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Original Contribution

NADPH oxidase is involved in angiotensin II-induced apoptosis in H9C2 cardiac muscle cells: Effects of apocynin

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Abstract

Angiotensin II stimulates NADPH oxidase activity in vascular cells. However, it is not fully understood whether angiotensin II, which plays an important role in heart failure, stimulates NADPH oxidase activation and expression in cardiac myocytes. Previous studies have shown that angiotensin II induces myocyte apoptosis, but whether the change is mediated via NADPH oxidase remains to be elucidated. In this study we proposed to determine whether angiotensin II stimulated NADPH oxidase activation and NADPH oxidase subunit p47-phox expression in H9C2 cardiac muscle cells. If so, we would determine whether the NADPH oxidase inhibitor apocynin prevented angiotensin II-induced apoptosis. The results showed that angiotensin II increased NADPH oxidase activity, p47-phox protein and mRNA expression, intracellular reactive oxygen species, and apoptosis in H9C2 cells. Angiotensin II elevated p38 mitogen-activated protein kinase (MAPK) activity, decreased Bcl-2 protein, and increased Bax protein and caspase-3 activity. Apocynin treatment inhibited angiotensin II-induced NADPH oxidase activation and increases in p47-phox expression, intracellular reactive oxygen species, and apoptosis. The effect of apocynin on apoptosis was associated with reduced p38 MAPK activity, increased Bcl-2 protein, and decreased Bax protein and caspase-3 activity. These results suggest that angiotensin II-induced apoptosis is mediated via NADPH oxidase activation probably through p38 MAPK activation, a decrease in Bcl-2 protein, and caspase activation.

Keywords: Apoptosis; Angiotensin II; NADPH oxidase; Signal transduction; Free radicals

Evidence has been accumulated that NADPH oxidases are critical determinants of redox state of vascular smooth muscle cells [1] and endothelial cells [2]. NADPH oxidase activation has been implicated in the pathophysiology of hypertension and atherosclerosis [3]. Recent studies have demonstrated that the four subunits of NADPH oxidase, including gp91-phox, p22-phox, p47-phox, and p67-phox, are identified in myocytes [4] and increased in pressure-overload-induced hypertrophy and heart failure in guinea pig [5]. Furthermore, NADPH oxidase activity is increased in human failing hearts examined by the increased translocation of the regulatory subunit, p47-phox, to myocyte membranes in failing myocardium [6,7]. Angiotensin II (Ang II) has been shown to activate NADPH oxidase in the endothelial cells [8,9], vascular smooth muscle cells [10,11], and fibroblast [12,13]. p47-phox plays a key role in Ang II-mediated NADPH oxidase activation [14]. However, it is not fully understood whether Ang II, which is implicated in the pathophysiology of heart failure [15,16], stimulates NADPH oxidase activation and expression in cardiac myocytes. Previous studies have shown that Ang II induces myocyte apoptosis [17], but it remains to be defined whether the change is mediated via NADPH oxidase activation.

Apocynin is a well-characterized inhibitor of NADPH oxidase [18]. It acts by impeding the assembly of the p47-phox and p67-phox subunits within the membrane NADPH oxidase complex [18,19]. Apocynin protects the endothelium from the initiating events of atherosclerosis by the inhibition of

Abbreviations: Ang II, angiotensin II; ROS, reactive oxygen species; SOD, superoxide dismutase; RT-PCR, reverse transcription polymerase chain reaction; carboxy-H_2DCFDA, 5-(and-6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate; 8-OHdG, 8-hydroxydeoxyguanosine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline.

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NADPH oxidase activity and reactive oxygen species (ROS) generation in vitro and vivo [19]. Apocynin reduces NADPH oxidase activity, p22-phox mRNA expression, and cardiac hypertrophy in aldosterone-infused rats [20]. Apocynin also prevents hyperglycemia-induced intracellular ROS elevation and myocyte dysfunction [21].

In this study, we proposed to determine whether Ang II stimulated NADPH activation and NADPH oxidase subunit p47-phox expression in H9C2 cardiac muscle cells. Because Ang II has been shown to induce myocyte apoptosis, we determined whether the increases in NADPH oxidase activity and expression were involved in Ang II-induced apoptosis by investigating the effects of the NADPH oxidase inhibitor apocynin. We also determined whether p38 mitogen-activated protein kinase (MAPK) was associated with Ang II-induced changes in Bcl-2 and Bax proteins, caspase-3 activity, and apoptosis by utilizing a p38 MAPK inhibitor, SB203580. We measured NADPH oxidase activity by superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction assay, NADPH oxidase subunit p47-phox protein expression by Western blot and immunocytochemistry, p47-phox mRNA by reverse transcription polymerase chain reaction (RT-PCR), intracellular ROS with the fluorescent probe 5-(and-6)-carboxy-2′7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), and total cellular oxidative stress as examined by cellular 8-hydroxydeoxyguanosine (8-OHdG) content, which is a sensitive and specific marker of oxidative stress, by enzyme-linked immunosorbent assay. We examined apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay and caspase-3 activity. We investigated apoptosis-related proteins p38 MAPK, Bcl-2, and Bax.

Materials and methods

Cell culture

The H9C2 embryonal rat heart-derived cell (cardiac muscle cell) line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in 75-cm^2 flasks in Dulbecco's modified Earle's medium (ATCC) containing 4.5 g/L D-glucose, 1.5 g/L sodium bicarbonate, and 110 mg/L sodium pyruvate, supplemented with 10% fetal bovine serum (ATCC) and penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified incubator with 95% air and 5% CO_2 at 37°C. The culture medium was changed every 2 or 3 days. After 4 to 5 days, cells were passaged at a 1:5 ratio and seeded at the density of 1.2×10^6 cells per 100-mm dish, 0.15×10^6 cells per 35-mm well of 6-well plates, 3×10^4 cells per 20-mm well of 24-well plates, or 0.64×10^4 cells per 6.4-mm well of 96-well plates. These cells were cultured for 4 days and then underwent treatments.

Cell treatment

H9C2 cells were treated with (a) Ang II (100 nmol/L) alone (Sigma–Aldrich, St. Louis, MO, USA), (b) apocynin (100 μmol/L) alone (Sigma), (c) SB203580 (10 μmol/L) alone (Calbiochem, La Jolla, CA, USA), (d) Ang II (100 nmol/L) and apocynin (100 μmol/L), (e) Ang II (100 nmol/L) and SB203580 (10 μmol/L), or (f) no drug for control (diluent only: phosphate-buffered saline containing 0.1% bovine serum albumin). Apocynin or SB203580 was added to H9C2 cells 1 h before exposure to Ang II. The cells were exposed to those drugs for 24 h. The doses used were based on our pilot studies and previous reports [21–24].

Measurements of cell viability

Cell viability was assayed by trypan blue (Gibco BRL, Grand Island, NY, USA) exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich) uptake assay [25,26]. For trypan blue staining, the H9C2 cells were incubated with 0.2% trypan blue for 5 min at room temperature. The cells were counted with a hemocytometer under an inverted microscope. The cells stained blue were considered nonviable cells, whereas the cells excluded from the stain were considered viable. The number of nonviable cells (stained cells) was expressed as a percentage of the total cells counted. For the MTT assay, cells were incubated with 0.5 mg/ml MTT for 4 h at 37°C. At the end of the incubation, the supernatants were discharged and the adherent cells were resolved in 0.04 M HCl in absolute isopropanol. The absorption of converted dye was measured on a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 570 nm.

NADPH oxidase activity

NADPH oxidase-dependent superoxide production was measured by SOD-inhibitable cytochrome *c* reduction as described previously [5,27]. H9C2 cell homogenates (final concentration 1 mg/ml) were distributed in 96-well flat-bottom culture plates (final volume 200 μl/well). Cytochrome *c* (500 μmol/L) and NADPH (100 μmol/L) were added in the presence or absence of SOD (200 U/ml) and incubated at room temperature for 30 min. Cytochrome *c* reduction was measured by reading absorbance at 550 nm on a microplate reader. Superoxide production in nanomoles per milligram of protein was calculated from the difference between absorbance with and without SOD and extinction coefficient for change of ferricytochrome *c* to ferrocytochrome *c,* i.e., 21.0 $mmol \cdot L^{-1} \cdot cm^{-1}$.

Western blot for NADPH oxidase subunit p47-phox protein

H9C2 cardiac muscle cells were washed in phosphate-buffered saline (PBS) once and lysed in a lysis buffer (Cell Signaling Technology, Beverly, MA, USA) on ice for 1 h and centrifuged at 12,000*g* for 15 min. The supernatant was used for protein determination and the pellets were discarded. Aliquots containing 50 μg of protein were separated by electrophoresis through 10–12% SDS–polyacrylamide gel. Proteins were transferred to a polyvinylidene fluoride