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The protective effect of lycopene on hypoxia/reoxygenation-induced endoplasmic reticulum stress in H9C2 cardiomyocytes

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Chemical compounds studied in this paper:
Lycopene (PubChem CID: 446925)
dimethyl sulfoxide (PubChem CID: 679)
thapsigargin (PubChem CID: 446378)
4-phenyl butyric acid (PubChem CID: 2733848)
methylthiazolyltetrazolium (PubChem CID: 64965)
lactic dehydrogenase (PubChem CID: 135297138)
sodium hydroxide (PubChem CID: 14798)
2, 4-dinitrobenzene hydrazine (PubChem CID: 3772977)

ABSTRACT

Nowadays, drugs protecting ischemia/reperfusion (I/R) myocardium become more suitable for clinic. It has been confirmed lycopene has various protections, but lacking the observation of its effect on endoplasmic reticulum stress (ERS)-mediated apoptosis caused by hypoxia/reoxygenation (H/R). This study aims to clarify the protective effect of lycopene on ERS induced by H/R in H9C2 cardiomyocytes. Detect the survival rate, lactic dehydrogenase (LDH) activity, apoptosis ratio, glucose-regulated proteins 78 (GRP78), C/EBP homologous protein (CHOP), c-Jun-N-terminal protein Kinase (JNK) and Caspase-12 mRNA and protein expression and phosphorylation of JNK (p-JNK) protein expression. LDH activity, apoptosis ratio and GRP78 protein expression increase in the H/R group, reduced by lycopene. The survival rate reduces in the H/R and thapsigargin (TG) groups; lycopene and 4-phenyl butyric acid (4-PBA) can improve it caused by H/R, lycopene also can improve it caused by TG. The apoptosis ratio, the expression of GRP78, CHOP and Caspase-12 mRNA and protein and p-JNK protein increase in the H/R and TG groups, weaken in the lycopene+H/R, 4-PBA+H/R and lycopene+TG groups. There is no obvious change in the expression of JNK mRNA or protein. Hence, our results provide the evidence that 10 μM lycopene plays an obviously protective effect on H/R H9C2 cardiomyocytes, realized through reducing ERS and apoptosis. The possible mechanism may be related to CHOP, p-JNK and Caspase-12 pathways.

1. Introduction

Myocardial ischemia-reperfusion injury (MIRI) refers to the phenomenon of the myocardial damage from restored blood flow after a period of myocardial ischemia, which is a kind of pathological phenomena (Çetin et al., 2001; Dongworth et al., 2014). In recent years, MIRI has increasingly caught the attention of clinicians (Çetin et al., 2001) because it can cause serious damage to patients' health (Brenner et al., 2014; King et al., 2014).

There are many mechanisms involved in the occurrence of reperfusion injury according to the literature (Bafakumar and Jagadeesh, 2011, 2010; Chong et al., 2003; Stamboul et al., 2015). An important role in I/R injury is played by ERS-induced apoptosis (Gajkowska et al., 2001; Logue et al., 2013). ERS refers to endoplasmic reticulum steady destruction, function disorder, causing a lot of protein misfolding and unfolding (Logue et al., 2013) and calcium imbalance.

Currently, many experiments have been conducted on protection against MIRI. Among them, although the myocardial protective effects of ischemic preconditioning (Murry et al., 1986) and ischemic postconditioning (Zhao et al., 2003) have been proved in many kinds of animal models, yet in actual clinical practice, they both have some difficulty in operation as they are relatively complex and thus not able to conform to current medical ethics. Therefore, drugs protecting against myocardial I/R are more

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suitable for clinical application, but until now researches on a variety of drugs have not got an ideal method to protect the myocardium (Van den Berg et al., 2014; Yin et al., 2013).

Lycopene, a carotenoid widespread in fruit and vegetables (Engelmann et al., 2011), is a natural antioxidant. It has various effects, such as improving the activity of various antioxidant enzymes (Hsu et al., 2008), maintaining normal cell metabolism (Maiani et al., 2009), regulating blood lipid metabolism (Ried and Fakler, 2011) and so on.

To date, research has confirmed that H/R can cause H9C2 cardiomyocyte ERS, increase the GRP78 protein expression, and lead to an increase in apoptosis of cardiomyocytes by activating the CHOP, p-JNK and Caspase-12 pathways. However, it is unknown whether lycopene can inhibit the CHOP, p-JNK and Caspase-12 pathways to decrease the expression of GRP78, CHOP, p-JNK and Caspase-12 protein to relieve ERS and protect H/R cardiomyocytes.

This study selected H9C2 cardiomyocytes to perform H/R injury and produce protection of cardiomyocytes with different concentrations of lycopene pretreatment. Methylthiazolyltetrazolium (MTT) was employed to test the survival rate of H9C2 cardiomyocytes. The apoptosis ratio of cardiomyocytes was detected by flow cytometry (FCM). Determination of GRP78, CHOP, JNK, Caspase-12 mRNA and protein expression and p-JNK protein expression by contrast with ERS inhibitor 4-PBA (Mimori et al., 2012) and specific ERS activator TG (Li et al., 2004) was used to clarify the protective effect and mechanism of lycopene on ERS in H/R H9C2 cardiomyocytes.

2. Materials and methods

2.1. H9C2 cardiomyocyte resuscitation, culture, disposal and cryopreservation

H9C2 cardiomyocytes (Cell Bank of the Chinese Academy of Sciences, China) were taken from liquid nitrogen and cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine blood, inoculated homogeneously into six-well plates and cultured for 24 h in a 5% carbon dioxide (CO_2) -humidified atmosphere at 37 °C in an incubator. We observed the status of the cardiomyocytes and then conducted fluid handling. After removing waste liquid, 2 ml phosphate buffer solution (PBS) was added into per well for cleaning cardiomyocytes and disposing of the waste liquid; cells continued to culture after addition of 2 ml fresh medium. The H9C2 cardiomyocytes were cleaned with 2 ml PBS while they grew to a density of about 90%; they were digested with 0.25% trypsin, and then the complete medium was subjected to the termination reaction once confluence was reached, and cells were taken for further experiments. When H9C2 cardiomyocytes grew to a density of 80–90%, in good status, in the logarithmic phase, the cardiomyocytes were placed in frozen storage tubes at −80 °C overnight then cryopreserved in a liquid nitrogen tank.

2.2. MTT cardiomyocyte survival assay

The survival rate of cardiomyocytes was determined by the MTT (Sigma, USA) colorimetric method. MTT (0.2 ml) was added to cardiomyocytes in each group that had reached the expected time, and incubated for 4 h at 37 °C in the incubator, then 200 μl dimethyl sulfoxide (DMSO) was added to dissolve the violet crystals formed by cardiomyocytes to determinate the absorbance value at 490 nm on the enzyme standard instrument. The percentage survival rate of cardiomyocytes = *light absorption value of experimental groups*/*light absorption value of control group* × 100%. Each experiment was performed for at least three times.

2.3. Best protective concentration of lycopene for H/R cardiomyocytes

In this part, cardiomyocytes were divided into eight groups. According to the experimental groups, H9C2 cardiomyocytes were cultured to a density of about 90% and incubated in 96-well culture plates at a density of 1×10^4/ml. The cardiomyocytes were cultured for 4 h in a 5% CO_2-humidified atmosphere at 37 °C in an incubator. After cultured cardiomyocytes were growing against the wall of culture plates, the liquid of seven groups was taken and changed for normal DMEM culture medium containing lycopene (Meilun, China), dissolved by DMSO, 0 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM and 20 μM respectively, cardiomyocytes were cultured for 4 h under the normal conditions, while globular adiponectin (gAd, Sigma, USA) group with the normal DMEM medium containing 2 μg/ml gAd under the normal conditions for 1 h. Then, the liquid in the 7 groups was exchanged for DMEM culture medium without glucose and serum in the tri-gas incubator (Shanghai, China) with a nitrogen (N_2) concentration of 95% and 5% CO_2 in a hypoxia incubator for 15 min in advance. Cardiomyocytes were cultured in the tri-gas incubator under hypoxia (37 °C, 95% N_2, 5% CO_2) for 4 h. Normal DMEM culture medium was replaced to make the cardiomyocytes reoxygenation (37 °C, 95% atmosphere, 5% CO_2) for 4 h in the tri-gas incubator. The control group was cultured under normoxic conditions, without being processed. The best protective concentration of lycopene filtered by MTT was *X* μM. Each experiment was performed for at least three times.

2.4. Experiment groups

Cardiomyocytes were divided into eight groups: (1) control group; (2) lycopene group: cardiomyocytes were incubated with *X* μM lycopene for 4 h under normoxic conditions; (3) H/R group: cardiomyocytes were treated under hypoxic conditions for 4 h then reoxygenation for 4 h (H4h/R4h); (4) lycopene+H/R group: cardiomyocytes were pretreated with *X* μM lycopene for 4 h before H4h/R4h; (5) gAd+H/R group: cardiomyocytes were pretreated with 2 μg/ml gAd for 1 h before H4h/R4h; (6) 4-PBA+H/R group: cardiomyocytes were pretreated with 1 mM 4-PBA (Sigma, USA), a classic ERS inhibitor, for 24 h before H4h/R4h; (7) TG group: cardiomyocytes were incubated for 24 h with 3 mM TG (Sigma, USA), a classic ERS inducer; and (8) lycopene+TG group: cardiomyocytes were pretreated with *X* μM lycopene for 4 h and then incubated with 3 mM TG for 24 h. The aim of groups 1–5 was to identify the relationship between the effect of the best protective concentration of *X* μM lycopene on H/R H9C2 cardiomyocytes and ERS, while the aim of groups 1–4 and 6–8 was to explore the protective mechanism under the specific concentration of lycopene which played a protective effect by reducing ERS induced by H/R on H9C2 cardiomyocytes.

2.5. Measurement of LDH activity

LDH activity was analyzed by LDH kits (Jiancheng, China), using a 2, 4-dinitrobenzene hydrazine colorimetry. After the experiments, culture supernatant was centrifuged at 1000 rpm for 5 min. Supernatant (20 μl) was taken, mixed with 25 μl 2, 4-dinitrobenzene hydrazine in a 37 °C thermostat aqueous bath for 15 min, and then placed in 250 μl 0.4 M sodium hydroxide (NaOH) to blend, then in a 37 °C thermostat aqueous bath for 15 min, keeping at room temperature for 5 min to determinate the absorbance at 490 nm on the enzyme standard instrument. All experiments were performed at least three times.