Pharmaceutica Analytica Acta

Shintani, Pharm Anal Acta 2013, 4:6
DOI: 10.4172/2153-2435.1000242

Short Communication Open Access

ROS (Reactive Oxygen Species)-Generating Systems in Mitochondria, Microsomes and Peroxisomes

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Keywords: Reactive Oxygen Species; Bovine Heart Submitcondrial particles; Microsomes; Liver; Malondialdehyde

Preparation of each fraction

Bovine heart submitchondrial particles (SMP)

Protocol: Keep the sample temperature below 4°C whenever possible.

1. Put fresh bovine heart obtained from a slaughter house in a vinyl bag and keep on ice during transfer.
2. Remove fat and endothelium, cut into 2-3 cm cubes and keep on ice in a vinyl bag until mincing.
3. Mince with an electric food cutter for ca 1 min and keep on ice in a vinyl bag. It is possible to stop experiments by freezing the mince in a vinyl bag at this step. For quick freezing and thawing, the mince should be spread thinly in the bag. Usually, 1-2 kg of the mince is used for the preparation of SMP.
4. Suspend in ice-cold 0.25 M sucrose-0.185% K_2HP0_4 (3 L/kg mince).
5. Adjust pH to ca 7.5 quickly by adding 6 M KOH (8-10 mL/kg).
6. Homogenize with a food mixer at maximum power for 2 min. The homogenates should be kept on ice until centrifugation.
7. Centrifuge at 2000 g for 10 min (Hitachi RPR 12-2 rotor, 4600 rpm).
8. Decant the supernatant and filter through four layers of gauze.
9. Centrifuge the supernatant at 11000 g for 40 min (Hitachi RPR 12-2 rotor, 10600 rpm).
10. Suspend the pellet in a small volume of sucrose (0.25 M, 40-80 mL) and homogenize with a Potter-Elvehjem type homogenizer (3-5 strokes). The homogenizer should be kept in the ice-water bath during homogenization.
11. Dilute the homogenate with sucrose (0.25 M, 400-800 mL).
12. Centrifuge at 11000 g for 40 min.
13. Repeat procedures 10-12 twice more.
14. Suspend in sucrose (0.25 M, 10-20 mL) containing EDTA (pH 7.5, 2 mM, 30 mg/mL).
15. Freeze at -20°C for at least 3 days.
16. Thaw quickly in flowing water.
17. Sonicate 10 mL of the mitochondrial sample six times for 45 s at 1 min intervals (Branson sonifier, cell disrupter 200, output 3.5-5.0 with a flat tip). The sample should be kept in the ice-water bath during and between sonications.
18. Centrifuge at 12000 g for 15 min (Hitachi RPR20 rotor, 13000 rpm).
19. Centrifuge the supernatant at 77 000 g for 1 h (Hitachi RP30 rotor, 30000 rpm).
20. Suspend the pellet in a small volume of sucrose (0.25 M) containing EDTA (2 mM) and homogenize with a Potter-Elvehjem type homogenizer (3-5 strokes).
21. Repeat procedures 18-20 twice more.
22. Finally suspend and homogenize in several mL sucrose (0.25 M) containing Hepes (5 mM), NaOH (5 mM), and EDTA (0.1 mM) (pH 7.5).
23. Store at -20°C.

Comments

1-2 g mitochondria are obtained from 1 kg mince. The recovery of sub-mitochondrial particles from the mitochondrial fraction is 20-30%.

Rat liver microsomes

Protocol

1. Perfuse rat liver with cold saline.
2. Cut into pieces with scissors and suspend in 8 m L/g wet weight of sucrose (0.25 M), Hepes (5 mM), NaOH (5 mM), and EDTA (0.1 mM) at pH 7.4.
3. Homogenize with a Potter-Elvehjem type homogenizer (3-5 strokes).
4. Centrifuge at 7000 g for 10 min (Hitachi RPR20 rotor, 10000 rpm).
5. Centrifuge the supernatant at 77000 g for 1 h (Hitachi RP30, 30000 rpm).
6. Suspend the pellet in sucrose (0.25 M), Hepes (5 mM), NaOH (5 mM), and EDTA (0.1 mM) at pH 7.4.
7. Homogenize with a Potter-Elvehjem type homogenizer (3-5 strokes).

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Received May 14, 2013; **Accepted** June 24, 2013; **Published** June 28, 2013

Citation: Shintani H (2013) ROS (Reactive Oxygen Species)-Generating Systems in Mitochondria, Microsomes and Peroxisomes. Pharm Anal Acta 4: 242. doi:10.4172/2153-2435.1000242

8. Centrifuge at 77 000 9 for 1 h.
9. Repeat procedures 6-8 twice more.
10. Suspend and homogenize in a small volume of sucrose (0.25 M), Hepes (5 mM), NaOH (5 mM), and EDTA (0.1 mM) at pH 7.4.
11. Store at -20ºC.

Results

Approximately 50 mg microsomes are obtained from 10 g wet weight liver.

Peroxisomes of rat liver

Protocol

1. Inject clofibrate (200 mg/kg) subcutaneously once a day consecutively for two weeks.
2. Perfuse rat liver with cold saline.
3. Cut into pieces with scissors and suspend in 4 mL/g of wet weight sucrose (0.25 M) containing ethanol (0.1% v/v). (Ethanol is added to prevent inactivation of catalase.)
4. Homogenize with a Potter-Elvehjem type homogenizer (3-5 strokes).
5. Centrifuge at 1000 g for 10 min (Hitachi RP20, 3700 rpm).
6. Homogenize the pellet in 2 vol. of the same buffer.
7. Centrifuge at 1000 g for 10 min.
8. Combine the supernatant from procedures 5 and 7.
9. Centrifuge the combined supernatant at 20000 g for 15 min (Hitachi RP20, 16600 rpm).
10. Suspend the pellet in the same buffer and homogenize.
11. Centrifuge at 20000 g for 15 min.
12. Repeat procedures 10 and 11.
13. Suspend and homogenize in a small volume of sucrose (0.25 M) containing ethanol (0.1% v/v).
14. Store at -20°C.

Results

50-100 mg peroxisomes are obtained from 10 g wet weight liver.

Measurement of the production of superoxide anion ($\cdot 0_2^-$)

$\cdot 0_2^-$ -is produced by complex 1 and ubisemiquinone in mitochondria, and by cytochrome P-450 in microsomes.

Mitochondria

Epinephrine (adrenaline) is reduced to adrenochrome by $\cdot O_2^-$ [1].

Protocol

1. Prepare reaction mixture: Hepes-NaOH (pH 7.5, 100 mM) containing sucrose (0.5 M), (500 µL; final concentration 50 mM/0.25 M); water (450 µL); rotenone in ethanol (100 µM, 10 µL; (final concentration 1 µM); epinephrine (200 mM, 5 µL; final concentration 1 mM); bovine heart SMP (20 mg/mL, 25 µL; final concentration 0.5 mg/mL); total volume 990 µL.
2. Preincubate at 37°C for 5 min.
3. Record the baseline at 485-575 nm (Hitachi spectrophotometer 557) for at least 1 min.
4. Start the reaction by adding NADH (20 mM, 10 µL) in Hepes-NaOH (pH 7.5, 50 mM) or in sodium succinate (pH 7.0, 2 M) (final concentrations 0.2 mM or 20 mM, respectively).
5. Add 10 µL 1 mg/mL superoxide dismutase (SOD) [2].

Results and calculations

The SOD inhibitable reduction of epinephrine is calculated as the reduction by $\cdot O_2^-$ (Figure 1). The absorbance coefficient for adrenochrome is 2.96 mM/cm.

Microsomes

Preparation of acetylated cytochrome-c.

The reduction of cytochrome-c by endogenous electron-transfer systems in microsomes is reduced to below 10% by acetylation of cytochrome-c.

Protocol

AII procedures should be performed at temperatures below 4°C.

1. Dilute saturated sodium acetate (2.5 mL) with distilled water (2.5 mL).
2. Add cytochrome-c(horse heart, Sigma; 50 mg) with stirring.
3. Add acetic anhydride (64 µL) slowly.
4. Allow the reaction to continue for 60 min.
5. Dialyse twice for 12 h in 2 L water at 4°C.
6. Determine the concentration and store at –20°C.

Measure the increase in absorbance at 550-540 nm in Hepes-NaOH (pH 7.5, 50 mM) after reduction by addition of a small amount of powdered $Na_2S_20_4$. The millimolar absorption coefficient is 19.1 mM/cm. Acetylated cytochrome-c is stable to several freeze thaw cycles.

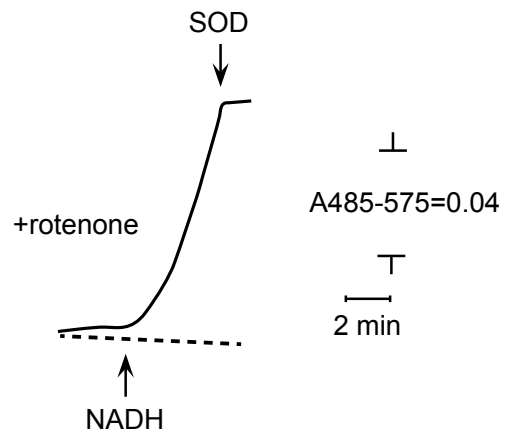

Figure 1: $\cdot O_2^-$ production by bovine heart SMP (submitcondrial particles) SOD: superoxide dismutase.

Measurements

Protocol

1. Reaction mixture: Hepes-NaOH (pH 7.7, 100 mM, 500 µL; final concentration 50 mM); water (280 µL); acetylated cytochrome-c (0.3 mM, 200 µL; final concentration 60 µM); rat liver microsomes (2 mg/mL, 10 µL; final concentration 20 µg/mL); total volume 990 µL.
2. Preincubate at 37°C for 5 min.
3. Record the baseline for ca 1 min at 550-540 nm (Hitachi spectro photometer 557).
4. Start the reaction by adding NADPH (20 mM, 10 µL) in Hepes-NaOH (pH 7.7, 50 mM) (0.2 mM final).
5. Add SOD (1 mg/mL, 10 µL).

Results and Calculations

The SOD-inhibitable part is calculated as the reduction of acetylated cytochrome-c by $\cdot O_2^-$ (Figure 2).

Measurement of the production of hydrogen peroxide (H_2O_2)

H_2O_2 results from the dismutation of $\cdot O_2^-$ in mitochondria and microsomes. In peroxisomes H_2O_2 is produced by acyl coenzyme A , an oxidase which is involved in the beta-oxidation of fatty acids.

Mitochondria-scopoletin method

Protocol

1. Reaction mixture: Tris-MOPS (pH 7.4, 60 mM) containing mannitol (0.46 M) and sucrose (0.14 M), 1000 µL (final concentration 30 mM, 0.23 M, and 0.07 M); water (740 µL); horseradish peroxidase (RZ > 3; Sigma; 100 µM, 20 µL; final concentration 1 µM); SMP (200 µL, final concentration 1 µM); scopoletin in ethanol (200 µM, 10 µL; final concentration 1 µM); total volume 2980 µL.
2. Preincubate at 37°C for 5 min.
3. Record baseline fluorescence (λex 365 nm, λem 450 nm).
4. Start the reaction by adding sodium succinate (1 M 20 µL; 10 mM final) and record the decrease in the fluorescence.

Microsomes-catalase Method

Protocol

1. Reaction mixture: Tris-HCI (pH 7.5, 100 mM) containing KCI (0.3 M) and $MgCl_2$ (20 mM, 3.75 mL); water (2.13 mL); trisodium isocitrate (80 mM, 750 µL; final concentration 8 mM); isocitrate dehydrogenase (Oriental; 100 units/mL, 25 µL; total amount 0.3 units); catalase (Boehringer-Mannheim; 13×10^5 units/mL, 5 µL; total amount 867 units); methanol (15 µL; final concentration 3 mM); rat liver microsomes (750 µL, 15 mg/mL; final concentration 1.5 mg/mL); total volume 7.425 mL.
2. Preincubate at room temperature for 5 min.
3. Start the reaction by adding NADPH (40 mM, 75 µL; 0.4 mM final).
4. Take aliquots (500 µL) every 2 min and add to ice-cold trichloroacetic acid (TCA; 15%, 500 µL) in an Eppendorf tube.
5. Centrifuge at 6000 g for 10 min (Tomy TMA-2, 10000 rpm).
6. Mix the supernatant (750 µL) with Nash reagent (750 µL)'.
7. Heat at 58°C for 10 min.
8. Cool to room temperature.
9. Measure absorbance at 412 nm.

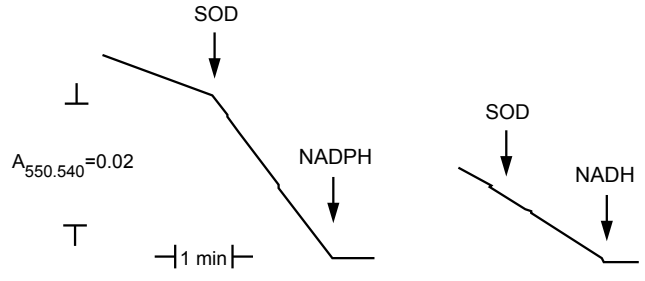

Figure 2: $\cdot O_2^-$ production by rat liver microsomes SOD: superoxide dismutase.

Peroxisomes-Catalase Method

Protocol

1. Reaction mixture: Tris-HCI (pH 7.5, 100 mM) containing KCI (0.3 M, 1.0 mL; final concentrations 50 mM and 0.15 M); water (896 µL); methanol (4 µL; final concentration 3 mM); rat liver peroxisomes (20 mg/mL, 50 µL; final concentration 0.5 mg/mL); total volume 1.95 mL.
2. Preincubate at room temperature for 5 min.
3. Start the reaction by adding palmitoyl-CoA (10 mM, 50 µL; dissolve before use, 0.2 mM final).
4. Take aliquots (500 µL) at 0, 1, and 2 min and add to ice-cold TCA (15%, 500 µL).
5. Proceed as for microsomes.

Lipid peroxidation

Ferric salts are reduced to the ferrous state by the mitochondrial or microsomal electron transport systems, and ferrous salts are more active in redox reactions leading to lipid peroxidation. Malondialdehyde (MDA), a degradation product resulting from lipid peroxidation reactions, is a widely used quantitative marker for lipid peroxidation.

Mitochondria MDA quantitation by HPLC

Preparation of standard MDA

1. Dissolve 1,1,3,3- tetraethoxypropane (Sigma; 0.1 mM) in HCl (18 mM, 10 m L).
2. Heat at 50°C for 1 h.
3. Dilute the sample (20 µL) to 10 mL with water and store at 4°C. The millimolar absorption coefficient at 267 nm is 31.8 mM/cm.

Lipid peroxidation reaction and quantitation of MDA

Protocol

1. Reaction mixture: Hepes-NaOH (pH 7.4, 100 mM, 250 µL; final concentration 50 mM); water (205 µL); bovine heart SMP (10 mg/mL, 15 µL; final concentration 0.3 mg/mL); ADP (100 mM,

10 µL; final concentration 2 mM); $FeCI_3$ (freshly prepared; 10 mM, 10 µL; final concentration 0.2 mM); rotenone in ethanol (100 µM, 5 µL; final concentration 1 µM); total volume, 495 µL.

2. Preincubate at room temperature for 5 min.
3. Start the reaction by adding NAD(P)H (10 mM, 5µL; 0.1 mM final).
4. Stop the reaction by adding acetonitrile (1 mL).
5. Leave to stand at room temperature for more than 5 min.
6. Centrifuge at 6000 g for 10 min (Tomy TMA-2, 10000 rpm).
7. Use the supernatant (50-100 µL) for HPLC analysis on a 4.6 mm i.d. × 150 mm Chemcopac Spherisorb-NH_2 column with 2:8 (v/v). Tris-HCI (pH 7.4, 30 mM)-acetonitrile as mobile phase at 2.0 mL/min detection wavelength 267 nm.

Result and Calculation

The amount of MDA in the sample is calculated by comparing its peak area with that from standard MDA (Figure 3).

Comments

When succinate is used as an electron donor, rotenone is replaced by 2-theonyltrifluoroacetone (TTFA, 1 mM final). The buffer solution used to suspend the SMP should be replaced by Hepes-NaOH (pH 7.4, 10 mM) if MDA is measured by the following TBA method.

Microsomes-thiobarbituric acid (TBA) method

Protocol

1. Reaction mixture: Hepes-NaOH (pH 7.4, 100 mM. 250 µL; final concentration 50 mM); water (220 µL); ADP (100 mM, 10 µL; final concentration 2 mM); $FeCI_3$ (freshly prepared; 10 mM, 10 µL; final concentration 0.2 mM); rat liver microsomes (20 mg/mL) in KCI (0.15 M, 5 µL; final concentration 0.2 mg/mL); total volume 495 µL.
2. Preincubate at room temperature for 5 min.
3. Start the reaction by adding NADPH (20 mM, 5 µL; 0.2 mM final).
4. Stop the reaction by adding ice-cold TCA (100%, 0.5 mL).
5. Add butylhydroxytoluene (10 mM, 5 µL) in ethanol to prevent non-enzymic lipid peroxidation in subsequent steps.
6. Stand on ice for at least 10 min.
7. Centrifuge at 6000 g for 10 min (Tomy TMA-2, 10000 rpm).
8. Add supernatant (0.5 mL) to TBA (0.375%, 0.5 mL)'.
9. Heat at 80°C for 15 min.
10. Cool to room temperature.
11. Measure absorbance at 535 nm.

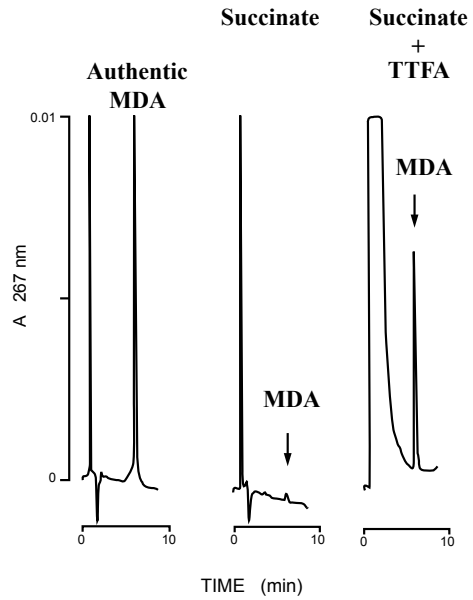

Figure 3: Lipid peroxidation in SMP (2) MDA: Malondialdehyde, TTFA:

Comments

The reaction of TBA is not specific for MDA. The absorbance values are, therefore, used for the quantitation of lipid peroxidation and expressed as TBA-reactive substances (TBARS).

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