

Acrolein, an Environmental Toxin, Induces Cardiomyocyte Apoptosis via Elevated Intracellular Calcium and Free Radicals

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Abstract Acrolein, an unsaturated aldehyde, is an environmental toxin known to inhibit mitochondrial electron transport chain in brain and induce lipid peroxidation and apoptosis. However, the nature of the effects of acrolein on cardiac function and myocardium is not known. The objective of this study is to examine whether acrolein induces apoptosis in cardiomyocytes and alters cytosolic calcium concentration and the intracellular oxygen free-radical levels. Adult mouse cardiomyocytes exposed to 1 $\mu\text{mol/l}$ of acrolein showed a marked increase in the intracellular oxygen free-radicals and calcium concentration, by 12- and 2-fold, respectively, compared to the resting value. Moreover, the cardiomyocyte viability decreased significantly in a dose-dependent manner by treatment with 25, 50, and 100 $\mu\text{mol/l}$ of acrolein compared to controls. Morphological changes and DNA laddering typical of apoptosis were found in acrolein-exposed cardiomyocytes. Our finding suggested that acrolein caused apoptotic death of adult mice cardiomyocytes by increasing intracellular oxygen free-radicals and calcium concentration.

Keywords Acrolein · Adult mice cardiomyocytes · Oxygen free-radical · Calcium · Apoptosis

Introduction

Acrolein (CH_2CHCHO), an unsaturated aliphatic aldehyde with potent cytotoxicity, exists in the environment as ubiquitous pollutant that is generated as the product of overheated organic materials [1]. In higher organisms, acrolein is endogenously produced by lipid peroxidation and is also identified as a metabolite of allylamine both in vitro and in vivo [2, 3]. It is demonstrated that acrolein induced intracellular generation of free radicals and lipid peroxidation [4].

Previous studies have demonstrated that acrolein is cytotoxic to various types of cells, and thus the cardiotoxic action of allylamine can be because of its metabolite [5, 6]. Other investigations indicated that acrolein inhibits electron transport and induces lipid peroxidation in mitochondria as a primary mechanism of its cardiotoxic action [7]. However, effects of acrolein on cardiomyocytes calcium concentration and apoptosis are still unknown.

In this study, we have used freshly isolated mouse cardiomyocytes to investigate the effects of acrolein on intracellular oxygen free-radicals and Ca^{2+} and cytotoxicity.

Materials and Methods

Drugs and Chemicals

Acrolein was purchased from Showa Corp (Japan). Collagenase (typeII) was purchased from Worthington Biochemical Corp (USA). DCF, RNase, and proteinase K were purchased from Sigma Chemicals. Fura-2AM was obtained from Biotium Corp. Laminin and minimal essential medium (MEM) were obtained, respectively, from BD Biosciences and GIBCO (USA). WST-8 was obtained from

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Kishida Chemical Corp (Japan). Glutamine and fetal calf serum (FCS) were purchased, respectively, from GIBCO and Hyclone. Primocin (1:500) (antibiotic) was obtained from Lonza cologne AG Corp (Switzerland).

Complete perfusion buffer contained following (mmol/l): NaCl (113); KCl (4.7); MgSO₄•7H₂O (1.2); NaH₂PO₄ (0.6); NaHCO₃ (12); KH₂PO₄ (0.6); KHCO₃ (10); Na-HEPES (10); Taurine (30) and BDM (2, 3-Butanedione Monoxime) (10).

Adult Mouse Cardiac Myocytes Isolation

The investigation was performed according to the guideline of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. The Japanese C57BL/6J mice (2-month-old; Japan Animal Co, Osaka, Japan) were used for isolation of viable adult mouse cardiac myocytes. We have modified Langendorff model of heart [8] using C57BL/6J mice (2-month-old). After opening the chest cavity, the aorta was quickly cannulated using 20G needle so that the tip of the cannula was just above the aortic valve. Then, the heart was removed and the aorta was perfused retrogradely with a constant flow (4 ml/min) at 37°C for 4 min, with a Ca²⁺-free bicarbonate-based buffer. The enzymatic digestion was initiated by adding collagenase (1.5 mg/ml) to the perfusion solution. After 2 min, 50 μmol/l Ca²⁺ was added to the enzyme solution. The cardiomyocytes were isolated, washed, and counted. Then, cardiomyocytes were cultured in the dishes precoated with 10 μg/ml mouse laminin in 2% CO₂ incubator at 37°C to allow myocyte attachment.

Measurements of Intracellular Oxygen Free-Radicals

To determine the oxygen free-radicals, isolated cardiomyocytes were washed with 3 ml of MEM, then centrifuged for 1 min at 1,000 rpm, and suspended in 3 ml of MEM. Cardiomyocytes were loaded with the 1.5 μl of 2,7-dichlorofluorescein diacetate (DCF; molecular probes) at room temperature for 30 min followed by washing in MEM. Relative levels of oxygen free-radicals were monitored with a confocal laser scanning microscope system (Diaphot, Nikon, Japan). After adding 1 μmol/l of acrolein, the fluorescence ratio signals were measured at room temperature [9].

Measurements of Intracellular Calcium

To monitor intracellular [Ca²⁺], the cellular suspension was centrifuged and the supernatants were withdrawn. The cardiomyocytes were resuspended in a HEPES-Tyrode's solution, then centrifuged for 1 min at 1,000 rpm, and

finally suspended in 2 ml of 0.5 mmol/l Ca²⁺ Tyrode's solution. Cardiomyocytes were loaded with 2 μl of Fura-2AM at room temperature for 15 min. The unincorporated dye was removed by washing the cells with 0.5 mmol/l Ca²⁺ Tyrode's solution. Cardiomyocytes were transferred to the stage of a fluorescent microscope (Diaphot, Nikon, Japan) at room temperature as described previously [10]. The fluorescent microscope was coupled to a dual-wavelength excitation spectrofluorometer. After adding acrolein 1 μmol/l, the fluorescent signals obtained at 340 (*F*₃₄₀) and 380-nm (*F*₃₈₀) excitation wavelengths were stored in a computer for data processing and analysis. The fluorescence ratio [*F*(340)/*F*(380)] was used to represent [Ca²⁺]_i changes in the cardiomyocytes.

Cardiomyocyte Viability Assay

Freshly isolated cardiomyocytes suspended in serum-free MEM were incubated with different concentrations of acrolein (0, 1, 5, 10, 25, 50, and 100 μmol/l) in 96-well plates and incubated in 2% CO₂ incubator at 37°C. After 1 h, FCS was added to the suspension of cardiomyocytes. After 12 h, the cell suspension was centrifuged for 1 min at 1,000 rpm. Then, the supernatant was removed and 100 μl of MEM and 10 μl of the dye WST-8 were added to the cells and incubations continued for 1 h. Cell viability was measured by multi-spectrophotometer (Viento, Japan) [11].

Detection of DNA Fragmentation

Freshly isolated cardiomyocytes suspended in serum-free MEM were incubated with different concentrations of acrolein (0, 5, 10, 25, and 50 μmol/l) in six-well plates and incubated in 2% CO₂ incubator at 37°C. After 1 h, FCS was added to the suspension of cardiomyocytes. After 3 h, the cell suspension was centrifuged at 1,000 rpm for 5 min. DNA fragmentation in cardiomyocytes was detected as described previously [12]. In brief, 20 μl of lysis buffer containing 50 mmol/l Tris-HCl (pH 7.4), 10 mmol/l EDTA-4Na, and 0.5% sodium-*N*-lauroylsarcosinate were added to the cell pellet. After suspending gently, 10 μl of RNase (0.5 mg/ml) was added to the supernatant and incubated at 50°C for 30 min followed by an additional incubation at 50°C for 1 h with 2 μl of proteinase K (10 mg/ml). Then, the fragments of DNA were precipitated with 20 μl of 5 M NaCl and 120 μl of 2-isopropanol and centrifuged at 15,000 rpm for 15 min. The supernatant was discarded and the samples were centrifuged again at 15,000 rpm for 5 min to remove 2-isopropanol completely. TE buffer (20 μl) was added and the samples were processed for DNA fragmentation analysis by 1.5% agarose gel electrophoresis.

Apoptotic Morphologic Change Assays

Cardiomyocytes (1.0 ml) were plated in complete MEM in a laminin-coated six-well plate and incubated in 2% CO₂ incubator at 37°C to achieve myocyte attachment. After 1 h culture, the medium was aspirated, 1.0 ml of fresh FCS-free medium was added to the plates, and then cardiomyocytes were treated with 1 and 10 μmol/l of acrolein. After 1 h treatment, 50 μl of FCS was added to the plates. Parallel controls were performed without acrolein. After 12 h, apoptotic morphologic changes were examined by Hoechst 33342 in the light microscopy.

Statistical Analysis

Values are expressed as mean ± SD. Statistical analyses were performed using one-factor analysis of variance (ANOVA) and Bonferroni/Dunn's method for multiple comparisons. Probability of $P < 0.05$ was considered statistically significant.

Results

Acrolein Alters Intracellular Oxygen Free-Radicals and Ca²⁺ Transients

In the DCF-loaded adult mouse ventricular myocytes, 1 μmol/l of acrolein caused a rapid increase of oxygen free-radicals starting within 30 s and reached a peak elevation by about 50 s to nearly 12-fold more than the resting values (Fig. 1a). This rapid increase was followed by a slow decline of oxygen radicals, reaching basal levels by 90 s (Fig. 1a). The oxygen free-radical rise was maintained for about 1 min.

Figure 1b shows Ca²⁺ transients of a Fura-2AM-loaded mouse ventricular myocytes. When the cells were exposed to 1 μmol/l of acrolein, the [Ca²⁺]_i increased markedly and reached twofold more than the resting value (Fig 1b). This rise in Ca²⁺ transients was maintained for 1–2 min with the

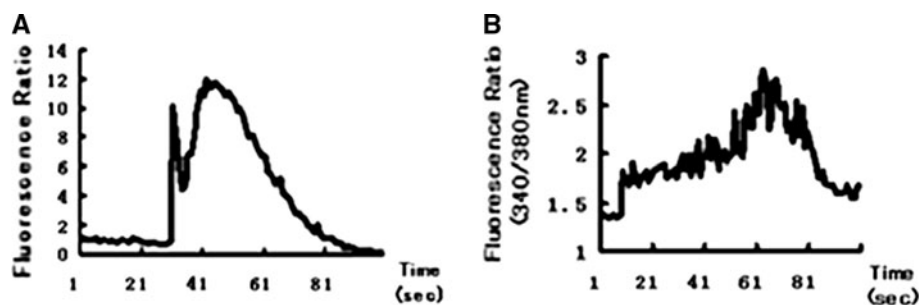


Fig. 1 Effect of acrolein treatment on oxygen free radicals and intracellular Ca²⁺ transient in cardiomyocytes. **a** DCF-loaded adult mouse ventricular myocytes were exposed to 1 μmol/l of acrolein, followed by immediate recording of fluorescence changes to measure

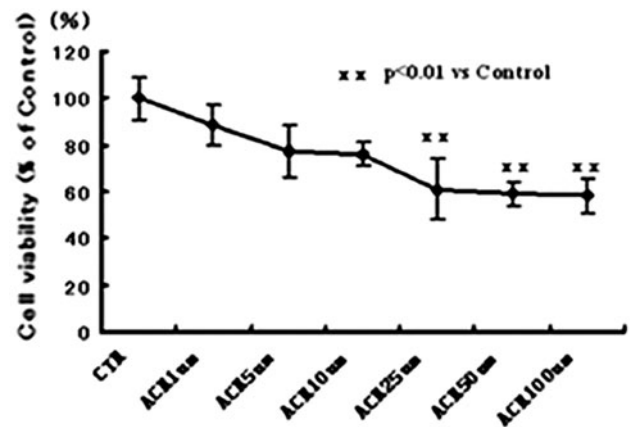


Fig. 2 Effect of acrolein on the viability of mouse cardiomyocytes. Cardiomyocytes were treated for 12 h with 0–100 μmol/l acrolein. And the viability was assessed using WST-8 assay, as detailed in Sect. Methods. The results are expressed as a percentage of the control. Values are mean ± SD. ** $P < 0.01$

oscillatory pattern and then declined to near basal level (Fig. 1b).

Effect of Acrolein on the Cardiomyocyte Viability

Cardiomyocyte viability decreased with increasing concentration of acrolein treatment for 12 h, from 1, 5, 10, and 25 μmol/l, but thereafter, no further increase in toxicity at 50 and 100 μmol/l acrolein was noticed (Fig. 2). Cardiomyocyte viability with 25, 50, and 100 μmol/l of acrolein treatment was significantly lower compared to controls ($P < 0.01$).

Apoptotic Changes in Cardiomyocytes on Acrolein Treatment

DNA Fragmentation

To examine whether acrolein induced apoptosis of cardiomyocytes, we examined the DNA fragmentation

the levels of oxygen free radicals. **b** Fura-2AM-loaded mouse cardiac myocytes were exposed to 1 μmol/l of acrolein and recording of fluorescence changes was started immediately, to assess the Ca²⁺ transients, as detailed in Sect. Methods

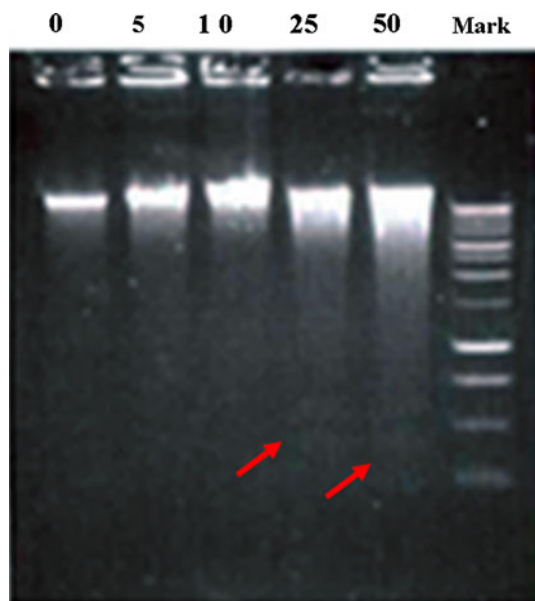


Fig. 3 Electrophoretic analysis of acrolein-induced DNA fragmentation in the cardiomyocytes. Cardiomyocytes were incubated with 0, 5, 10, 25, and 50 $\mu\text{mol/l}$ of acrolein for 3 h. *Arrows* indicates DNA ladder pattern in *Mark* and cardiomyocytes treated with 25 and 50 $\mu\text{mol/l}$ of acrolein

pattern, a hallmark of apoptosis, after acrolein treatment for 3 h. As seen in Fig. 3, DNA ladder pattern was observed in the cardiomyocytes treated with 25 and 50 $\mu\text{mol/l}$ of acrolein suggesting the occurrence of apoptosis. In contrast, no significant DNA fragmentation was observed in control and cardiomyocytes treated with 5 and 10 $\mu\text{mol/l}$ of acrolein. However, the DNA fragmentation was not observed in the cardiomyocytes treated with 25 and 50 $\mu\text{mol/l}$ of acrolein for 12 h (data not shown).

Morphological Changes

Figure 4 shows the morphological changes caused by the incubation of cardiomyocytes in the presence of 1 and

10 $\mu\text{mol/l}$ of acrolein for 12 h. Acrolein induced cell shrinkage, nuclear chromatin condensation, and segmentation of the nucleus. Rod-shaped cardiomyocytes shortened or became rounded after exposure to acrolein. No such morphological features were noted in control cells (Fig. 4).

Discussion

Acrolein-Induced Intracellular Generation of Oxygen Free-Radicals

Earlier experiments of *in vitro* liposomes incubated with acrolein showed a proportional increase in lipid peroxidation of liposomal membrane [13]. A major cause of allylamine's cardiovascular toxicity is because of its product, acrolein, which is known to induce lipid peroxidation, especially in mitochondria [14]. In this study, we found that acrolein increased intracellular oxygen free-radicals and such increase may have significant clinical relevance in human cardiotoxicity situations. In addition, previous investigations demonstrated the acrolein-mediated intracellular generation of free radicals and lipid peroxidation, either through metabolism of acrolein or its adduct with glutathione (GSH) (glutathionyl-propionaldehyde) or by rapid depletion of cellular GSH. The enzymes including xanthine oxidase and aldehyde dehydrogenase were found to interact with glutathionylpropionaldehyde to produce superoxide radical ($\text{O}_2^{\bullet-}$) and hydroxyl radical (HO^{\bullet}). Acrolein was oxidized by xanthine oxidase producing acroleinyl radical and $\text{O}_2^{\bullet-}$. Aldehyde dehydrogenase can also metabolize acrolein to form superoxide [4].

Acrolein-Induced Intracellular Ca^{2+} Transients

Allylamine is a cardiovascular-specific toxin that causes aortic, valvular, and myocardial lesions in many species

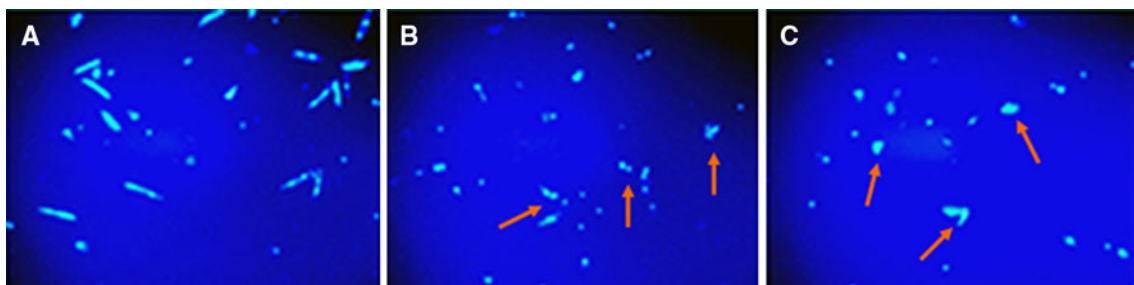


Fig. 4 Acrolein induces apoptotic morphological features in the cardiomyocytes. **a** Control; **b** ACR, 1 $\mu\text{mol/l}$; and **c** ACR, 10 $\mu\text{mol/l}$. **a** Cardiomyocytes were incubated in the laminin-coated six-well plates without acrolein. Apoptotic cardiomyocytes were not observed. **b** and **c** Cardiomyocytes were incubated in the laminin-coated six-well plates with 1 and 10 $\mu\text{mol/l}$ of acrolein for 12 h. Note the

acrolein-induced morphological changes of apoptotic cell such as cell shrinkage, nuclear chromatin condensation, and segmentation of the nucleus. Rod-shaped mouse cardiac myocytes shortened or became rounded after exposure on acrolein (*arrows* indicate fragmented nuclei; original magnification $\times 10$)

[15, 16]. Acute toxicity of allylamine is believed to involve its metabolism to highly reactive acrolein [17]. In addition, these investigations demonstrated that allylamine and acrolein weakly inhibited mitochondrial electron transport and interfered with the energy production in cultured myocytes. However, the precise mechanism of acrolein-mediated injury to myocytes is not clear.

Because of the known effect of acrolein on mitochondria and energy production, in this study we investigated if acrolein-mediated toxicity and cell death in cardiomyocytes involves altered intracellular Ca^{2+} levels. Previous studies reported that oxygen free-radicals can cause calcium loading and cellular injury in the cardiomyocytes [18]. In this study, we noticed a marked elevation of intracellular Ca^{2+} levels on acrolein treatment in cardiomyocytes. Importantly, the increase in oxygen free-radicals after the addition of acrolein preceded the elevation in $[\text{Ca}^{2+}]_i$ by nearly 30 s (see Fig. 1). This suggested that the intracellular calcium in acrolein-exposed cardiomyocytes is likely increased by the augmented production of oxygen free-radicals ($\text{HO}\cdot$ and $\text{O}_2\cdot$). The mechanisms of Ca^{2+} increase include enhanced sarcoplasmic reticulum calcium release and calcium influx through the voltage-gated calcium channels of sarcolemmal membrane during exposure to oxygen free-radicals [19].

Oxygen free-radicals induce calcium loading probably by suppressing the sarcolemmal Ca^{2+} -pump ATPase and Na^+ - Ca^{2+} exchange activity and also by reducing Ca^{2+} extrusion from cytosol to extracellular space [20]. Furthermore, it has also been shown that the Ca^{2+} -pump ATPase activity and steady-state Ca^{2+} uptake are decreased by oxygen free-radicals in the cardiac sarcoplasmic reticulum [20]. Thus, it is possible that the acrolein-induced intracellular calcium loading by oxygen free-radicals in cardiomyocytes is related to reduced sarcolemmal Ca^{2+} -pump ATPase activity.

Ca^{2+} overloading can have serious consequences for both reversible and irreversible myocyte damage by the activation of a variety of proteases, lipases, phospholipases, and ATPases [21]. Peroxidation of membrane phospholipids and intracellular calcium loading induced by oxygen free-radicals were supposed to be major mechanisms of ischemia–reperfusion injury in cardiac tissue [22]. Moreover, elevation of Ca^{2+} can promote the formation of oxygen free-radical by activating lipases.

Acrolein-Induced Cardiomyocytes Apoptosis

It was reported that acrolein caused apoptosis in the colon cancer cells by production of a cellular stress response [23, 24]. In this study, we demonstrated that acrolein can cause cardiac myocytes injury and cell death in concentration-dependent manner. Moreover, typical DNA fragmentation

was observed in the cardiomyocytes treated with 25 and 50 $\mu\text{mol/l}$ of acrolein for 3 h, strongly suggesting apoptotic cell death. The absence of DNA fragmentation in cells treated for 12 h with 25 and 50 $\mu\text{mol/l}$ of acrolein indicated that prolonged exposure of cardiomyocytes with higher doses of acrolein induces necrotic cell death.

Collectively, our data indicate that acrolein causes a dose-dependent oxygen free-radical production, calcium loading, and apoptosis in the adult mice cardiomyocytes. Thus, we suggest that acrolein-induced cardiomyocytes apoptosis is likely because of increased intracellular oxygen free-radicals and calcium concentration.

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