Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.3, No.3, 121-125, 2016

Letter

Methyl cinnamate increases cell vulnerability to oxidative stress induced by hydrogen peroxide in rat thymocytes

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(Received April 16, 2016; Accepted April 22, 2016)

ABSTRACT — Methyl cinnamate (MC) and essential oils containing MC possess beneficial antimicrobial, antifungal, and insecticidal effects, among others. Such effects are related to the biocidal action of MC. The antioxidant activity of MC has also been reported elsewhere. It has been suggested that MC may be cytotoxic to cells exposed to oxidative stress. To test this possibility, the effect of MC on rat thymocytes was examined while the cells were subjected to oxidative stress induced by hydrogen peroxide (H_2O_2). Flow cytometric techniques with appropriate fluorescent probes were used for quantification. MC increased cell vulnerability to oxidative stress *via* acceleration of the cell death process and/or potentiation of oxidative stress. The use of MC is widespread because of its beneficial actions, and thus further attention should be paid to whether MC is effective under oxidative stress.

Key words: Methyl cinnamate, Hydrogen peroxide, Cytotoxicity, Lymphocytes, Oxidative stress

INTRODUCTION

Methyl cinnamate (MC) is a methyl ester of cinnamate. It is one of the major components of essential oils extracted from herbal plants (Politeo *et al*., 2007; Gilles *et al*., 2010). MC and essential oils containing MC possess antimicrobial, antifungal, insecticidal, and antioxidant effects (Peterson *et al*., 2000; El-Massry *et al*., 2002; Peretto *et al*., 2014; Vieira *et al*., 2014). The cytotoxic action of essential oils containing MC on tumor cells has been reported (Ferraz *et al*., 2013; Shirazi *et al*., 2014). All the effects of MC, except for its antioxidant activity, are related to its biocidal action. Therefore, it is suggested that MC could exert cytotoxic action on mammalian cells exposed to oxidative stress, despite being an antioxidant. In the present study, to test this possibility, the effect of MC on rat thymocytes was examined while the cells were subjected to oxidative stress induced by hydrogen peroxide (H_2O_2). Flow cytometric techniques with appropriate fluorescent probes were used for quantification. The use of MC is widespread; it can be used as a food flavoring, cosmetic fragrance, and for management of obesity (Bhatia *et al*., 2007; Chen *et al*., 2012). Therefore, this study may provide insights into the toxicological profile of MC for its safe use.

MATERIALS AND METHODS

Cell preparation

This study was approved by the Committee for Animal Experiments of Tokushima University, Tokushima, Japan (No. 14124). The cell suspension was prepared as previously reported (Chikahisa *et al*., 1996). In brief, the thymus glands dissected from ether-anesthetized rats were sliced under cold conditions. The slices were triturated in Tyrode's solution to dissociate the thymocytes. The cell suspension was incubated at 36-37°C for 1 hr before the experiment.

Chemicals

MC was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Annexin V-FITC, propidium iodide, and 5-chloromethylfluorescein diacetate (5CMF-DA) were obtained from Molecular Probes Inc., Invitrogen (Eugene, OR, USA). Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) unless otherwise mentioned.

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Measurements of cellular parameters

To assess cell lethality (the population of dead cells), propidium iodide was added to the cell suspension to a final concentration of 5 μM. Exposure of phosphatidylserine on the outer surface of cell membranes is a marker of the early stages of apoptosis, and was detected using 10 μL/mL annexin V-FITC (Koopman *et al*., 1994). 5-CMF-DA at a concentration of 1 μM was used to monitor changes in cellular content of non-protein thiols, such as glutathione (Chikahisa *et al*., 1996). The excitation wavelength for the fluorescent probes was 488 nM. Fluorescence of FITC and 5-CMF was detected at 530 ± 20 nm. Propidium fluorescence was detected at 600 ± 20 nm. 5-CMF fluorescence was monitored only for living cells that did not exhibit propidium fluorescence.

Statistical analysis

Statistical analyses were performed by ANOVA with post-doc Tukey's multivariate analysis. P-values of less than 0.05 were considered significant. Results (including columns and bars in figures) are expressed as the mean and standard deviation of four samples.

RESULTS AND DISCUSSION

Changes in cell lethality by MC and H_2O_2

As shown in Fig. 1A, the incubation of thymocytes with 300 μM MC for 3 hr did not increase the population of cells exhibiting propidium fluorescence, which indicates that 300 μM MC did not increase the population of dead cells. However, incubation with 300 μM H_2O_2 significantly increased the population of dead cells (Fig. 1A). Thus, 300 μM H_2O_2 is suggested to be cytotoxic to rat thymocytes. The simultaneous application of 300 μM H_2O_2 and 300 μM MC further increased the population of dead cells. This further increase was also observed in the case of 100 μM MC, but not with 30 μM MC. We thus concluded that 100-300 μM MC potentiates the cytotoxicity of 300 μM H_2O_2 (Fig. 1B).

The incubation of thymocytes with 100-300 μM H_2O_2 for 3 hr significantly increased the population of dead cells. The simultaneous application of 300 μM MC with 100-300 μM H_2O_2 further induced an increase in the dead cell population (Fig. 1C). The difference in cell lethality between the control group and the group of cells simultaneously treated with 30 μM H_2O_2 and 300 μM MC was statistically significant. There was no statistically significant difference between the group of cells treated with 30 μM H_2O_2 alone and the group of cells treated with 30 μM H_2O_2 and 300 μM MC. It is unlikely that MC potentiates the action of 30 μM H_2O_2.

Effects of MC, H_2O_2, and their combination on process of cell death

The effect of 300 μM MC on the process of cell death induced by 300 μM H_2O_2 was examined using propidium iodide and annexin V-FITC. The effect was examined at 2 hr after drug application. The populations of intact living cells (exhibiting neither propidium fluorescence nor FITC fluorescence), annexin V-positive living cells (FITC fluorescence but not propidium fluorescence), and dead cells (propidium fluorescence) were not altered by treatment with 300 μM MC (Fig. 2A). However, the incubation of thymocytes with 300 μM H_2O_2 significantly decreased the population of intact living cells and increased those of annexin V-positive living cells and dead cells. The combination of MC and H_2O_2 further increased the population of dead cells, as seen in the decrease of the population of annexin V-positive living cells. MC is suggested to accelerate the transition from annexin V-positive living cells to dead cells.

Changes in cellular content of nonprotein thiols by MC, H_2O_2, and their combination

To determine if MC augments the oxidative stress induced by H_2O_2, the effects of MC, H_2O_2, and a combination of the two on the cellular content of nonprotein thiols, mainly glutathione, were examined using 5CMF fluorescence (Chikahisa *et al*., 1996). The incubation of thymocytes with 300-100 μM MC for 3 hr did not change the intensity of 5CMF fluorescence. MC at 300 μM slightly attenuated 5CMF fluorescence (Fig. 2B). This slight attenuation was statistically significant. H_2O_2 at 30 μM increased the intensity of 5CMF fluorescence, while a more significant reduction of 5CMF fluorescence was observed at 100-300 μM H_2O_2. In previous studies (Kinazaki *et al*., 2011; Fukunaga *et al*., 2013), we suggested that intracellular Zn^{2+} release in response to oxidative stress is a trigger for the restoration of the cellular content of nonprotein thiols that is decreased by oxidative stress. H_2O_2 at higher concentrations predominantly reduces cellular thiol content. Simultaneous application of either 30 or 100 μM H_2O_2 and 300 μM MC greatly reduced the intensity of 5CMF fluorescence (Fig. 2C). These results suggest that the combination MC and H_2O_2 reduces the cellular content of nonprotein thiols, although H_2O_2 at low concentrations tends to elevate cellular thiol levels. It is likely that MC potentiates H_2O_2-induced oxidative stress with influential consequences.

Taken together, MC increases the vulnerability of rat thymocytes to oxidative stress induced by H_2O_2, and either accelerates the cell death process induced by H_2O_2 and/or potentiates H_2O_2-induced oxidative stress. The use

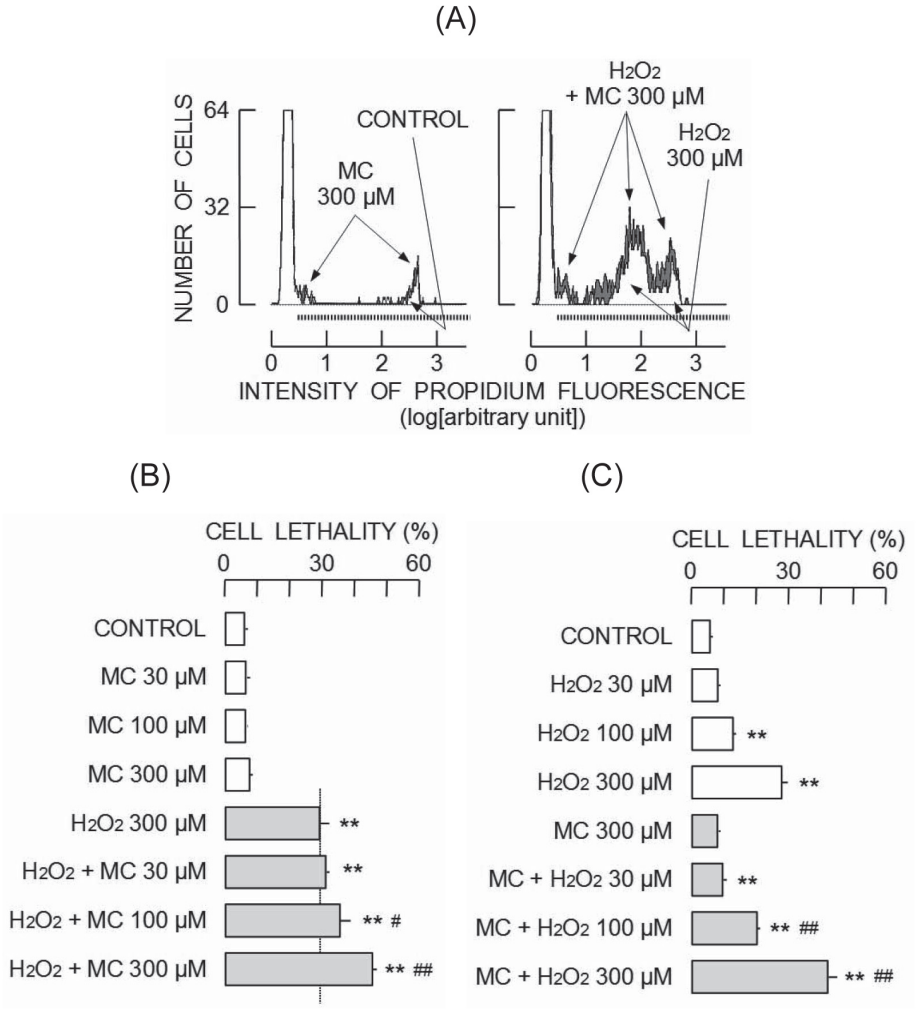

Fig. 1. Changes in population of cells exhibiting propidium fluorescence (cell lethality) by MC, H_2O_2, and the combination of the two. (A) Changes in histograms of propidium fluorescence. Each histogram was constructed with 2,000 cells. Effects were examined at 3 hr after drug application. (B) Changes in cell lethality after application of 300 μM H_2O_2 and 30-300 μM MC. Asterisk (**) indicates a significant difference between the control group (CONTROL) and test groups. Symbols (#, ##) show a significant difference ($P < 0.05$, $P < 0.01$) between the group of cells treated with 300 μM H_2O_2 and the group of cells simultaneously treated with H_2O_2 and 30-300 μM MC. (C) Changes in cell lethality after application of 30-300 μM H_2O_2 and 300 μM MC. Asterisk (**) indicates a significant difference ($P < 0.01$) between the control group (CONTROL) and test groups. Symbol (##) shows a significant difference ($P < 0.01$) between the group of cells treated with 30-300 μM H_2O_2 and the group of cells simultaneously treated with H_2O_2 and 300 μM MC.

of MC is widespread owing to its many beneficial effects, and thus further attention should be paid to whether MC is effective under oxidative stress.

ACKNOWLEDGMENTS

This study was supported by the Grant-in-Aid for Scientific Research (C26340039) from the Japanese Society for the Promotion of Science (Tokyo, Japan).

Conflict of interest---- The authors declare that there is no conflict of interest.

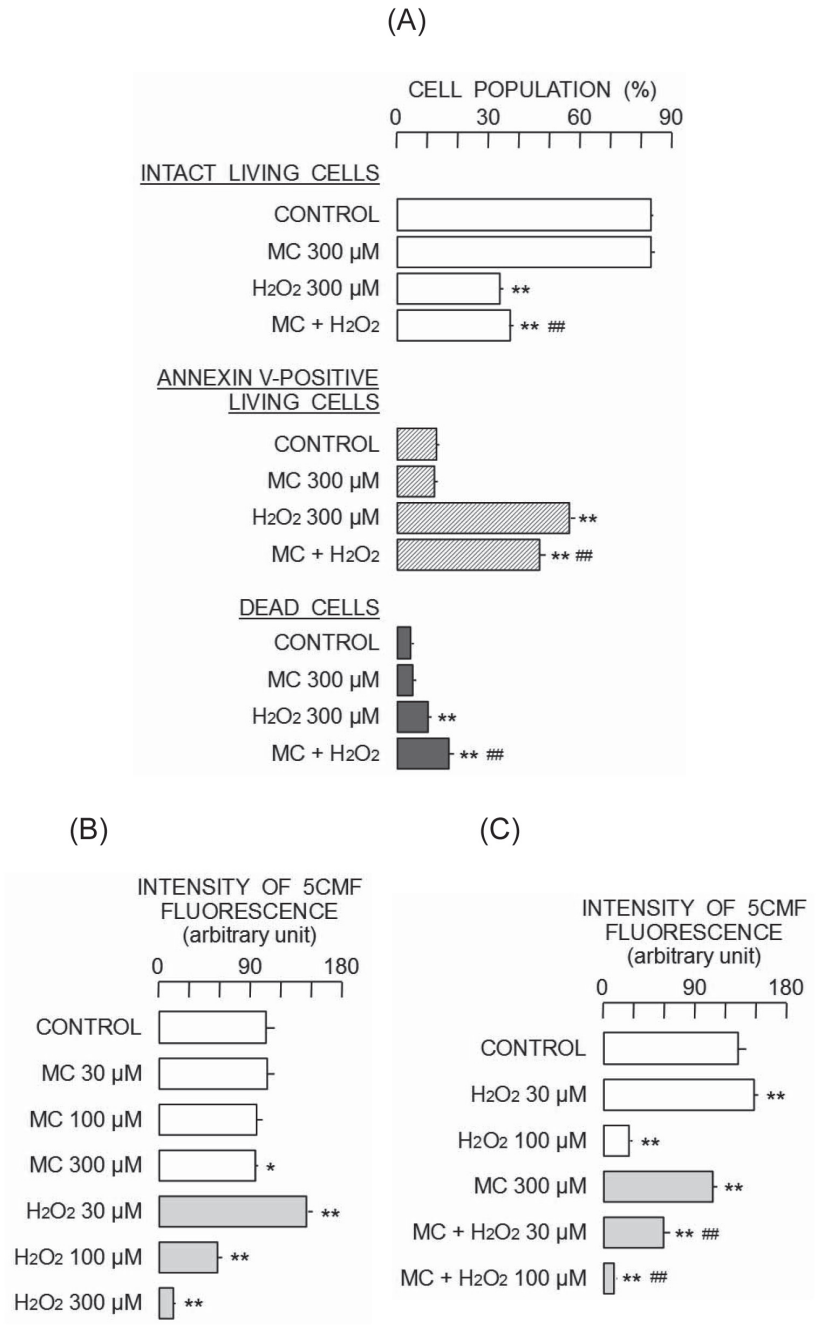

Fig. 2. Changes in cellular parameters by MC, H_2O_2, and the combination of the two. (A) Changes in cell population classified by propidium iodide and annexin V-FITC. The cells exhibiting neither propidium fluorescence nor FITC fluorescence were defined as intact living cells (INTACT LIVING CELLS). The cells exhibiting FITC fluorescence but not propidium fluorescence were the living cells positive to annexin V (ANNEXIN V-POSITIVE LIVING CELLS). The dead cells (DEAD CELLS) were stained with propidium, and exhibited propidium fluorescence. Asterisk (**) indicates a significant difference ($P < 0.01$) between the control group (CONTROL) and test groups. Symbol (##) shows a significant difference ($P < 0.01$) between the group of cells treated with H_2O_2 alone and that with H_2O_2 and MC. (B) Changes in 5CMF fluorescence by 30-300 μM MC or 30-300 μM H_2O_2. Asterisks (*, **) indicate a significant difference ($P < 0.05$, $P < 0.01$) between the control group (CONTROL) and test groups. (C) Changes in 5CMF fluorescence by the combination of MC and H_2O_2. Asterisk (**) indicates a significant difference ($P < 0.01$) between the control group (CONTROL) and test groups. Symbol (##) shows a significant difference ($P < 0.01$) between the group of cells treated with MC alone and with MC and H_2O_2 together.

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