Effect of Amiodarone on Release of Cytokines from Mouse Alveolar Macrophages Pretreated with Eicosapentaenoic Acid

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ABSTRACT—I studied the effect of amiodarone, a cationic amphiphilic drug, on the cytokine release and protein kinase C (PKC) activity of mouse alveolar macrophages. In addition, I examined the relationship between amiodarone and eicosapentaenoic acid (EPA) with respect to the cytokine release. The decrease in cell number caused by amiodarone was depressed by pretreatment of the macrophages with EPA for 2 days and co-treatment for 1 day. These changes reflected the potency of EPA to protect against the cell injury elicited by amiodarone. As regards to the cytokine release, amiodarone caused an increase in interleukin (IL)-1α, IL-1β and tumor necrosis factor (TNF)α release from macrophages. As EPA suppressed this increase in cytokine levels, I considered that the protective effect of EPA may be extended to the acute release of cytokines. PKC activity was increased by amiodarone, and this increase was depressed by EPA. These changes were well-related to the results on cytokine levels in this study, indicating that amiodarone firstly activated PKC, leading to the stimulation of release of cytokines and that pretreatment with EPA prevented these effects. I conclude that mouse alveolar macrophages treated with amiodarone show activated release of cytokines and that EPA depresses these increases, thereby demonstrating EPA’s anti-inflammatory effect and protective action against injury of alveolar macrophages.

Keywords: Alveolar macrophage, Amiodarone, Cytokine, Eicosapentaenoic acid, Protein kinase C

I have been studying the toxicity caused by cationic amphiphilic drugs (CADS); These drugs consist of a basic amine group attached via a short side chain to a hydrophobic moiety, and they include amantadine, amiodarone, imipramine, mianserin, tripelennamine and more than 30 other compounds (1). Although these drugs vary widely in their principal pharmacological actions, CADs have been shown to cause lipidosis in humans, animals and cultured cells (2). Hostetler and Matsuzawa found that several CADs inhibited the in vitro activity of lysosomal phospholipases A and C, enzymes that are active toward most of the phosphoglycerides (3). In addition, high doses of these CADs damage cell membranes and cellular function as well as reduce cell viability (1). Morphologically, the lipid accumulation appears to be a consequence of changes in the metabolism of phosphatidylinositol and sphingomyelin; that is, components of the intracellular signal transduction system induced by several drugs (4).

Amiodarone is an anti-arrhythmic drug and holds great promise for the treatment of supraventricular and ventricular arrhythmias, particularly those associated with the Wolff-Parkinson-White syndrome (5, 6). However, the use of amiodarone is accompanied by a variety of adverse reactions, the most serious being a dose-dependent pulmonary toxicity (7). The mechanism by which pulmonary toxicity develops in patients receiving amiodarone is unknown, although it has been suggested that alterations in pulmonary phospholipid metabolism are causally related to the amiodarone-induced toxicity (8).

Eicosapentaenoic acid (EPA) has proved to have a beneficial effect on lipid metabolism, and it is used for the treatment of hypercholesterolemia and atherosclerosis (9, 10). It is also reported that EPA is effective toward active inflammation and in modulating the immune system (11). Terano and his co-workers reported that EPA has potent inhibitory activity toward inflammation (12), Lefkowith et al. described the effect of EPA on acute inflammation (13), and others reported the improvement of acute immune disease in NZB/NZW mice treated with EPA (14). In addition, Endres et al. proposed that the anti-inflammatory effect of EPA and other n-3 fatty acids might be mediated by their inhibitory effect on the release of interleukin-1 (IL-1) and tumor necrosis factor (TNF) (15).
Alveolar macrophages in the lung cavity are involved in local defense against a variety of pathogenic and particulate entities that gain entrance via the airway. These cells play a role in the early stages of inflammation and in controlling infection by releasing cytokines, e.g., IL-1 and TNF. Alveolar macrophages have a central role in chronic granulomatous conditions such as sarcoidosis, silicosis and asbestosis (16). The present study examined the effects of amiodarone on the release of IL-1 and TNF by mouse alveolar macrophages and those of EPA on the release of these cytokines. I determined the protein kinase C (PKC) activity of macrophages to define the mechanism responsible for cytokine release induced by amiodarone.

MATERIALS AND METHODS

Test materials

Amiodarone hydrochloride (#A8423) and cis-5,8,11,14,17-eicosapentaenoic acid sodium salt (EPA, #E6627) were purchased from Sigma Co., Ltd. (St. Louis, MO, USA). Recombinant mouse interleukin-1α (IL-1α, #1920-01), recombinant mouse interleukin-1β (IL-1β, #1921-01) and recombinant mouse tumor necrosis factor α (TNFα, #TNFM) and their respective polyclonal antibodies (generated in rabbits), i.e., anti mouse IL-1 antibody (#IP-110), anti mouse IL-1β antibody (#80-3188-01) and anti mouse TNFα antibody (#IP-400), were obtained from Genzyme Co., Ltd. (Cambridge, UK). Other reagents used in this study were special grade. Amiodarone was dissolved and diluted in 50% ethanol. EPA was dissolved and diluted in phosphate-buffered saline (pH 7.4, PBS) and used as soon as possible thereafter.

Isolation and cultivation of mouse alveolar macrophages

Alveolar macrophages were isolated by the adhesion method (17). In brief, male Slc:ICR mice (8-week-old, SPF) were obtained from Japan SLC Co., Ltd. (Shizuoka). These animals were anesthetized by intraperitoneal injection with sodium pentobarbital at a dose of 25 mg/kg, and after exsanguination, the trachea of each animal was opened. After a tracheal catheter had been attached, warm MEM medium (Dainippon Pharmaceutical Co., Ltd., Osaka) was gently introduced into the trachea via a syringe until the lung had expanded to give an almost 80% increase in lung volume. Thereafter, amiodarone solution was added to the well at a dose of 0.02, 0.2, 2 or 20 μM; and the plate was then incubated for 24 hr under the above culture conditions. The number of viable cells was counted microscopically after trypsinization and staining with trypan blue (Wako Pure Chemical, Inc., Osaka) solution. Ethanol (0.1% v/v) was used as the control for amiodarone treatment, and PBS was used as the vehicle control for EPA.

Determination of cytokine levels

Determination of cytokine contents in the supernatant was carried out by the TR-FIA method (Pharmacia Biotech Co., Ltd., Uppsala, Sweden) (18, 19). After amiodarone (0.02, 0.2, 2 or 20 μM) had been added to wells containing mouse alveolar macrophages previously treated with EPA for 2 days according to the method used for the cytotoxicity test, the plate was incubated for 24 hr at 37°C. Then the medium was collected, and its supernatant was obtained by centrifugation (3000 x g for 5 min at 4°C). To each microwell (96-well microplate; Nunc, Naperville, IL, USA) that had been previously coated with anti rabbit IgG antibody solution, anti murine IL-1α antibody, anti murine IL-1β antibody or anti murine TNFα antibody solution was added and the microplate shaken. After incubation for 1 hr at room temperature, each microwell was washed three times with DELFIA washing solution (Pharmacia Biotech Co., Ltd.), and then blocked with Block Ace (Dainippon Pharmaceutical Co., Ltd.). Determination of cytokine contents in the supernatant was carried out by the TR-FIA method (Pharmacia Biotech Co., Ltd., Uppsala, Sweden) (18, 19). After amiodarone (0.02, 0.2, 2 or 20 μM) had been added to wells containing mouse alveolar macrophages previously treated with EPA for 2 days according to the method used for the cytotoxicity test, the plate was incubated for 24 hr at 37°C. Then the medium was collected, and its supernatant was obtained by centrifugation (3000 x g for 5 min at 4°C). To each microwell (96-well microplate; Nunc, Naperville, IL, USA) that had been previously coated with anti rabbit IgG antibody solution, anti murine IL-1α antibody, anti murine IL-1β antibody or anti murine TNFα antibody solution was added and the microplate shaken. After incubation for 1 hr at room temperature, each microwell was washed three times with DELFIA washing solution TM (Pharmacia Biotech Co., Ltd.), and then blocked with Block Ace TM (Dainippon Pharmaceutical Co., Ltd.). After inoculating 1 ml of alveolar macrophages (8 x 10^4 cells) into each well of a 24-well plastic culture plate (FalconTM), EPA solution was added to each well at a dose of 0.1, 1 or 10 μg/ml; and the plate was incubated for 2 days at 37°C under an atmosphere of 5% CO2 and 95% air. Thereafter, amiodarone solution was added to the well at a dose of 0.02, 0.2, 2 or 20 μM; and the plate was then incubated for 24 hr under the above culture conditions. The number of viable cells was counted microscopically after trypsinization and staining with trypan blue (Wako Pure Chemical, Inc., Osaka) solution. Ethanol (0.1% v/v) was used as the control for amiodarone treatment, and PBS was used as the vehicle control for EPA.

Cytotoxicity test

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Determination of cytokine levels

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cal Co., Ltd.). To each well, the supernatant obtained above and each europium-labeled cytokine were added, and the plate was shaken at room temperature for 1 hr. Next, the wells were washed with the DELFIA washing solution™, and 100 µl of DELFIA enhancement solution™ (Pharmacia Biotech Co., Ltd.) was added to each well, and the plate was then shaken at room temperature for 5 min. The fluorescence of the solution was measured with a DELFIA fluorometer (Model 1232; Pharmacia Biotech Co., Ltd.); and the cytokine concentration was calculated from a previously prepared standard curve. The test materials were previously found not to interfere with this determination system.

**Determination of PKC activity**

PKC activity was measured with an NRPK™ (Non-radioisotopic protein kinase) kit (MBL Co., Ltd., Nagoya) modified for TR-FIA using the DELFIA™ system (20, 21). Briefly stated, these procedures were as follows: In wells (96-well microplate) using synthetic peptide G1, the homogenates of cells treated with drugs (6.25 µl) were incubated with 12.5 µl of 1 mM ATP solution for 5 min at 25°C for the phosphorylation of G1. After incubation, the reactions were stopped by the addition of 100 µl of 20% H₃PO₄ solution. Then, after each well had been washed, 100 µl of anti-YC antibody (that is, antibody toward phosphorylated G1 peptide) was added to the well; and the plate was incubated at 25°C for 30 min with shaking. Again, after washing, 100 µl of europium-labeled anti mouse IgG antibody solution was added to each well, and incubation was carried out at 20°C for 60 min with shaking. After further washing, 100 µl of the DELFIA enhancement solution™ was added to each well; following stirring of the solution for 5 min, the fluorescence of europium was measured with the DELFIA fluorometer. The activity of PKC was calculated from a previously prepared standard curve. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of phosphorylated peptide per minute.

**Statistical analyses**

Results are expressed as the mean ± S.E. of nine dishes. Statistical analyses of the data were carried out by analysis of variance and Dunnett’s test. A difference from the respective control or vehicle control at the P < 0.05 level was regarded as statistically significant.

**RESULTS**

**Cytotoxicity test (Fig. 1)**

Mouse alveolar macrophages treated with 2 µM amiodarone or more decreased in number in a dose-dependent manner. By pretreatment with EPA at 1 or 10 µg/ml, the decrease in cell number was significantly reversed, that is, recovered, in a dose-related manner. Because this EPA effect was not seen when EPA was added at the same time as amiodarone (data not shown), the following study was performed under the condition of EPA pretreatment for 2 days before amiodarone treatment.

![Fig. 1. Effects of amiodarone on viability of mouse alveolar macrophages treated with EPA for 24 hr. Data are means ± S.E. (n = 9). Data were analyzed by analysis of variance. Significant differences from the vehicle: *P < 0.05, **P < 0.01. Significant differences from the control: *P < 0.05, **P < 0.01. EPA: ○ 0 µg/ml (control), ● 0.1 µg/ml, □ 1 µg/ml, ■ 10 µg/ml.](image-url)
Contents of cytokines

IL-1α level (Fig. 2): The content of IL-1α in the medium was significantly increased by treatment with amiodarone at a concentration of 0.2 μM or more in a dose-dependent manner in the absence of EPA or in the presence of 0.1 μg/ml EPA. Treatment with 1 μg/ml of EPA or...
more significantly depressed the increase in the IL-1α level of cultures treated with amiodarone at a dose of 0.2 μM or greater. In addition, a decrease in the release of this cytokine by 10 μg/ml EPA treatment was observed at 0.02 μM amiodarone and in the absence of amiodarone (vehicle group).

Fig. 4. Effects of amiodarone on tumor necrosis factor α (TNFα) production of mouse alveolar macrophages treated with EPA. Data are means±S.E. (n=9). Data were analyzed by analysis of variance. Significant difference from the vehicle: **P<0.01. Significant difference from the control: *P<0.01. EPA: □ 0 μg/ml (control), ◦ 0.1 μg/ml, □ 1 μg/ml, ■ 10 μg/ml.

Fig. 5. Effects of amiodarone on protein kinase C (PKC) activity of mouse alveolar macrophages treated with EPA. Data are means±S.E. (n=9). Data were analyzed by analysis of variance. Significant differences from the vehicle: *P <0.05, **P <0.01. Significant difference from the control: *P<0.01. EPA: □ 0 μg/ml (control), ◦ 0.1 μg/ml, □ 1 μg/ml, ■ 10 μg/ml.
IL-1β level (Fig. 3): The IL-1β level was also significantly increased by treatment with amiodarone at a dose of 0.02 μM or more in the absence of EPA or in its presence at 0.1 μg/ml. On the other hand, EPA suppressed the increases in IL-1β stimulated by amiodarone as it did for IL-1α; in addition, it was also effective at 1 μg/ml in the presence of 0.02 μM amiodarone.

TNFα level (Fig. 4): The contents of TNFα in the supernatant of macrophages treated with amiodarone at the dose of 0.2 μM or more was significantly increased. The treatment with EPA at 1 or 10 μg/ml suppressed the TNFα increases caused by amiodarone, and the degrees of suppression were almost the same as those in the case of IL-1α and IL-1β.

PKC activity (Fig. 5)

Treatment with amiodarone at a dose of 0.02 μM or more significantly increased PKC activity in a dose-dependent manner in the absence of EPA (control group) or in its presence at 0.1 μg/ml. On the other hand, treatment with 1 μg/ml of EPA or more significantly depressed the increase in PKC activity in these macrophages. These changes in PKC activity were similar to those for IL-1α, IL-1β and TNFα release.

DISCUSSION

I examined the effects of amiodarone and EPA on the cytokine release and PKC activity of mouse alveolar macrophages. Firstly, the decrease in the number of macrophages treated with amiodarone was depressed by pretreatment with EPA for 2 days and co-treatment for 1 day; Thus, these data indicate the potency of EPA to protect against the cell injury caused by amiodarone. Because EPA, a polyunsaturated fatty acid found in fish oil, is a component of cell membranes and organellar membranes (22, 23), I speculated that EPA might improve intracellular lipid metabolism and protect against cell injury. Although the intracellular concentrations of amiodarone and EPA were not determined, the dosing levels of amiodarone and EPA used in this study should be sufficient to cause pharmacological effects in vivo (24–27).

Secondly, with respect to cytokine release, amiodarone caused an increase in IL-1α, IL-1β and TNFα release from macrophages. As EPA also suppressed the increase of cytokine levels, I considered that the protective effect of EPA extended to the acute release of cytokines. In fact, it is known that EPA suppresses inflammation both in vitro and in vivo (12, 28, 29). It was reported that delayed-type hypersensitivity in mice was reduced by the injection of n-3 polyunsaturated fatty acids including EPA (7) and that EPA had a depressive effect on the activity of natural killer cells present among human peripheral blood lymphocytes (30). In addition, several researchers have demonstrated the EPA suppresses humoral responses and phagocytic functions involving primary and secondary granule release from leukocytes in vivo and in vitro (31). Furthermore, a decrease in IL-1 and TNF production levels by mononuclear cells treated with EPA was noted (15). These results agree with the present result that EPA depressed the release or production of inflammatory cytokines by macrophages stimulated with amiodarone.

Next, because it was reported that IL-1 secretion is closely related to the activation of PKC (4), I determined PKC activity to examine the relationship between the levels of cytokines released from macrophages and their PKC activity. Because PKC activity was increased by amiodarone and depressed by EPA and these changes closely reflected the results on the cytokine levels, I considered that amiodarone activated the PKC, leading to the release of cytokines and that EPA prevented these effects. Although the detailed mechanism responsible for these changes remains unclear, the relationship between the release of cytokines and the change in PKC activity of alveolar macrophages caused by amiodarone and EPA should be associated with the toxicological and pharmacological effects of these drugs. The current study indicates that the activation of PKC by amiodarone may play an important role in the cytokine release.

I conclude that mouse alveolar macrophages treated with amiodarone show activated release of cytokines and that EPA can reverse these increases. Thus EPA can be considered to have an anti-inflammatory effect and to act as an protector against injury of alveolar macrophages.

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