Effects of Antiarrhythmic Drugs on Apoptotic Pathways in H9c2 Cardiac Cells

Shojiro Isomoto1,2,*, Atsushi Kawakami3, Tatsuya Arakaki1, Shunichi Yamashita4, Katsusuke Yano2, and Katsushige Ono1

1Department of Cardiovascular Science, Oita University School of Medicine, 1-1 Idaigaoka, Hasama, Yufu 879-5593, Japan
Divisions of 2Cardiovascular Medicine and 3Immunology, Endocrinology and Metabolism, Department of Translational Medical Sciences, Course of Medical and Dental Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
4Division of Radiation Biology, Department of Radiology and Radiation Biology, Course of Life Sciences and Radiation Research, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Received November 25, 2005; Accepted June 22, 2006

Abstract. Antiarrhythmic drugs may induce cellular apoptosis in the heart. By using representatives of 5 different categories of antiarrhythmic drugs, that is, pilsicainide, propranolol, nifekalant, verapamil, and amiodarone, we investigated whether these ion channel blockers or β-antagonists affect cardiac apoptosis in cell cultures. Cultured H9c2 cells were treated with the drugs at varying concentrations. To determine the degree of apoptosis, the percentage of hypodiploid cells, mitochondrial transmembrane potential (∆Ψm), and activities of caspases were measured quantitatively. At 24 h after administration, only amiodarone induced apoptosis in the H9c2 cells. Amiodarone at a concentration of 14.8 µM or higher decreased ∆Ψm and activated caspase-2 within 3 h of administration, and it caused the appearance of hypodiploid cells and activation of caspases-3 and -9 at 6 h or later. Thus, amiodarone, but none of the other antiarrhythmic drugs tested, possesses a pro-apoptotic effect, mainly via the mitochondrial pathway, suggesting that this effect is distinct from the blocking action of Na+, K+, and Ca2+ channels or the β-adrenergic receptor. Furthermore, induction of apoptosis in a dose-dependent manner by amiodarone indicates the importance of monitoring the serum concentration in order to avoid its adverse effects.

Keywords: antiarrhythmic drug, amiodarone, apoptosis, H9c2 cell

Introduction

Apoptosis is an active process of cell death, morphologically and biochemically distinct from necrosis, which occurs under both physiologic and pathophysiologic conditions (1). The pathological activation of apoptosis is now thought to contribute to a variety of disease processes including cancer, neurodegenerative disease, and autoimmune disease (2). Apoptosis is also observed in the human cardiovascular system, for example, in cardiomyocytes in end stage heart failure or after myocardial infarction and in vascular smooth muscle cells in atherosclerotic plaques or after arterial injury (3–5). Moreover, several chemotherapeutic agents that are well known to lead to cardiac damage have been shown have cardiotoxicity, in part, through the induction of apoptosis (6, 7). Much attention has thus been devoted to the role of apoptosis in the pathogenesis of drug-induced cytotoxicity.

Antiarrhythmic drugs are a diverse group of drugs that affect various cardiac ionic channels or receptors. The standard classification of antiarrhythmic drugs was developed by Singh and Vaughan Williams, based upon the drug’s electrophysiological mechanisms of action (8). This classification scheme is relatively simple and is still in use, although an alternative classification (the Sicilian Gambit), which is based on molecular actions

*Corresponding author. isom@med.oita-u.ac.jp
Published online in J-STAGE: August 5, 2006
doi: 10.1254/jphs.FP0050951
and vulnerable parameters involved in arrhythmias, has recently been proposed (9). In the former scheme, class I drugs are those with local anesthetic properties that block Na+ channels, class II drugs are β-adrenergic blocking agents, class III drugs prolong action potential duration by blocking outward K+ conductance, and class IV drugs are Ca2+ entry blockers. Some of these drugs, such as amiodarone and bepridil, have multichannel blocking actions. Amiodarone is unique in that it possesses properties belonging to all four of the Singh and Vaughan Williams classes. Recent studies have shown that the action of compounds with antiarrhythmic effects is involved in the apoptotic pathway of several tissues and that apoptotic effects of the compounds differ in different cell types depending on dosage (10-15). However, although antiarrhythmic drugs are chiefly cardioactive, their pro-apoptotic effects on cardiac cells are poorly understood.

We hypothesized that some antiarrhythmic drugs may cause cardiotoxicity through the induction of apoptosis. The aim of this study was to investigate using the H9c2 cell, a clonal myogenic cell line derived from embryonic rat ventricle, whether apoptosis contributes to cardiac cytotoxicity caused by these antiarrhythmic drugs at their individual clinically achievable concentrations, and, if so, by what mechanism. In this context, we examined the effects of representatives of five different categories of antiarrhythmic drugs, that is, pilsicainide (a pure class I), propranolol (a pure class II), nifekalant (a pure class III), verapamil (a pure class IV), and amiodarone (a multichannel blocker).

**Materials and Methods**

**Cell culture**

H9c2 cells were obtained from the American Type Culture Collection (16). Cells were cultured on 60-mm-diameter dishes in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco). The culture medium was exchanged every 2 days, and the H9c2 cells were maintained as a monolayer culture at 37°C in a 5% CO2 atmosphere at 95% humidity for 3 or 4 days until the cells had grown almost confluent.

**Administration of antiarrhythmic drugs**

Pilsicainide was provided by Daiichi Pharmaceutical Co., Ltd. (Tokyo), and nifekalant by Nihon Schering KK (Osaka). Propranolol, verapamil, and amiodarone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). To make up a stock solution of each drug, amiodarone was dissolved in dimethyl sulfoxide (DMSO, Sigma). The other antiarrhythmic drugs were dissolved in distilled water. Finally, identical concentrations of DMSO were added to each drug tested in serum-free DMEM. Since high concentrations of DMSO have been shown to cause apoptosis (17), its final concentration in the medium was maintained at 0.099%, a level at which induced apoptosis has not been reported. Control incubations were performed in the presence of the diluent, DMSO, used alone at the same concentration as the test drugs.

The cultured H9c2 cells were incubated up to 24 h with the antiarrhythmic agents at varying concentrations. Because each drug has its own pharmacological potency, the drug concentrations tested in this study were adapted based on their clinical concentrations. Pilsicainide and nifekalant are often used clinically in the treatment of several types of tachyarrhythmias in Japan, in which the upper limit of therapeutic concentration has been suggested to be 2.8 µM and 2.2 µM, respectively (18, 19). The therapeutic serum concentration ranges of propranolol, verapamil, and amiodarone have been reported differently by different authors and researchers (19, 20). Referring to the reports, we judged that the upper limit of therapeutic concentration for propranolol is 0.80 µM, 0.60 µM for verapamil, and 3.7 µM for amiodarone. We exposed the H9c2 cells to the five test antiarrhythmic drugs at a range of from 1 to 8 times the upper limit of their individual therapeutic concentrations.

**Determination of H9c2 cell apoptosis**

After cultivation, apoptotic cell death of H9c2 cells was quantitatively evaluated by DNA fragmentation, loss of mitochondrial transmembrane potential (ΔΨm), and activities of caspases-2, -3, -8, and -9, as has been previously described (21).

DNA fragmentation in the H9c2 cells was expressed as the percentage of hypodiploid DNA cells. In brief, the H9c2 cells were fixed with 70% ethanol, treated with RNAase (100 µg/ml, Sigma), and then stained with propidium iodide (100 µg/ml, Sigma) for 30 min on ice. The stained cells were analyzed by a flow cytometer (Becton Dickinson, San Jose, CA, USA) to detect the cells with hypodiploid DNA.

Mitochondrial perturbation in the H9c2 cells was examined by ΔΨm. The cells were incubated at 37°C for 15 min with 40 nM of the potential-sensitive fluorescence dye, 3,3′-dihexyl-oxacarbocyanine iodide [DiOC6(3); Fluoreszenz Technologie, Grottenhofstr, Austria], and then analyzed by flow cytometry.

The enzymatic activities of caspases-2, -3, -8, and -9 were estimated through the use of a colorimetric protease assay kit (MBL, Nagoya), following the manufacturer’s protocol. The peptide substrates conjugated
with \( p \)-nitroanilide (\( p \)NA) for caspase-2 (VDVAD-\( p \)NA), caspase-3 (DEVD-\( p \)NA), caspase-8 (IETD-\( p \)NA), and caspase-9 (LEHD-\( p \)NA) were used in the testing. Briefly, cell lysates from the H9c2 cells were mixed with each substrate at 37°C for 60 min. After incubation, the activity of caspases was evaluated by a spectrophotometer (Multiskan JX; Labsystems, Tokyo) at an O.D. of 405 nm.

Statistical analysis

Data were expressed as the mean ± S.E.M., and were analyzed with one-way ANOVA followed by a post hoc Scheffé F test. Differences were considered significant at \( P<0.05 \).

Results

Effects of antiarrhythmic drugs on apoptotic parameters in H9c2 cells

To examine whether the antiarrhythmic drugs tested induce apoptosis in H9c2 cells, the cells were incubated over 24 h with each drug at the concentration 8 times higher than its maximum therapeutic concentration.

As shown in Fig. 1, a and b, at 8 times the upper limit of therapeutic concentration of each drug, amiodarone (29.6 \( \mu \)M) but not other drugs increased the percentage of hypodiploid cells. The percentage of hypodiploid cells was significantly increased by 11.3-fold in the cells treated with amiodarone compared with vehicle-treated control cells (47.3 ± 12.9\% vs 4.2 ± 0.9\%, \( P<0.01 \)). Likewise, at 8 times the concentrations, only the cells treated with amiodarone showed reduced DiOC\(_{6}\)(3) staining (Fig. 1c). Compared with vehicle alone, treatment with amiodarone resulted in a significant (7.9-fold) increase in the percentage of cells exhibiting a loss of \( \Delta \Psi_m \) (44.8 ± 2.5\% for amiodarone vs 5.7 ± 0.5\% for vehicle alone, \( P<0.01 \)) (Fig. 1d). In addition, at 8 times the upper limit of therapeutic concentration for each drug, only amiodarone significantly enhanced caspase-3 activity, by 3.7-fold, compared to vehicle alone (OD 0.197 ± 0.037 vs 0.053 ± 0.001, \( P<0.01 \)) (Fig. 1e). All the drugs tested including amiodarone failed to induce apoptosis at the upper limit of the therapeutic concentration range (data not shown).

Time- and dose-dependent manners of amiodarone-induced apoptosis in H9c2 cells

Because DNA fragmentation, loss of \( \Delta \Psi_m \) and activation of caspase-3 were observed in the H9c2 cells treated with amiodarone 29.6 \( \mu \)M for 24 h, we examined the time- and dose-dependent manner of amiodarone-induced apoptosis. The apoptotic parameters were monitored in at 3, 6, 12, and 24 h following administration of amiodarone at concentrations of 0, 3.7, 7.4, 14.8, and 29.6 \( \mu \)M. The apoptotic parameters were not significantly altered during the time-course studies for the DMSO vehicle used as a control (data not shown).

As shown in Fig. 2a, in comparison with the control vehicle for each of the other drugs, the percentage of hypodiploid cells was significantly increased at 6 h after administration of amiodarone at 29.6 \( \mu \)M and at 24 h using a concentration of 14.8 \( \mu \)M. At 6 and 12 h after addition of amiodarone at 14.8 \( \mu \)M, the hypodiploid cells were slightly but not significantly increased, by over 2-fold, compared with each vehicle control. The number of cells exhibiting a loss of \( \Delta \Psi_m \) was increased as early as 3 h after addition of amiodarone at 14.8 or 29.6 \( \mu \)M, although the increase was not significant at 3 h with the addition of amiodarone at 14.8 \( \mu \)M (Fig. 2b).

The time-course of activation of caspases-2, -3, -8, and -9 in the apoptosis induced by amiodarone at 29.6 \( \mu \)M is shown in Fig. 3a. At 3 h post-amiodarone treatment, only caspase-2 activity was significantly enhanced. Caspase-3 and -9 activities were highly significantly enhanced at 6 h or later after amiodarone treatment, as was caspase-8 activity at 24 h. The effects of varying concentrations of amiodarone on the activity of caspases are shown in Fig. 3b. Amiodarone at concentrations of 14.8 or 29.6 \( \mu \)M, but not 7.4 \( \mu \)M or less, enhanced the activities of caspases-2, -3, and -9 at 6 h after addition, when DNA fragmentation could be detected.

Discussion

To the best of our knowledge, the present study is the first one to systematically investigate the pro-apoptotic effects of clinically available antiarrhythmic drugs on cardiac cells. Our study demonstrates that amiodarone, but not pilsicainide, propranolol, nifekalant, or verapamil, possesses pro-apoptotic effect at a concentration of 4 times or more the upper limit of the therapeutic range for each drug.

Although selective blockers for Na\(^+\), K\(^+\), and Ca\(^{2+}\) channels or the \( \beta \)-adrenergic receptor tested in this study failed to induce apoptosis in H9c2 cardiac cells, apoptosis induced by compounds belonging to the same categories as the blockers tested in this study has been observed in certain cell and tissue types. For example, clofilium, a potent K\(^+\) channel blocker, induced apoptosis in human promyelocytic leukemia cells at a concentration of 10 \( \mu \)M (10), but conversely, attenuated apoptosis induced by hypoxia at 0.1 \( \mu \)M in cultured cortical neurons (11). Verapamil-induced apoptosis was observed in rat thymus in vivo and in Chinese hamster
Antiarrhythmic Drugs and Apoptosis 321

Ovaryl cells in vitro (12, 13). On the other hand, verapamil alleviated apoptosis induced by angiotensin II in cardiomyocytes (14) and that by simvastatin in vascular smooth muscle cells (15). It was demonstrated that apoptosis was induced by 2 $\mu$M verapamil in the ovary cells (13), but not in H9c2 cells, as was shown in our study. From these observations, it would appear that the various antiarrhythmic drugs have distinct actions leading to apoptosis, depending on the specific target cell type or on their concentration. Individual studies are thus needed to clarify the apoptotic effects of specific drugs on specific tissues or cells. Our observations in this study demonstrated that pilsicainide, propranolol, nifekalant, and verapamil failed to induce apoptosis in

Fig. 1. Effects of antiarrhythmic drugs on apoptotic parameters in H9c2 cells. H9c2 cells were cultured with antiarrhythmic drugs for 24 h at 8 times the upper limit of therapeutic concentration of each drug. DNA fragmentation, expressed as the percentage of hypodiploid DNA cells, and mitochondrial transmembrane potential ($\Delta\Psi_m$) were analyzed by flow cytometry as described in Material and Methods. Caspase-3 activity was estimated by colorimetric protease assay. a: Histograms of the hypodiploid DNA cells (indicated by arrows). b: Summarized data for DNA fragmentation. c: Histograms of the cells exhibiting a loss of $\Delta\Psi_m$ (indicated by arrows). d: Summarized data for the percentage of cells exhibiting a loss of $\Delta\Psi_m$. e: Summarized data for caspase-3 activity in the cells. Data are the mean ± S.E.M. from four separate experiments. **$P<0.01$ vs vehicle control.
H9c2 cells even at a relatively high concentration, suggesting that these drugs may not induce cardiac apoptosis in clinical situations. In addition, it is suggested that the blocking actions on Na\(^+\), K\(^+\), and Ca\(^{2+}\) channels and the \(\beta\)-adrenergic receptor, which are electrophysiological relevancies of amiodarone, are not essential for the generation of cardiac apoptosis.

Amiodarone is the most promising drug in the treatment of life-threatening tachyarrhythmias, and consequently the prevention of sudden cardiac death. However, amiodarone has been shown to result in a number of relatively frequently adverse effects, for example, pulmonary toxicity, thyroid dysfunction, and liver dysfunction (22, 23). Recent studies have demonstrated that amiodarone-induced cytotoxicity in the alveolar epithelial cells, thyroid cells, and hepatocytes are associated with apoptosis (24 – 27). In contrast to noncardiac cells, amiodarone has been shown to have little toxic potentials for cardiac myocytes at therapeutic concentrations (22, 23), even though adding this drug to cardioplegic solutions led to dose-dependent myocardial damage in pig hearts exposed to cardiac surgery (28). One of the explanations for this lower cardiac toxicity of amiodarone may be due to the differences in tissue sensitivity to the drug. Assuming that cardiac myocytes are less sensitive to amiodarone than other cell types, an attempt must be made to discontinue the drug due to noncardiac adverse effects before occurrence of cardiac toxicity. In fact, amiodarone at the concentration that did not induce apoptosis in H9c2 cells has been shown to increase apoptotic events in other cell types such as alveolar epithelial cells and hepatocytes (24, 27). The differences in tissue distribution of amiodarone, and its exerted cardioprotective effects by the inhibition of the Na\(^+\)/Ca\(^{2+}\) exchanger (29), or by directly scavenging oxygen free radicals that cause oxidative stress-mediated...
injury (30) may also explain the lower cardiac toxicity of the drug.

An increase in the value of the apoptosis-related parameters during amiodarone treatment in H9c2 cells was time-dependent, offering elucidation of its apoptotic pathways. There are two main pathways leading to apoptosis: the participation of mitochondria and the interaction of death receptors with their specific ligands (31, 32). Mitochondrial damage, that is, loss of $\Delta \Psi_m$, leads to activation of caspase-9. In contrast, ligation of death receptors activates caspase-8. These initiator caspases then propagate the cascade of downstream effector caspases, including caspase-3. The activation of caspase-3 leads to DNA fragmentation and cleavage of cytoplasmic substrates for the manifestation of apoptotic morphological changes. We demonstrated that loss of $\Delta \Psi_m$ was observed prior to DNA fragmentation with the expression of activated caspases-3 and -9 during the apoptotic process. This finding indicates that one possible mechanism for amiodarone-induced apoptosis is via the mitochondrial pathway. Among caspases tested in this study, only caspase-2 was activated within 3 h after administration of amiodarone. Caspase-2 has been recognized to be activated downstream of caspase-9 and caspase-3 (31) and to act upstream of mitochondria as an initiator caspase (32). The present study clearly indicates that caspase-2 acts as an initiator rather than as an effector during the amiodarone-induced apoptosis. Caspase-8 was also activated in amiodarone-treated cells. However, substantial activation of caspase-8 was not observed until 24 h, which was delayed compared to the activation of caspase-3 and the occurrence of DNA fragmentation. This observation suggests that the death receptor-mediated pathway is not the main mechanism of amiodarone-induced apoptosis. Caspase-8 has been shown not only to be an initiator caspase of the death receptor-mediated pathway, but also to be activated downstream of caspase-3 in a positive feedback loop enhancing cell death (31). Therefore, the present time-course study suggests that the most proximate event in amiodarone-induced cardiac apoptosis is activation of caspase-2, followed by activation of the mitochondrial pathway that involves the activation of caspase-9 and its downstream caspase-3 prior to the occurrence of DNA fragmentation, and ultimately by activation of caspase-8.

Admittedly, our study has limitations. We acknowledge that it may be difficult to extrapolate the results of the present in vitro study to in vivo conditions. Moreover, because this study was of short duration, the intracellular accumulation of the drugs with chronic exposure could not be assessed. To settle these issues, it will be necessary to carry out analyses under in vivo conditions using animals receiving long term treatment with these drugs. Another disadvantage of this study is that we did not assess the effects of antiarrhythmic drugs on the possible anti-apoptotic, caspase-independent apoptotic, and necrotic pathways (33). Despite these limitations, the results obtained from our systematic study suggest, at a minimum, that amiodarone can induce apoptosis in cardiac myocytes in a dose- and time-dependent manner and that amiodarone is more cardiotoxic than the other antiarrhythmic drugs tested.

In conclusion, the antiarrhythmic drugs tested, other than amiodarone, appear to have a low cardiac toxicity through apoptosis. Because amiodarone caused the significant apoptotic effect at the concentration 4 times higher than its maximum therapeutic concentration, it should be cautiously used for the patients with arrhythmia or congestive heart failure which make cardiac myocytes prone to apoptosis. Our data demonstrate the importance of monitoring the serum amiodarone concentration in order to avoid the adverse effects including cardiotoxicity.

Acknowledgment

This work was supported by Japan Foundation of Cardiovascular Research.

References

9 The Sicilian gambit. A new approach to the classification of antiarrhythmic drugs based on their actions on arrhythmogenic


