Free fatty acids act as endogenous ionophores, resulting in Na\(^+\) and Ca\(^{2+}\) influx and myocyte apoptosis

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Aims Disturbances in lipid metabolism have been suggested to play an important role in myocardial damage. Marked accumulation of free fatty acids (FFAs), including arachidonic acid (AA), palmitic acid, oleic acid, and linoleic acid, occurs during post-ischaemia and reperfusion (post-I/R). Possible cellular mechanisms of AA/FFAs-induced myocyte apoptosis were investigated.

Methods and results In neonatal rat ventricular myocytes, AA/FFAs activate a novel non-selective cation conductance (NSCC), resulting in both intracellular Ca\(^{2+}\) and Na\(^+\) overload. AA caused sustained cytosolic [Na\(^+\)]\(_{cyt}\) and [Ca\(^{2+}\)]\(_{cyt}\) overload, resulting in mitochondrial [Na\(^+\)]\(_{m}\) and [Ca\(^{2+}\)]\(_{m}\) overload, which induced caspase-3-mediated apoptosis. Similar apoptotic effects were seen using Na\(^+\)-ionophore cocktail/Ca\(^{2+}\)-free medium, which induced [Na\(^+\)]\(_{cyt}\) and [Na\(^+\)]\(_{m}\), but not [Ca\(^{2+}\)]\(_{cyt}\) and [Ca\(^{2+}\)]\(_{m}\) overload. Electron microscopy showed that inhibition of [Na\(^+\)]\(_{m}\) overload prevented disruption of the mitochondrial membrane, showing that [Na\(^+\)]\(_{m}\) overload is an important upstream signal in AA- and FFA-induced myocyte apoptosis. Conclusion AA and FFAs, which accumulate in the myocardium during post-I/R, may therefore act as naturally occurring endogenous ionophores and contribute to the myocyte death seen during post-I/R.

1. Introduction

Disturbances in lipid metabolism have been shown to play an important role in irreversible myocardial damage. During ischaemic reperfusion, not only arachidonic acid (AA) is markedly released from the myocardium, but there is also a 10- to 15-fold increase in other types of free fatty acids (FFAs), including palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2).\(^1,3\) Of these FFAs, AA has been most studied, since it and its metabolites have many cellular effects.\(^4,5\) for example, AA induces apoptosis in a variety of non-excitable cells\(^6\) and is suggested to be involved in post-ischaemia and reperfusion (post-I/R)‐induced myocyte death by an undefined mechanism.\(^7\) Although it is difficult to determine the actual intracellular concentration of AA during post-I/R, a concentration of 40 \(\mu\)M has been suggested in the heart.\(^7,8\)

The key events in the mitochondrial‐mediated apoptotic pathway are opening of the permeability transition pore (PTP) and release of cytochrome c (cytC) and other apoptotic proteins, resulting in caspase-3‐dependent chromatin condensation and fragmentation (for review refs 9,10). Cyclosporin A (CsA) has a marked protective effect against I/R‐induced PTP opening\(^11,12\) and myocyte death.\(^13,14\)

In excitable cells, voltage‐gated Ca\(^{2+}\) and K\(^+\) channels are modulated by AA at concentrations of 10–100 \(\mu\)M.\(^4,15\) However, studies on non‐excitable cells have shown that AA opens a Ca\(^{2+}\)‐selective channel, the AA‐regulated Ca\(^{2+}\) (ARC) channel.\(^16,17\) By inhibiting all known AA‐modulated ion channels, we showed for the first time in myocytes that AA induced both cytosolic and mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)) and Na\(^+\) ([Na\(^+\)]\(_{cyt}\)) overloads by activating a non‐selective cation conductance (NSCC). The present study also showed that, in addition to the [Ca\(^{2+}\)]\(_{m}\) overload, the [Na\(^+\)]\(_{m}\) overload is an important upstream signal in PTP opening, mitochondrial membrane disruption, and caspase‐3‐dependent myocyte apoptosis. Since palmitic acid, oleic acid, and linoleic acid also induced a dose‐dependent Ca\(^{2+}\) and Na\(^+\) increase and nuclear condensation,
AA and FFAs possibly act as endogenous ionophores and participate in the myocyte death seen during post-I/R.

2. Methods

2.1 Preparation of neonatal and adult ventricular myocytes

All procedures were performed in accordance with the guide for Use of Laboratory Animals published by National Taiwan University. In brief, one-day-old Wistar rats were killed by decapitation, and the ventricular myocyte cultures were prepared. For isolation of adult myocytes, guinea-pigs (~250 g, both sexes) were killed by cervical translocation and single myocytes were isolated using combination of enzymatic digestion (collagenase type 1, 1 mg/mL; protease type XIV, 0.1 mg/mL) and mechanical dispersion.18

2.2 Electrophysiology

Patch-clamp was performed at room temperature (RT) with the whole-cell configuration using a Dagan 8900 amplifier and PCLAMP 6 software.18 The holding potential was held at ~40 mV to inactivate the Na+ channel. Cells were bathed in a solution consisting of (in mM): NaCl, 130; Cs aspartate, 4.5; MgCl2, 1.2; CaCl2, 2; glucose 10; and HEPES–NaOH buffer, 10 (pH 7.4) containing nifedipine (10 μM), paxilline (1 μM), glibenclamide (30 μM), KB-R7943 (10 μM), and ouabain (100 μM) to block contamination by Ica2, Ica3, and Ikatp, the Na+-Ca2+ exchange, and Na+-K+ ATPase currents. Pipettes were filled with solution containing (in mM): Cs aspartate, 140; Na creatine, 5; MgCl2, 5; CaCl2, 1.0 (+11 mM EGTA, free [Ca2+]i = 100 nM); MgATP, 5; HEPES–KOH buffer, 10 (pH 7.2).

2.3 Intracellular fluorescence measurement of the [Ca2+]i, or [Na+]i

The [Ca2+]i concentration was continuously monitored using fura 2 AM- or Na+-binding benzofuran isothipan (SBFI) AM-loaded myocytes, respectively. In vivo calibration was performed as described in Supplementary material. To prevent possible contamination of AA-activated ion channels demonstrated in previous studies,4,10 nifedipine (10 μM), paxilline (1 μM), and glibenclamide (30 μM) were always present to block any contamination by Ica2, Ica3, and Ikatp.

2.4 Simultaneous measurement of cytosolic and mitochondrial Ca2+ or Na+ changes using time-lapse confocal microscopy in live myocytes

Thin optical sectioning (182.3 μm) was used to obtain independent cytoplasmic and mitochondrial Ca2+ ([Ca2+]i) and [Ca2+]m and Na+ ([Na+]i and [Na+]m) fluorescent signals19,20 using a Leica SP confocal laser scanning imaging system equipped with a 63X oil-immersion objective. Cells were loaded with Rhod-2 AM (5 μM, a Ca2+-sensitive probe) or with MitoTracker Green (MTG; 200 nM). Time-lapse Rhod-2 images were taken every 10 s, whereas MitoTracker Green images were recorded every 20 s, since this dye is easily bleached. The X-Y plane images were 512 by 512 pixels. Because variability in dye loading causes differences in absolute fluorescence between different myocytes, changes in [Ca2+]i and [Na+]i were simultaneously measured as peak/basal fluorescence ratios $F_{peak}/F_{basal}$.19,20 In vivo calibration of both the [Na+]i and [Na+]m was performed using a set of five calibration solutions containing different Na+ concentrations (10, 30, 60, 100, and 140 mM) and an Na+ ionophore cocktail (5 μM gramicidin D, 40 monensin, and 100 strophanthidin). The best-fit two straight lines are then respectively fitted to the plot of $F_{peak}/F_{basal}$ vs. [Na+]i (10, 30, 60, 100, and 140 mM). The [Na+]i and [Na+]m in each experiment were calculated using the equations:

\[ [Na^+]_i = \frac{18.65 \cdot F_{peak}/F_{basal} - 27.02}{0.998}, \]
\[ [Na^+]_m = \frac{36.95 \cdot F_{peak}/F_{basal} - 43.47}{0.997}. \]

2.5 In situ detection of apoptotic machinery

(i) Tetra-methyl rhodamine-methyl ester (10 nM) was used to measure the persistent depolarization of the mitochondria, which is suggested to be an indicator of PTP opening.10 Dynamically changes in the $\Delta \Psi_m$ were then monitored by time-lapse confocal microscopy with excitation at 568 nm and emission at >580 nm.

(ii) cytc release. After various treatments, the cells were fixed, permeabilized, then stained for 1 h at 37°C with mouse monoclonal anti-cytc antibody (1:500 dilution). They were then incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody and for 15 min at RT with Hoechst 33342 (2 μg/mL), then examined by confocal microscopy.

(iii) In situ labelling of activated caspase-3. Caspase activation was examined using FITC-DEVD-fmk, which binds irreversibly to the catalytic site of activated caspase 3 in cells.

(iv) DNA condensation or fragmentation. After various treatments, nuclei were labelled with Hoechst 33342 (chromatin condensation) or TUNEL (DNA fragmentation) according to the manufacturer's instructions.

2.6 Statistics

In fluorescence measurement and patch-clamp experiments, all results are expressed as the mean ± SEM for the stated number of animal preparations (n), each tested in duplicate. In immunostaining experiments, 200 cells from 10 randomly selected fields were scored on each coverslip, at least four animal preparations were used (n = 4) and duplicated in each test. Statistical differences were compared using the Mann–Whitney U test, taking a P-value of <0.05 as significant.

3. Results

3.1 Free fatty acids induce an increase in both intracellular Ca2+ and Na+ levels

The resting level of intracellular Ca2+ ([Ca2+]i, measured by Fura 2) and Na+ ([Na+]i, measured by SBFI) in neonatal myocytes was 95 ± 13 mM (n = 55) and 9.98 ± 1.3 mM (n = 36), respectively. Addition of 1 μM AA had little effect on the [Ca2+]i or the [Na+]i, and the myocytes still showed normal spontaneous Ca2+ transients (Figure 1A). After addition of 5–50 μM AA, both the [Ca2+]i and [Na+]i slowly increased to peak levels within 30–40 min (Figure 1A and B). The EC50 of the AA-induced [Ca2+]i and [Na+]i overload was 8 and 5 μM, respectively (Figure 1C and D). The spontaneous Ca2+ transients slowly disappeared, probably due to an inhibitory effect of AA on Ca2+-induced Ca2+ release.3 Although AA has been suggested to accumulate to a concentration of 40 μM in ischemic hearts,1,2 the commonly used concentration of 10 μM was used in the following experiments.

After addition of AA for ~30 min, the [Ca2+]i and the [Na+]i peaked, with little recovery in the absence of inhibitors of Ca2+-ATPase and Na+/K+-ATPase (Figure 1E–G). The [Ca2+]i (Figure 1H) and [Na+]i (Figure 1F) overloads were
AA induces $[\text{Ca}^{2+}]_{i}$ and $[\text{Na}^+]_{i}$ overload. (A–D) Dose response curves. AA concentrations of 1–50 μM were applied. (E–G) AA metabolism (E) and activation of the reverse mode of the Na$^+$/Ca$^{2+}$ exchanger (rNa$^+$/Ca$^{2+}$ exchanger) (G) are not involved in the $[\text{Ca}^{2+}]_{i}$ and $[\text{Na}^+]_{i}$ overload. (H) A statistical analysis. AA-induced Ca$^{2+}$/Na$^+$ influx resulting in $[\text{Ca}^{2+}]_{i}$ and $[\text{Na}^+]_{i}$ overloads were both abolished by 100 μM La$^{3+}$ ($n=8$) in the absence of Ca$^{2+}$-ATPase and Na$^+$-K$^+$-ATPase inhibitors (F). A mixture of metabolic inhibitors (MI, E) was used (see text). Na$^+$-free medium (G) inhibited the rNa$^+$/Ca$^{2+}$ exchanger. Intracellular $[\text{Ca}^{2+}]_{i}$ and $[\text{Na}^+]_{i}$ were measured by fura-2 AM and SBFI-AM, respectively. *$P<0.05$ by the Mann–Whitney U test (H).
totally abolished by 100 μM La³⁺, indicating that they were probably due to influx of extracellular Ca²⁺ and Na⁺. The rapid recovery from [Ca²⁺]cyt and [Na⁺]cyt overload in the presence of La³⁺ (Figure 1F) was probably due to an activation of Ca²⁺-ATPase and Na⁺/K⁺-ATPase. Using the patch-clamp technique, AA was found to activate a NSCC (see Supplementary material online, Figure S1), which may result in both the [Ca²⁺]m and the [Na⁺]m overloads seen in Figure 1.

The conversion of AA to active metabolites that may induce the [Ca²⁺]m and [Na⁺]m overload is unlikely. ETYA (10 μM), a non-metabolized analogue of AA, had a similar effect to AA (Figure 1E and H). Moreover, a mixture of metabolic inhibitors (MI, Figure 1E and H) of cyclo-oxygenase (indomethacin, 100 μM), 5- and 12- lipooxygenases (MK-886 and baicalein, both 10 μM), and cytochrome P450 (SK&F 96365, 30 μM) did not inhibit the entry of either Ca²⁺ or Na⁺. Each of these metabolic inhibitors used alone also had no inhibitory effect on the [Ca²⁺]m overload (not shown). The [Ca²⁺]m overload (Figure 1E) was not caused by the [Na⁺]m overload (Figure 1D), since, under conditions when the reverse mode of the Na⁺/Ca²⁺ exchange was inhibited by Na⁺-free medium, little inhibitory effect on the [Ca²⁺]m overload was observed (Figure 1G). CsA had little effect on the [Ca²⁺]m overload (Figure 1H). Overproduction of ROS (H₂O₂, OH⁻) also seems not to be involved in the AA-induced [Ca²⁺]m overload, since catalase, phenanthroline, tiron, and superoxide dismutase did not inhibit the [Ca²⁺]m overload (see Supplementary material online, Figure S2).

In addition to AA accumulation, a marked increase in levels of palmitic acid, oleic acid, and linoleic acid is seen in post-I/R. On addition of each FFA (10 μM), little change was seen in the [Ca²⁺]m, or [Na⁺]m (Figure 2G and H), whereas addition of either 30 or 50 μM FFA resulted in a marked and sustained increase in both (Figure 2A–H). Addition of 100 μM La³⁺ had again a significant inhibitory effect on the oleic acid- or linoleic acid-, but not palmitic acid-, induced Ca²⁺ and Na⁺ influxes (Figure 2G and H). Palmitic acid may activate a La³⁺-insensitive NSCC which also induces non-selective cation influx.

3.2 Simultaneous measurement of cytosolic and mitochondrial Ca²⁺+ Na⁺ changes: arachidonic acid causes a marked increase in the [Ca²⁺]cyt, [Na⁺]cyt, Ca²⁺/Na⁺ exchange, and [Ca²⁺]m overload

Since the nuclear membrane does not represent a barrier to cytosolic ion movement,²⁰ averaging the signal over a small nuclear optical section (endoplasmic reticulum- and mitochondria-free) or a mitochondrion (identified by MTG; nuclear optical section (endoplasmic reticulum- and nuclear optical section) allows dynamic changes in the [Ca²⁺]cyt and [Ca²⁺]m to be recorded (F/F₀, peak/basal fluorescence ratios). The basal level of the [Ca²⁺]cyt and [Ca²⁺]m was both F/F₀ = 1 (Figure 3B and E).

Photobleaching of Rhod-2 did not occur in the two myocytes shown in Figure 3A, since, in the absence of AA, little change in the [Ca²⁺]cyt and [Ca²⁺]m was seen (-AA in Figure 3B). When AA was added at 420 s, both the [Ca²⁺]cyt (i.e. the [Ca²⁺]nucleus) and [Ca²⁺]m started to increase at 9.5 ± 1.2 min (n = 12). After exposure to AA for >30–40 min, a sustained increase in the [Ca²⁺]cyt and [Ca²⁺]m was seen (Figure 3A and B), and AA had little effect on either the [Ca²⁺]cyt or the [Ca²⁺]m under Ca²⁺-free conditions (Figure 3E).

Activation of the Ca²⁺-ATPase in isolated heart mitochondria,²¹,²² markedly inhibited the AA-induced [Ca²⁺]m overload (Figure 3D and E), confirming that the Ca²⁺ influx occurred mainly via the Ca²⁺-unipporter.⁹,²³ Note that RU360 had little effect on the AA-induced [Ca²⁺]cyt increase (Figure 3E), indicating that RU360 treatment has little effect on the NSCC.

CGP37157 is a specific and potent blocker of the Na⁺–Ca²⁺ exchanger in isolated heart mitochondria,²⁴ but seems to have a variable inhibitory effect in adult intact myocytes.²⁵ We examined its effect on cardiac myocytes and found that 10 μM CGP37157 largely prevented the recovery of the [Ca²⁺]m induced by 40 mM KCl (no Na⁺ influx is induced by KCl stimulation) (data not shown), indicating that it effectively inhibits the exchanger. Interestingly, CGP37157 inhibited the AA-induced [Ca²⁺]m overload (Figure 3E; see Supplementary material online Figure S3A, see below).

CoroNa Red was used to simultaneously measure changes in the [Na⁺]cyt and [Na⁺]m. After exposure to AA in normal medium (Figure 4A), the [Na⁺]cyt and [Na⁺]m started to increase at 7.3 ± 0.7 min (n = 8) and peaked at ∼30–40 min (Figure 4B). There was little increase in the [Na⁺]cyt and [Na⁺]m when AA was applied in Na⁺-free medium (bottom trace, Figure 4B), indicating that CoroNa Red is a Na⁺-sensitive and Na⁺-selective probe and that photobleaching of CoroNa Red did not occur in these experiments. Interestingly, RU360 markedly, but not completely, prevented the [Na⁺]m overload, but not the [Na⁺]cyt overload (Figure 4C); the residual [Na⁺]cyt uptake is probably caused by the Na⁺–Ca²⁺ exchanger in the inner membrane.²³,²⁶ Both CGP37157 (inhibition of the AA-induced [Ca²⁺]m overload; Figure 3E; see Supplementary material online Figure S3A) and Ca²⁺/Na⁺-free/EGTA medium (presumably inhibiting the mitochondrial Na⁺–Ca²⁺ exchanger) had little inhibitory effect on the AA-induced [Na⁺]m overload (Figure 4E; see Supplementary material online Figure S3C and D), therefore suggest that part of the AA-induced [Ca²⁺]m overload is probably caused by activation of the reverse mode of the Na⁺–Ca²⁺ exchanger (Na⁺/Ca²⁺ exchange for Ca²⁺). One possible explanation for activation of the reverse mode is that the slightly earlier uptake into the matrix of Na⁺ ions compared with Ca²⁺ ions (7 vs. 9 min after AA exposure) results in a reduction in the transmembrane electrochemical Na⁺ gradient and activation of the reverse mode, which is also observed under conditions of ischaemia-reperfusion.²⁷

The Na⁺-ionophore cocktail/Ca²⁺-free medium clamps the [Na⁺]m at the level of the [Na⁺]cyt, (i.e. [Na⁺]m = [Na⁺]cyt, see Supplementary material). Using this cocktail medium containing 60 mM Na⁺, the peak [Na⁺]cyt and [Na⁺]m were seen at a Na⁺ concentration of ∼60 mM (Figure 4E), and RU360 again markedly, but not completely, prevented the [Na⁺]m overload, but not the [Na⁺]cyt overload (Figure 4E). A non-specific inhibitory effect of RU360 cannot therefore be ruled out at present.

There is evidence that [Ca²⁺]m overload is one of the major causes of PTP opening, including in heart cells.⁹,¹⁴,²³,²⁸ The following experiments investigated whether the [Ca²⁺]m and/or [Na⁺]m overload played a role in activation of the PTP, resulting in myocyte apoptosis.
FFAs also induce \([\text{Na}^+]_i\) and \([\text{Ca}^{2+}]_i\) overload. (A and B) Palmitic acid, (C and D) oleic acid, or (E and F) linoleic acid induces \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\) overload. All FFAs were 50 μM. (G and H) Dose response for the \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\) overload induced by 10–50 μM FFAs. La³⁺ (100 μM) inhibits the oleic acid- and linoleic acid-, but not palmitic acid-, induced \([\text{Ca}^{2+}]_i/\text{Na}^{+}\) influx. The values shown in G and H are the mean ± SEM for at least four different animals. **P < 0.05 by the Mann–Whitney U test.
Figure 3  AA induces cytosolic [Ca$^{2+}$]$_{cyt}$ and mitochondrial [Ca$^{2+}$]$_{m}$ overload and RU360 markedly inhibits the [Ca$^{2+}$]$_{m}$ overload. (A and B) 10 μM AA induced an increase in the [Ca$^{2+}$]$_{m}$ and [Ca$^{2+}$]$_{cyt}$ in two myocytes, using nuclear levels to represent cytosolic Ca$^{2+}$ levels ([Ca$^{2+}$]$_{cyt}$). (C and D) RU360 significantly inhibited the AA-induced [Ca$^{2+}$]$_{m}$ overload, but not the [Ca$^{2+}$]$_{cyt}$ overload (compare Ci and Cii). RU360 (10 μM) was used as a 60 min pretreatment and was present during recording. The bottom right panels in A and C show the localization of mitochondria (MTG, green; 'N' for nucleus) and the numbers indicate the position of the mitochondria analysed. The colour scale is shown below the left column in A and C. The arrows (A and C) indicate the time of AA addition. (E) Summary histogram (data from A–D). CGP37157 (10 μM, n = 6) inhibited the [Ca$^{2+}$]$_{m}$ overload (also see Supplementary material online, Figure S3A and B). In each experiment, the mean value for five responding mitochondria in each of four myocytes was calculated. The values shown are the mean ± SEM for five or six different experiments. *P < 0.05 by the Mann–Whitney U test.
Figure 4  AA induces [Na\(^+\)]\(_{\text{cyt}}\) and [Na\(^+\)]\(_{\text{m}}\) overload and the [Na\(^+\)]\(_{\text{m}}\) overload is markedly inhibited by RU360. (A-D) Recording methods were similar to those in Figure 3, but measuring Na\(^+\). The arrow indicates the time of addition of 10 \(\mu\)M AA (A and C), (C and D) RU360 (10 \(\mu\)M) significantly inhibited the AA-induced [Na\(^+\)]\(_{\text{m}}\) overload, but not the [Na\(^+\)]\(_{\text{cyt}}\) overload (compare Ci and Cii). (E) Summary histogram (data from A-D). Note that Ca\(^{2+}\)-free/EGTA \((n = 6)\) or 10 \(\mu\)M CGP37157 treatment (\(n = 5\); see also see Supplementary material online, Figure S3C-E) had little or no inhibitory effect on the [Na\(^+\)]\(_{\text{cyt}}\) or [Na\(^+\)]\(_{\text{m}}\) overload.

Fatty acids induce myocyte apoptosis
3.3 The [Na\(^+\)]\(_{\text{m}}\) overload is involved in permeability transition pore opening and mitochondrial swelling, resulting in cytochrome c release and activation of the caspase-3-dependent apoptotic machinery

A potent proton ionophore, FCCP, almost immediately and completely collapses the mitochondrial membrane potential (100% \(\Delta V_{\text{m}}\) depolarization)\(^1,2\) (first column in Figure 5Ai), and this effect has been shown to be CsA-insensitive.\(^3\) In myocytes, AA induced CsA-sensitive PTP opening, resulting in \(\Delta V_{\text{m}}\) depolarization (Figure 5Ai). The half maximal AA-induced depolarization was seen after 34 \(\pm\) 2.7 min (\(n = 5\)) exposure to AA, at which time peak levels of the [Ca\(^{2+}\)]\(_{\text{m}}\) and [Na\(^+\)]\(_{\text{m}}\) were usually observed (see Figures 1, 3 and 4). La\(^3+\), which inhibited the AA-induced Ca\(^{2+}\) and Na\(^+\) influxes (Figure 1F and H), or RU360, which inhibited the AA-induced [Ca\(^{2+}\)]\(_{\text{m}}\) and [Na\(^+\)]\(_{\text{m}}\) overloads (Figures 3 and 4), markedly inhibited the \(\Delta V_{\text{m}}\) depolarization (Figure 5Ai), indicating that the [Ca\(^{2+}\)]\(_{\text{m}}\) and/or [Na\(^+\)]\(_{\text{m}}\) overloads may play a role in the CsA-sensitive opening of the PTP.

We then found that AA-induced cytC release and caspase-3 activation (Figure 5Bii and Cii), both of which were markedly decreased by pretreatment with CsA, RU360, Na\(^+\)-free medium, Na\(^+\)- and Ca\(^{2+}\)-free medium, or La\(^3+\) medium (Figure 5Bvi and Cvi). Na\(^+\)-ionophore/ Ca\(^{2+}\)-free/EGTA medium also induced RU360-sensitive cytC release and caspase-3 activation (Figures 5Bvi and Cvi). Under Ca\(^{2+}\)-free conditions, surprisingly, only a small (10–12%), but significant, inhibition was seen of the effects of AA on cytC release (Figure 5Bvi) and caspase-3 activation (Figure 5Cvi). CGP37157, which inhibited the [Ca\(^{2+}\)]\(_{\text{m}}\) overload (Figure 3E), but not the [Na\(^+\)]\(_{\text{m}}\) overload (Figure 4E), had little inhibitory effect on cytC release (Figure 5Bvi), indicating that the [Na\(^+\)]\(_{\text{m}}\) overload is an important factor in cytC release. A caspase-3 inhibitor, Z-DEVD.fmk, inhibited the AA-induced caspase-3 activation (Figure 5Cvi), but not cytC release (Figure 5B), indicating that cytC release occurs upstream of caspase-3 activation. A potent Ca\(^{2+}\)-ionophore, ionomycin (15 \(\mu\)M in 2 mM Ca\(^{2+}\)-free medium), induced both cytC release and caspase-3 activation (Figure 5Bvi and Cvi). The above results indicate that not only the [Ca\(^{2+}\)]\(_{\text{m}}\) overload, but also the [Na\(^+\)]\(_{\text{m}}\) overload plays a role in the induction of PTP opening, resulting in cytC release and caspase-3 activation. Since AA induced ROS overproduction (see Supplementary material online, Figure S2), which is suggested to inhibit the mitochondrial respiratory chain, resulting in mitochondrial depolarization,\(^4,8\) a cocktail of ROS inhibitors (see Supplementary Material) was applied and found to have no inhibitory effect on either cytC release or caspase-3 activation (Figure 5Bvi and Cvi).

After exposure to AA, significant chromatin condensation (arrows and inset in Figure 6Aii, Hoechst staining) and DNA fragmentation (Figure 6Bii, green for TUNEL (+) staining) were seen. These effects were significantly inhibited by treatment with CsA, Na\(^+\)-free medium, or Z-DEVD.fmk, but not CGP37157 or a mixture of ROS inhibitors (Figure 6A and B). Marked inhibition of the AA-induced nuclear condensation and fragmentation was seen in Ca\(^{2+}\)-free medium (Figure 6Aiii and Biii) (see Discussion). RU360, which markedly inhibited both the AA- and Na\(^+\)-ionophore cocktail-induced [Na\(^+\)]\(_{\text{m}}\) and [Ca\(^{2+}\)]\(_{\text{m}}\) overload, largely inhibited condensation and fragmentation (Figure 6A and B). Again, addition of 15 \(\mu\)M ionomycin/Na\(^+\)-free medium induced marked nuclear fragmentation (Figure 6Bvii). Addition of 5–50 \(\mu\)M AA, palmitic acid, oleic acid, or linoleic acid resulted in dose-dependent nuclear condensation (Figure 6Aviii), which correlated with changes in the [Ca\(^{2+}\)]\(_{\text{m}}\) and [Na\(^+\)]\(_{\text{m}}\) (Figure 2H). La\(^3+\) (100 \(\mu\)M) had a significant inhibitory effect on AA-, oleic acid-, or linoleic acid-, but not palmitic acid-, induced nuclear condensation (Figure 6Aviii). The La\(^3+\)-insensitive palmitic acid-induced nuclear condensation was correlated with its small inhibitory effect on palmitic acid-induced Na\(^+\) and Ca\(^{2+}\) influx (Figure 2G and H).

Electron microscopy (EM) studies in animal models of ischaemic perfusion have shown TUNEL-positive nuclear fragmentation and mitochondrial hernia, with a loss of cristae in I/R-induced myocyte apoptosis.\(^3,0,31\) EM was therefore used to determine whether the AA- and NSCC-induced [Na\(^+\)]\(_{\text{m}}\) and/or [Ca\(^{2+}\)]\(_{\text{m}}\) overload played a role in the disruption of the outer mitochondrial membrane (OMM). After 60 min exposure to AA and washing for 60 min, disruption of the OMM, hernia, and cristae was seen (arrows in Figure 6Cb). Importantly, little disruption of the OMM and cristae was seen using AA/Ca\(^{2+}\) in Na\(^+\)-free medium (arrows in Figure 6Cc). Since Ca\(^{2+}\)-free medium had only a very small protective effect (Figure 6Cd) and the [Na\(^+\)]\(_{\text{m}}\) overload induced by AA was not inhibited by Ca\(^{2+}\)-free medium (see Figure 4E), the protective effect of Na\(^+\)-free medium on the integrity of the OMM indicates that [Na\(^+\)]\(_{\text{m}}\) overload plays an important role in AA- and NSCC-induced OMM disruption, resulting in cytC release (Figure 5Bii).

4. Discussion

It is well-documented that [Ca\(^{2+}\)]\(_{\text{m}}\) and [Ca\(^{2+}\)]\(_{\text{i}}\) overloads induce post-I/R myocardial injury. One major cause of the [Ca\(^{2+}\)]\(_{\text{m}}\) overload is that ischaemia-induced intracellular acidosis results in reperfusion-induced Na\(^+\)-H\(^+\) exchanger activation and causes [Na\(^+\)]\(_{\text{m}}\) overload, which may facilitate the subsequent influx of Ca\(^{2+}\) via the Na\(^+\)-Ca\(^{2+}\) exchanger in the plasma membrane.\(^3,2,33\) Application of inhibitors of either the Na\(^+\)-H\(^+\) exchanger, Na\(^+\)-Ca\(^{2+}\) exchanger, or Na\(^+\) channel prevents the [Ca\(^{2+}\)]\(_{\text{m}}\) overload and limits infarct size.\(^3,4,36\) However, the accumulation of FFAs in the myocardium is also suggested to be involved in post-I/R injury,\(^1,3\) since a time-dependent degradation of membrane phospholipids associated with an increase in membrane Ca\(^{2+}\) permeability has been observed in the ischaemic canine myocardium.\(^3,7\)

The present study showed that, in myocytes, after inhibition of all possible AA-modulated ion channels, AA and FFAs caused (i) La\(^3+\)-sensitive and/or La\(^3+\)-insensitive influx of extracellular Na\(^+\) and Ca\(^{2+}\) ions (Figures 1 and 2), possibly via a novel NSCC (see Supplementary material online, Figure S1), (ii) sustained Na\(^+\) and Ca\(^{2+}\) influx, resulting in accumulation of Ca\(^{2+}\) and Na\(^+\) in both the cytosol and mitochondrial matrix (Figures 3 and 4), and (iii) Ca\(^2+\)-sensitive PTP opening, OMM disruption, cytC release, caspase-3 activation, and induction of myocyte apoptosis (Figures 5 and 6). A summary diagram is shown in Figure 7.

Figure S1 (see Supplementary material online) shows that some of the biophysical properties of the AA-activated NSCC in the myocyte were different from those of the ARC in...
Both AA and FFAs induce myocyte apoptosis, resulting from intracellular [Ca\textsuperscript{2+}] and [Na\textsuperscript{+}] overload. (A) (i–iii) 10 \text{mM} AA induced both RU360- (first arrow, ii) and CsA-sensitive mitochondrial \Delta{}\Psi\text{m} depolarization (iii). The complete depolarization of the mitochondrial potential (100\% \Delta{}\Psi\text{m}, red arrows, i and ii) caused by 1 \text{mM} FCCP was CsA (1 \text{mM})-insensitive (iii). (B and C) Cells were exposed to 10 \text{mM} AA and labelled with either anti-cytC antibody (B) or FITC-DEVD-fmk (C). The AA- ionomycin (15 \text{mM})- or Na\textsuperscript{+}-ionophore-induced cytC release (reduction in green fluorescence in mitochondria, Bii and vii) and caspase-3 activation (green in Cii and vii) were both markedly inhibited by 1 \text{mM} CsA, 10 \text{mM} RU360, Na\textsuperscript{+}-free-, Na\textsuperscript{+}-Ca\textsuperscript{2+}-free, or 100 \text{mM} La\textsuperscript{3+} treatment, but not by CGP37157 (10 \text{mM}) or ROS inhibitors (catalase, phenanthroline, SOD, and tiron). Myocytes were identified using antibody A12 (red in C). The control experiment (first bar, Bvii and Cvii) was performed in the presence of 0.02\% of ethanol as vehicle. The values shown are the mean ± SEM for at least five experiments. *P < 0.05 compared with the AA group by the Mann–Whitney U test.
Figure 6  AA, FFAs, or Na⁺-ionophore cocktail induces Na⁺-sensitive mitochondrial membrane rupture, nuclear condensation, and DNA fragmentation. (A and B) [Ca²⁺]ᵢ- and [Na⁺]ᵢ-dependent nuclear changes. Cells exposed to 5–50 μM AA or FFAs showing Z-DEVD.fmk-sensitive chromatin condensation (inset, arrows in Aii, Hoechst staining) and DNA fragmentation (Bii, TUNEL). La³⁺(100 μM)-insensitive palmitic acid-induced nuclear condensation was also shown in Aviii. CGP37157 or a mixture of ROS inhibitors had little inhibitory effect. The cells were double-labelled with (A) Hoechst 33342 (blue) and antibody A12 (red) or (B) TUNEL reagent (green) and antibody A12 (red). *P < 0.05 compared with the AA, FFAs, or Na⁺-ionophore group by the Mann–Whitney U test. (C) AA-induced Na⁺-sensitive mitochondrial (Mt) membrane rupture observed by electron microscopy. After exposure to 10 μM AA for 60 min and washing for 60 min, mitochondrial swelling, disruption of the mitochondrial membrane, and cristae were observed (arrows in Cb). Na⁺-free treatment markedly reduced membrane disruption (arrows inCc), but not the matrix swelling.
non-excitable cells, i.e. (i) the I/V curve of the NSCC was linear without rectification and its reversal potential close to zero, and (ii) the Ca\(^{2+}\) to Na\(^{+}\) permeability ratio of the NSCC was lower (Ca\(^{2+}\)/Na\(^{+}\) = 0.26) than that of the ARC channel, which shows little Na\(^{+}\) permeability in the presence of divalent ions.\(^{16,17}\) However, we suggest that the AA-induced NSCC is not a protein channel, as it behaves like a non-selective cation 'pore' which can be opened when AA or FFAs accumulates, as occurs in certain pathological conditions,\(^{1,2,7}\) since (i) no current inactivation of the NSCC was seen within the 400 ms voltage-clamp period (see Supplementary material online, Figure S1Ab), (ii) the current density of the AA-induced NSCC measured at −80 mV (see Supplementary material online, Figure S1Biiii) was much higher than that normally seen for NSCCs, and (iii) the AA- or FFA-induced intracellular Ca\(^{2+}\) and Na\(^{+}\) overload was long-lasting (Figures 1 and 2). However, the NSCC is not a leak current, since the conductance was markedly reduced after Na\(^{+}\) removal (see Supplementary material online, Figure S1Ad). The Ca\(^{2+}\) and Na\(^{+}\) influx was not caused by membrane disruption, since there were no fluorescent probe leaks during the 50–60 min of AA or FFAs application (Figures 1 and 2). Comparison with known Ca\(^{2+}\)- and Na\(^{+}\) ionophores (Figures 4–6) showed that AA (≥5 μM, Figure 1A) or FFAs (≥30 μM) may behave like endogenous ionophores, which accumulate during post-I/R, resulting in marked Ca\(^{2+}\)/Na\(^{+}\) influx. Importantly, these naturally occurring ionophores (AA and FFAs) may induce [Ca\(^{2+}\)]\(_{m}\) and [Na\(^{+}\)]\(_{m}\) overload with different potencies, resulting in dose-dependent and La\(^{3+}\)-sensitive or insensitive-nuclear condensation (Figure 6Aviii). These results strengthened our finding that the Na\(^{+}\) and Ca\(^{2+}\) influxes are important in myocyte condensation.

We also found that, as well as the [Ca\(^{2+}\)]\(_{m}\) overload, the [Na\(^{+}\)]\(_{m}\) overload was an important upstream signal for AA-induced mitochondrial-mediated apoptosis, since (i) RU360 (which markedly inhibited both the [Na\(^{+}\)]\(_{m}\) and [Ca\(^{2+}\)]\(_{m}\) overload), Na\(^{+}\)-free medium, or La\(^{3+}\)-markedly inhibited AA- or FFA-induced apoptosis, including CsA-sensitive PTP opening, cytC release, caspase-3 activation, and nuclear condensation/fragmentation (Figures 5 and 6), (ii) Na\(^{+}\) ionophore/Ca\(^{2+}\)-free medium also caused cytC release and caspase-3-dependent apoptosis (Figures 5 and 6), indicating that [Na\(^{+}\)]\(_{m}\) overload per se (in the absence of [Ca\(^{2+}\)]\(_{m}\) overload) could cause PTP opening, resulting in cytC release, and (iii) importantly, AA-induced OMM disruption was markedly abolished in Na\(^{+}\)-free/AA (+2 mM Ca\(^{2+}\)) medium (Figure 6Cc), again indicating that [Na\(^{+}\)]\(_{m}\) overload is an upstream signal for PTP opening.

The molecular mechanisms proposed for AA-induced CsA-sensitive PTP opening in non-excitable cells or isolated mitochondria are (i) AA induces Ca\(^{2+}\) release from the ER, which is followed by mitochondrial Ca\(^{2+}\) uptake,\(^{38}\) triggering persistent PTP opening, and (ii) in the absence of [Ca\(^{2+}\)]\(_{m}\) overload, AA directly activates the voltage sensor in the PTP.\(^{39}\) In myocytes, however, AA-induced ER Ca\(^{2+}\) release was negligible, since there was little increase in the [Ca\(^{2+}\)]\(_{cyt}\) or [Ca\(^{2+}\)]\(_{m}\) when Ca\(^{2+}\)-influx was inhibited under Ca\(^{2+}\)-free conditions (Figure 3E). Direct activation of the voltage sensor in the PTP by AA also seems unlikely, since La\(^{3+}\) inhibited the AA-induced Ca\(^{2+}\) and Na\(^{+}\) influx (Figure 1H) and RU360 or Na\(^{+}\)-free medium inhibited the AA-induced [Ca\(^{2+}\)]\(_{m}\) and [Na\(^{+}\)]\(_{m}\) overloads (Figures 3 and 4), and all of these treatments largely inhibited the AA-induced PTP opening (Figure 5Aiiii) and cytC release (Figure 5Biiii and iv). Moreover, CsA, a potent PTP inhibitor (Figure 5Aiiii), did not

Figure 7  Schematic diagram. Endogenous ionophores (AA or FFAs) open a La\(^{3+}\)-sensitive or La\(^{3+}\)-insensitive NSCC, resulting in cytosolic and mitochondrial [Na\(^{+}\)] and [Ca\(^{2+}\)] overload and in delayed myocyte apoptosis. The RU360-sensitive [Na\(^{+}\)]\(_{m}\) and [Ca\(^{2+}\)]\(_{m}\) overloads activate the apoptotic machinery, including membrane disruption, resulting in CsA-sensitive PTP opening, cytC release, and caspase-3-dependent nuclear condensation.
inhibit the AA-induced Ca\(^{2+}\) influx (see Figure 1H), indicating that AA-induced Ca\(^{2+}\) influx was an upstream event of PTP opening.

One hypothesis regarding cytC release resulting from disruption of the OMM is that persistent PTP opening induces water influx, resulting in matrix swelling and OMM disruption.\(^{12-40}\) Since Na\(^+\) is an important osmolyte, it is possible that the [Na\(^+\)]\(_{\text{m}}\) overload may contribute to the mitochondrial swelling and OMM disruption, resulting in cytC release. An important unanswered question is how matrix Na\(^+\) overload directly or indirectly activates persistent PTP opening followed by mitochondrial membrane disruption (Figure 6Cc vs. Cb). Clearly, additional experiments are warranted to elucidate the mechanism.

Ca\(^{2+}\)-free/EGTA medium had a small, but significant, inhibitory effect (~10%) on AA-induced cytC release and caspase-3 activation (Figure 5Bvii and Cvii), but a marked inhibitory effect (~55%) on nuclear condensation and fragmentation (Figure 6Avii and Bvii). One possible explanation is that the apoptotic effectors downstream of the nuclear changes are Ca\(^{2+}\)-dependent, for example, Ca\(^{2+}\)-dependent endonuclease activation.\(^{41}\) It is also intriguing to note that Ca\(^{2+}\)-free medium had very little protective effect on AA-induced OMM disruption (Figure 6Cd). Since the Ca\(^{2+}\) to Na\(^+\) permeability ratio of the NSCC is low, the [Ca\(^{2+}\)]\(_{\text{m}}\) overload induced by AA may not be high enough to disrupt the OMM.

In summary, the present study shows that AA and FFAs activate La\(^{3+}\)-sensitive and/or La\(^{3+}\)-insensitive Na\(^+\) and Ca\(^{2+}\) influxes, resulting in both cytosolic and mitochondrial Na\(^+\) and Ca\(^{2+}\) overload, followed by activation of the mitochondrial-mediated caspase-3-dependent apoptotic pathway. AA and FFAs, which accumulate in the myocardium during post-I/R, may therefore act as naturally occurring endogenous ionophores and contribute to the myocyte death seen during post-I/R.

SUPPLEMENTARY MATERIAL
Supplementary material is available at Cardiovascular Research online.

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