Published in final edited form as:

Methods Mol Biol. 2017 ; 1614: 61–67. doi:10.1007/978-1-4939-7030-8_5.

Use of a fluorescent substrate to measure ACE2 activity in the mouse abdominal aorta

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Abstract

The use of fluorogenic substrates to measure enzymatic activity is widely used to understand function within different experimental models. ACE2 is important in understanding the balance between AngII and Ang-(1–7) and how this balance could then in turn influence hypertension or other disease outcomes. Here we describe a method to measure ACE2 activity in abdominal aorta of hyperlipidemic mice under both saline and AngII infusion.

Keywords

angiotensin converting enzyme 2; aorta; activity; enzyme; fluorescence

1. Introduction

Angiotensin converting enzyme 2 (ACE2) is a monocarboxypeptidase that cleaves angiotensin II (AngII) to angiotensin-(1–7)(Ang-(1–7)).(1) ACE2 is known to have other substrates, however it has been best characterized in the catabolism of AngII.(2–4) Our lab uses iodinated AngII (¹²⁵I-AngII) and separates out the angiotensin peptides by high performance liquid chromatography (HPLC) in order to measure Ang-(1–7) levels in tissues.(5,6) This method has the benefit of using AngII as the substrate; however this method requires dedicated lab equipment and the use of radiolabels which may not be practical for all labs. Recently, more labs are using fluorescently quenched compounds to measure enzymatic activity in cells and tissues.(7,8) This principle uses a quencher molecule, in this case dinitrophenol (Dnp), to block the fluorescence of methoxycoumarin (Mca). This interaction is abolished however when the enzyme cleaves the proline-lysine residue and allows for the Mca to then emit light at a given wavelength. These fluorogenic compounds offer more flexibility since more samples can be screened in a relatively shorter amount of time (e.g. higher throughput). ACE2 is a metalloproteinase therefore it requires a divalent cation positioned at the active site in order to perform catalysis. Optimal pH balance is also important for ACE2 and inhibition of angiotensin converting enzyme 1 (ACE1) and other endopeptidases (ex. neprilysin and thimet oligopeptidase) are necessary in order to prevent the influence of these enzymes on Ang-(1–7) formation. Within this protocol, we will use

the abdominal aorta as an example of how to measure ACE2 activity in hyperlipidemic mice that have been infused for 28 days with either saline or AngII.

2. Materials

Prepare all solutions using ultrapure water (18M Ω at 25°C). Store all reagents at room temperature (unless otherwise indicated) and protect fluorescent substrates from light when working with them at the lab bench. Also, dispose of all wastes properly and according to hazardous waste regulations.

2.1 Stock solutions

1. 1 M Tris-HCl, pH 7.5: Measure out 75 mLs of water and place in beaker with a stir bar. Weigh out 15.76 grams of Tris-HCl and place in beaker and mix to dissolve. Adjust the pH with 1 M NaOH to get to a pH of 7.5. Make up to 100 mLs with water.
2. 5 M NaCl solution: Measure out 100 mLs of water and place in beaker with a stir bar. Weigh out 29.2 grams of NaCl and place in beaker and mix to dissolve.
3. 1 M ZnCl₂ solution: Measure out 75 mLs of water and place in beaker with a stir bar. Weigh out 13.6 grams of ZnCl₂ and place in beaker and mix. Then add 3 mLs of 1 N HCl (*see Note 1*) to solution and then add water to make up 100 mLs.

2.2 ACE1, ACE2, and Prolyl endopeptidase inhibitors

1. 100 mM stock of captopril (ACE1 inhibitor): Weigh out a small amount (10–20 milligrams (mgs) or 0.01–0.02 g) and divide this amount by 0.02173 g (Molecular weight (MW) of captopril is 217.3) in order to determine the amount of water needed (*see Note 2*). Next, do a 10-fold serial dilution twice, to get a final concentration of 1 mM.
2. 10 mM solution of MLN-4760 (ACE2 inhibitor, MW 472.3): Weigh out 4.7 mgs and place in 1 mL of water to get this stock solution. Aliquot stocks into eppendorf tubes at 100 microliters and store at –20°C. MLN-4760 is used to determine any activity that is not ACE2 dependent (*see Note 3*). You may also use ACE2-deficient cells or tissues as a negative control.
3. 50 mM stock of Z-Pro-Prolinal (Prolyl endopeptidase inhibitor, MW 376.45): Weigh 18.8 mgs and dissolving in 1 mL of 50% methanol. Aliquot stocks into eppendorf tubes at 25 microliters and store at –20°C.
4. 1 mg of Mca-APK (Dnp)(ACE2 substrate, MW 696.7): Dilute 1 mg into 143 microliters of DMSO to get a 10 mM stock solution (*see Note 4*). Store solution at –20°C and protect from light at all times.
5. Make 10 mLs of a 1X ACE2 buffer with the following components: 750 μ Ls of 1 M Tris-HCl, pH 7.5, 2 mLs of 5 M NaCl, 5 μ Ls of 1 M ZnCl₂, 100 μ Ls of 1 mM captopril, 20 μ Ls of 50 mM Z-Pro-Prolinal, and 7 mLs of ultrapure H₂O. Make 1

mL of a 10X ACE2 buffer by using 750 μ Ls of 1 M Tris-HCl, pH 7.5, 5 μ Ls of 1 M ZnCl_2 , and 245 μ Ls of ultrapure H_2O .

6. In order to make up the fluorescent substrate, make the following master mix solution: 265 μ Ls of 1X ACE2 buffer, 10 μ Ls of 10X ACE2 buffer, 20 μ Ls of 5 M NaCl, and 5 μ Ls of fluorescent substrate (10 mM stock, final concentration is 50 μ M)(*see* Note 5).

3. Methods

Carry out all procedures on ice unless otherwise specified.

1. Add a Complete “Mini” protease inhibitor tablet (EDTA-free) into the 10 mLs of 1X ACE2 buffer and vortex until dissolved (*see* Note 6).
2. Take 5 mLs of the 1X ACE2 buffer and 25 μ Ls of Triton-X in order to get a 0.5% solution. The other 5 mLs of 1X ACE2 buffer will be used for the enzymatic assay.
3. Homogenize the tissue using a Dounce homogenizer (glass-on-glass) in the 0.5% Triton-X 1X ACE2 buffer. For abdominal aorta, we weigh the tissue and use the weight to figure out how much buffer to add (*see* Note 7).
4. Centrifuge samples at 12,000 g's for 15 minutes at 4°C. Take clarified supernatant to measure the protein concentration using the Bicinchoninic acid (BCA) assay (*see* Note 8). If you have enough protein, perform duplicates for all samples.
5. Take 100 μ gs of abdominal aortic protein from each sample for loading into the assay (*see* Note 9). From the given volume amount subtract it from 75 μ Ls to figure out how much 1X ACE2 buffer to use as a diluent. Load 70 microliters of this solution into each well and allow the inhibitors to incubate with the samples for at least 10 minutes before loading any substrate. Include a “blank” that contains only 1X ACE2 buffer and no protein in your 96-well plate.
6. Before adding the master mix, make sure to turn on spectrophotometer and allow it to warm up for at least 10 minutes. During this time, you can edit your plate, and set your excitation and emission filters (*see* Note 10)(Figure 1).
7. Once everything is ready, add 30 μ Ls of master mix using a multi-channel pipettor to all wells and take readings at 5 minutes for 2.5 hours. This will give you a total of 31 readings.

Notes:

1. A slight amount of hydrochloric acid is needed in order to get ZnCl_2 into solution. Heating the solution will not help with solubility of ZnCl_2 .
2. In order to not waste these compounds, we typically weigh out the smallest amount possible and then use the formula weight to divide to get a certain molarity of the compound. If we weighed out 0.01 grams of captopril, then we

would need 0.46 mLs of water to get a 100 mM stock. Captopril is very stable so you can keep at room temperature or at 4°C for 3–6 months.

3. We use MLN-4760 and we incubate tissues or cells with this compound for 30 minutes at 37°C before addition of any ACE2 substrate. There are other ACE2 inhibitors, such as DX-600, however this inhibitor may have different affinities to human versus mouse ACE2.(7,8)
4. In our hands, Mca-APK(Dnp) works the best in both ACE2-proficient and ACE2-deficient tissues. There is another fluorescent substrate, Mca-YVADAPK (Dnp), however this substrate shows a higher background level in ACE2 deficient tissues (unpublished data) and is also reactive to other enzymes, such as caspase-1 and ACE1.
5. You may need to test the amount of substrate needed for your given reactions. We have found that 50 μ M works best, however other labs have used less (1–30 μ M).(9,7,10) It is important to make sure that the substrate is in excess during the entire incubation time. This master mix is enough for 9 reactions. If there are more samples, then you can recalculate for your given number of samples.
6. There are numerous methods for inhibiting proteases and it is important to recognize that the literature has not conformed to one single method. Some labs, including ours, use numerous inhibitors such as pepstatin (renin inhibitor), bestatin (aminopeptidase inhibitor), benzyl succinate (carboxypeptidase inhibitor), and thiorpan/phosphoramidon (neprilysin inhibitors) to inhibit these enzymes. Depending on the tissue type, you may need to test different combinations of inhibitors to find the one that best fits your needs.
7. For abdominal aorta, it is good to use the least amount of buffer necessary to homogenize the tissue. For example, if the abdominal weighs 10 mgs, then we will use 100 μ Ls of the homogenization buffer (1 mg per 10 μ Ls of buffer).
8. It should be noted that ACE2 can reside on the cell membrane and within the cell.(11) If cell membrane ACE2 is what is preferred, we would recommend using a high centrifugation rate of 25,000–30,000 g's at 4°C for 20 minutes with 1X ACE2 buffer only without Triton-X. Once you have the pellet, then you resuspend the pellet in 0.5% Triton-X ACE2 buffer. Allow this to incubate overnight and then perform a 5,000 g's spin at 4°C for 5–10 minutes to get membrane ACE2. This assay is to look at total ACE2 from abdominal aorta.
9. Abdominal aorta and fat tissue(5,12) have much lower ACE2 activity than kidney, heart, or lungs, therefore it is necessary to load a higher amount of protein. It is best to keep this volume as low as possible. The protein lysate should not be any more than 20–25% of the total reaction volume as other proteins could interfere with the assay. It is also wise to try different amounts of protein lysate to determine what protein concentration will work best for this assay.
10. You may need to test the amount of substrate needed for your given reactions. We have found that 50 μ M works best, however other labs have used less (1–30

μM).(9,7,10) It is important to make sure that the substrate is in excess during the entire incubation time. This master mix is enough for 9 reactions. If there are more samples, then you can recalculate for your given number of samples.

11. Make sure that the spectrophotometer can incubate the plate at 37°C. Also, we do not use the well mixing function before each reading. This can influence the absorbance reading and so the incubation time of 2.5 hours may need to be shortened if this function is performed. Also, we do use the auto-cutoff function for performing all spectrophotometry readings at 420 nm. It is best to use a confined emission wavelength as other proteins or substrates could produce background fluorescence within the samples.

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Figure 1.

Abdominal aortic ACE2 activity in saline and angiotensin II (AngII) infused mice (28-day infusion protocol). Note that the ACE2 $-/-$ LDLR $-/-$ mice have slopes that are close to zero, whereas the ACE2 $+/-$ LDLR $-/-$ mice have a slope of 4.8 and a slope of 13.7 (10^{-3} RFU/100 µg protein/5 min) under saline and AngII infusion, respectively. This represents a 2.9-fold increase in ACE2 activity under AngII infusion. Each data point represents an average of 3–5 mice per group.