Squid ink polysaccharide reduces cyclophosphamide-induced testicular damage via Nrf2/ARE activation pathway in mice

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ABSTRACT

Objective(s): Cyclophosphamide (CP) toxicity on testis was hampered by squid ink polysaccharide (SIP) via restoration of antioxidant ability in our previous investigations. This study investigated roles of Nrf2/ARE signal pathway in testis of treated mice.

Materials and Methods: Male Kunming mice were employed to undergo treatment with SIP and/or CP. Protein levels of Nrf2, keap-1, histone deacetylase 2 (HDAC2), quinone oxidoreductase 1 (NQO-1), and heme oxygenase 1 (HO-1) and phosphorylation level of protein kinase C (PKC) in testis were evaluated by Western blotting.

Results: Data showed that SIP elevated expressions of NQO-1 and HO-1 genes, two downstream target molecules of Nrf2, via activating Nrf2 to play preventive roles on CP-treated testis, and further discovered that upstream regulators of Nrf2, keap-1, HDAC2, and PKC, were concerned with the regulation of Nrf2.

Conclusion: These results suggest that SIP could effectively weaken CP-associated testicular damage via Nrf2/ARE signal pathway.

Introduction

Chemotherapy is known to be a critical remedy for tumors, and cyclophosphamide (CP) is used as a common chemotherapeutic drug for cancer and immunosuppression for nephritic disorders and lupus erythematosus, while negative roles of CP on male reproductive functions have been widely recognized for many years, which result in dose reduction of antineoplastic drugs and leads to a decline of chemotherapeutic effects (1-3). Hence, weakening toxic damage of CP on normal tissues and increasing dose of CP for elevation of therapeutic effects in patients have been taken as purposes for developing cytoprotectors to be used as assistant drugs for chemotherapy.

In recent years we proved squid ink polysaccharides (SIP), a type of glycosaminoglycan with a unique structure -[3GlcAβ1-4(GalNAcα1-3)-Fucα1]n-(4, 5), to have antioxidative activities and chemoprotective roles in vitro and/or in vivo (6-10). Our previous investigations revealed that the chemoprotective activities of SIP were observed in liver, lung, heart, kidney, marrow, and testis of model animals exposed to CP through improving antioxidant abilities of the organs (6), which implies that SIP can prevent testis from CP-induced oxidative stress damage.

Nrf2 (NF-E2-related factor 2) is an important transcription factor that binds to AREs that are important gene regulatory elements of many phase II drug-metabolizing/detoxification enzymes as well as cellular defensive enzymes and regulates anti-oxidative stress and plays a concernful role in eliminating intracellular superfluous reactive oxygen species (ROS) and improves antioxidant ability (11). Some bioactive substances have been proved to activate Nrf2 to relieve or prevent diseases correlated to oxidative stress, such as astaxanthin (12) and curcumin (13, 14). However, it is still unknown whether SIP impairs CP-caused testicular damage through activating Nrf2 to upregulate expression of antioxidative enzyme genes and phase II enzyme genes subjected to maintaining redox equilibrium. In this study, mice were employed to prove the hypothesis in vivo.

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Materials and Methods

Preparation of SIP

Live squids purchased from a local aquatic products market were sacrificed to harvest fresh ink sacs that were then stored at -28 °C for future use. According to our previous methods (6), ink collected from sacs thawed at 4 °C and was suspended with pH 6.7 PBS, and was then ground and ultrasonically treated. The resultant ink solution was stored at 4 °C for 24 hr and was then centrifuged at 14000 g for 1 hr at 4 °C. The supernatant was subjected to enzymolysis with 1% papain in PBS (pH 6.7) at 60 °C for 24 hr, and was then mixed with a 1/4 volume liquid mixture of chloroform and n-butanol (v/v, 4/1) followed by stirring for 30 min on a magnetic stirrer plate. After centrifugation at 5000 g for 15 min, the supernatant was re-digested with papain, the digestion process was performed twice. SIP in the resulting supernatant was precipitated with four volumes of absolute alcohol, and was subjected to freeze-drying in a vacuum. Solid powder SIP was stored at 4 °C before use.

Animal experimental scheme

Following habituation for 1 week, sexually mature male Kunming mice purchased from the Experimental Animal Centre of Guangdong Medical College were allocated to four groups (ten mice per group): a control group, a CP-treated group, a SIP-treated group, and a co-treated group (SIP and CP). The SIP dose was 80 mg/kg body weight, once a day for a continuous ten week period, and the CP dose was 15 mg/kg body weight, once a week (again for a continuous ten week period).

Western blotting analysis

Testes were collected from mice that were sacrificed by breaking neck vertebrae and were quickly cleaned with ice-cold normal saline. Testis was minced and homogenated in ice-cold normal saline with a glass homogenizer. Testis homogenate was centrifugated at 3000 rpm for 10 min at 4 °C. The harvested supernatant was denatured in protein sample buffer for 5 min in boiling water. After SDS-PAGE, protein was transferred to nitrocellulose membrane and then probed with monoclonal antibody, against Nrf2 (Cell Signaling), β-actin (Cell Signaling), keap-1 (Cell Signaling), HDAC2 (Cell Signaling), p-PKC (Cell Signaling), NQO-1 (abcam), or HO-1 (abcam), which would be captured by the secondary antibody conjugated with horseradish peroxidase (Santa Cruz). The membrane was visualized with SuperSignal West Pico chemiluminescence detection system (Pierce). These protein levels were normalized with β-actin.

Statistical analysis

Value is expressed as mean±standard error. Statistical analysis was performed using ANOVA by JMP 7.0 software. Differences were separated by Duncan’s multiple range test. Significance was considered at P<0.05 or P<0.01.

Results

To determine roles of Nrf2 in mice testis treated with SIP and/or CP, in this study we detected the expression level of Nrf2 gene in testis and the results were presented in Figure 1. The data showed that CP obviously decreased protein content of Nrf2 in testes of model mice. Our data declared apparently an insignificant result that PKC was not phosphorylated in testes of mice exposed to CP. However in SIP-treated mice degree of PKC phosphorylation was markedly improved, meanwhile expression level of Nrf2 gene was increased sharply in testes, the similar positive effects of SIP were observed in testes of CP-administered mice.

![Figure 1](image-url)
SIP reduces CP-induced testicular damage

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Figure 2. Contents of keap-1 and histone deacetylase 2 (HDAC2) proteins in testes of mice. Values with different lowercase superscripts mean significant difference ($P<0.05$), those with different capital letters are extremely different ($P<0.01$)

In comparison with CP-treated mice, a marked increase of expression of keap1 gene in testes from SIP-exposed and co-treated mice. For a regulatory enzyme that can stabilize Nrf2 protein through deacetylation, expression content of HDAC2 gene was not affected by CP in mice testes, but obvious increases of the protein content of HDAC2 were observed under stimulation of SIP that is presented in mice of both SIP- and co-treated groups (Figure 2).

As shown in Figure 3, data showed that CP did not obviously change expression level of HO-1 gene but sharply declined the content of NQO-1 protein in testes, and that SIP not only markedly improved contents of HO-1 and NQO-1 in normal mice, also significantly increased levels of the two proteins in CP-treated mice.

Discussion

Acrolein, a cyclophosphamide’s metabolite induces body to produce superfluous ROS that cause tissues to undergo oxidative stress and/or apoptosis (15). To avoid the damages induced by CP or other oxidative stressors, cells develop a series of signal regulative mechanisms to keep intracellular redox equilibrium (16), Nrf2/ARE is one of their important pathways. Under normal conditions, Nrf2 is kept fairly low level in cytoplasm through binding to keap1 or rapid degradation. However stimulation by drugs or other oxidative stressors can elevate dissociative content of Nrf2, release from keap1 or up-regulating expression of Nrf2 gene, which enters the nucleus to bind ARE of the downstream genes and then to stimulate expression of the genes, such as phase II enzyme genes, antioxidant enzyme genes and protein kinase genes, which results in immediate detoxication and maintaining intracellular homeostasis. Nrf2 widely distributed in any of various organs plays a concerned role in protection of the organs, deletion or active disturbance of the factor should lead cells to more sensitivity to oxidative stress (17). This study revealed that CP reduced protein content of Nrf2 in testis which was in accordance with Tripathi’s report (18). Presently, it is known that Nrf2 is phosphorylated by PKC to be

Figure 3. Contents of heme oxygenase 1 (HO-1) and quinone oxidoreductase 1 (NQO-1) proteins in testes of mice. Values with different lowercase superscripts mean significant difference ($P<0.05$), those with different capital letters are extremely different ($P<0.01$)
activated (19, 20). Although PKC failed to be phosphorylated in CP-exposed mice testis, it was activated under treatment together with SIP. Nrf2 was regulated by other upstream molecules besides PKC, such as keap1 and HDAC2. Keap1 is identically considered to be an inhibitor of Nrf2. Under normal conditions, Nrf2 is degraded through ubiquitin-proteasome pathway after the protein is bound in the Neh 2 domain by keap1 (21), and is separated from keap1 for reasons such as phosphorylation of PKC on Nrf2 (19, 20), to play biological roles. In this paper we found SIP increased content of keap1 protein, which may be originated from positive feedback regulation by increase of Nrf2 protein content, which would be subjected to binding and decreasing dissociative Nrf2 protein in cytoplasm and combined Nrf2 protein bound to AREs in nucleus (22). The results suggested that SIP not only improved expression of keap1 gene in testes of normal mice, but also positively affected contents of the protein in testes of chemotherapeutic mice, which was subjected to resisting expression increase of Nrf2 gene. HDAC2 is an enzyme that can deacetylate Nrf2 protein to harvest stabilized protein that can activate the downstream effective molecules (23, 24), such as antioxidative enzymes and phase II enzymes. According to the data, CP did not change HDAC2 content in testes, but SIP significantly improved the expression level of HDAC2 gene in both SIP- and co-treated mice.

Although our data have proved upstream regulatory factors of Nrf2 were affected by stimulation of SIP, the regulation was just acted on Nrf2 protein. This paper discovered that SIP interfered with contents of Nrf2 protein as determined by Western blotting, which was similar to present reported antioxidants, such as green tea (25), astaxanthin (12) and curcumin (13, 14), and the metallic element zinc (26). The increase in Nrf2 protein may be originated from improvement of expression of Nrf2 gene caused by SIP.

From above mentioned information, activated Nrf2 protein plays roles through activating downstream effective molecules, which contain many enzymes, such as heme oxygenase 1 (HO-1) and quinone oxidoreductase 1 (NQO-1), which are two important enzymes in antioxidation and detoxification. Background researchers reported that Nrf2 binds to ARE through using bZIP (basic leucine zipper, a domain found in many DNA binding eukaryotic proteins) to form dimers with Maf (a transcription factor) and then activate the downstream genes, HO-1 and NQO-1; expression levels of the two genes were positively correlated with the Nrf2 gene (27-29). This study showed that SIP effectively changed effects of CP on expression levels of HO-1 and NQO-1 genes in testes.

Summarily, in this study we firstly found that SIP employed the Nrf2/ARE signal pathway to activate downstream target genes, HO-1 and NQO-1, to exert preventive roles against CP-induced damage on mice testis, and that PKC acted as an upstream regulator that participated in the regulative process on Nrf2. In addition, two other upstream molecules, keap1 and HDAC2, were also vital proteins in the process of regulation of SIP on activity of Nrf2. Thus, we can conclude that SIP triggers Nrf2/ARE signal cascades via regulating PKC, keap1 and HDAC2, whereas we are still ignorant of the accurate regulative mechanisms of SIP on PKC, keap1 and HDAC2, which would be an important content in our future research.

Conclusion

This study showed that SIP reduces CP-induced testicular damage via Nrf2/ARE activation pathway in mice. To our knowledge this study is the first to report that intervention of SIP on CP-mediated testicular damage is connected with Nrf2/ARE signalling pathway, which suggests that Nrf2/ARE may be an important signalling pathway that is used by SIP to weaken chemotherapeutic drugs-induced damage of male reproductive ability. Further investigation is necessary to learn more details about the regulatory mechanisms, the results would be helpful to developing SIP as a potential chemotherapeutic adjuvant drug.

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Conflict of interests

The authors declare no potential conflict of interests.

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