reaches the nanosecond level. Protein fluorescence lifetime change was used to judge the type of p38MAPK fluorescence quenching caused by drugs. There are two main types of fluorescence quenching, namely; dynamic quenching and static quenching. Dynamic quenching means that both the fluorescent substance and the quencher have molecular diffusion behavior, and some are caused by collision. However, static quenching is mainly due to the interaction between the quencher and the fluorescent substance to form a stable compound [12,13]. A more effective way to distinguish between static quenching and dynamic quenching is to measure the fluorescence lifetime of the system. Normally, static quenching will not bring about changes in the survival time of fluorescent substances. The presence of parecoxib hardly affects the fluorescence quenching of p38MAPK, and the combination of parecoxib and p38MAPK forms a stable complex (static quenching). Circular dichroism spectroscopy has been widely used in biochemistry, organic chemistry, pharmaceuticals, and coordination chemistry and has gradually become a more important method in experimental research of three-dimensional configuration of organic compounds [14-16]. Circular dichroism spectrum curve is mainly distinguished according to the absorption level of circularly polarized light when the active material is left-handed and right-handed, and the difference between the wavelength (λ) and the absorbance obtained at various wavelengths is fitted as a curve relationship. By analyzing the specific structure of the protein molecule, each part of the peptide chain can obtain a very specific structure, such as α-helix, β-turn, and β-sheet three-dimensional structures. The decrease in the alpha-helix content of p38MAPK indicates that bound parecoxib changed the secondary structure of p38MAPK, which may affect the physiological function of p38MAPK. Parecoxib causes the p38MAPK protein backbone to unfold, become loose, and increase the hydrophobicity of the microenvironment of its tryptophan residues [17]. The combined parecoxib changed the secondary structure of p38MAPK and reduced the alpha-helix content.

Ultraviolet–visible absorption spectrum is obtained by partially widening the absorption band. Ultraviolet–visible spectrophotometry analyze the bone valence structure of proteins and studies the microenvironment changes in the presence of some amino acid residues. For proteins, results show that in 280 nm wavelength range, the strong absorption position of aromatic amino acids remains unchanged. At a wavelength of 280 nm, tryptophan has a very distinctive absorption peak. The peptide bond absorption band of the protein backbone is formed at 190 – 230 nm. Generally, the corresponding absorption peak appears at 210 nm [18,19]. With the continuous addition of parecoxib to p38MAPK solution, the weak absorption peak at approximately 279 nm appeared as a hypochromic effect, indicating that the interaction of parecoxib and p38MAPK unfolded the tertiary structure of p38MAPK, and aromatic ring caused by microenvironment amino acid residues increased the corresponding hydrophobicity. This phenomenon shows that the microenvironment of the three amino acid residues changed to a certain extent, and tertiary structure of the protein changed correspondingly.

CONCLUSION

The results show that parecoxib has a very significant effect on the structure of p38MAPK. Furthermore, parecoxib reduces COX-2 protein expression. In addition to explicitly inhibiting COX-2 and blocking arachidonic acid synthesis of prostaglandins, it inhibits the pathway involved in p38MAPK.

DECLARATIONS

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Funding

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities

pertaining to claims relating to the content of this article will be borne by the authors. Xuejie Li conceived and designed the study, and drafted the manuscript. Kun Huang collected, analyzed, and interpreted the experimental data. Shan Zhong revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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