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Protective effects of veskamide, enferamide, becatamide, and oretamide on H_2O_2-induced apoptosis of PC-12 cells

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ABSTRACT

Veskamide, enferamide, becatamide, and oretamide are phenolic amides whose analogues are found in plants. In this study, the four amides were prepared by chemical synthesis and their protective effects on H_2O_2-induced apoptosis in PC-12 cells were investigated. The syntheses were relatively simple and the yields were more than 43%. Using NMR spectroscopic methods, the chemical structures of veskamide, enferamide, becatamide, and oretamide were confirmed. The decreasing order of the protective effects on H_2O_2-induced apoptosis was becatamide > enferamide ≥ oretamide > veskamide. In fact, becatamide suppressed H_2O_2-induced mitochondrial membrane depolarization in a dose-dependent manner. At the concentration of 10 μM, becatamide maintained mitochondrial membrane depolarization at 16% compared to 51% in H_2O_2-treated PC-12 cells ($P < 0.05$). Also, at the same concentration, becatamide inhibited H_2O_2-induced caspase-9 activation and caspase-independent chromatin condensation by 68% ($P < 0.05$) and 73% ($P < 0.05$), respectively. This is the first report about the chemical synthesis of becatamide and its potential biological activity to inhibit H_2O_2-induced apoptosis of PC-12 cells via protecting mitochondrial membrane integrity, thereby suppressing caspase-9 activation and chromatin condensation.

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Introduction

Certain neurodegenerative diseases progressively deteriorate the structure and/or function of neurons (e.g., soma, axon, dendrites) in the central nervous system, eventually leading to cell death (Reeve et al. 2008; Martinelli and Rugarli 2010; Nakamura and Lipton 2009; Glass et al. 2010). In actual fact, neurodegenerative processes are very much responsible for several neuronal diseases such as Alzheimer's, Parkinson's, and Huntington's diseases (Duyckaerts et al. 2009; Winklhofer and Haass 2010; Bates 2003). Although there are several mechanisms involved in neuronal cell death, a most common mechanism is through the well-known intrinsic mitochondrial apoptotic pathway (Ribe et al. 2008; Rohn and Head 2008). This pathway regulates the activation of caspase cascade systems by releasing cytochrome c from the mitochondria premeabilized by the pro-apoptotic Bcl-2 members such as Bax and Bak (Jourdain and Martinou 2009). Reactive oxygen species (ROS) can be produced as normal by-products from several sources of human body. Among them, mitochondria are a major source of ROS (Wei et al. 1998; Richter et al. 1995). However, along with insufficient antioxidant defense systems and/or some types of disease conditions, the elevated levels of ROS can cause detrimental damage to cellular components such as lipids, proteins, and DNA leading to apoptosis (Cutler et al. 2005; Mena et al. 2009). A number of studies suggest that ROS-induced apoptosis in neuronal cells is associated with a series of cellular events such as mitochondrial depolarization, cytochrome c release, and activation of caspases and caspase-independent chromatin condensation (Mena et al. 2009; Kirkland and Franklin 2003; Du and Yan 2010). Therefore, the protection of mitochondria in neuronal cells from ROS is considered as a very promising way in treating and/or preventing neurodegenerative and other related disorders (Du and Yan 2010; Ramassamy 2006; Sun et al. 2008).

Veskamide, enferamide, becatamide, and oretamide are *N*-phenylethylbenzoylamide-type phenolic amides, whose analogues are found in plants such as *Aniba riparia*, *Begonia nantoensis*, *Haplophyllum tuberculatum*, and *Houttuynia cordata* (Thomas et al. 1994; Wu et al. 2004; Al-Rehaily et al. 2001; Chou et al. 2009). Most likely, these four amides are to be found in numerous plant sources. In fact, during the course of our study, becatamide was subsequently isolated from *Houttuynia cordata* and its structure (houttuynamide A) was published (Chou et al. 2009). Currently, there is great interest regarding potential biological activities of these amides, but their potential effects have not been investigated much, probably due to the scarcity of the amides. Therefore, in this paper, the four phenolic amides were prepared using a chemical synthesis method and their protective effects on H_2O_2-induced apoptosis of rat pheochromocytoma PC-12 cells were studied by investigat-

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ing mitochondrial membrane depolarization, caspase-9 activation, and chromatin condensation, in order to search potential compounds with anti-neurodegenerative activity. This study clearly indicates that out of the four amides, becatamide is the most potent compound able to inhibit H_2O_2-induced apoptosis of PC-12 cells via maintaining mitochondrial membrane polarization, suppressing caspase-9 activation, and inhibiting caspase-independent chromatin condensation.

Experimental

Materials

Benzoic acid, protocatechuic acid, vanillic acid, syringic acid, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle's Medium was purchased from ATCC (Manassas, Virginia). The Mito Flow fluorescent dye monitoring mitochondria membrane potential was purchased from Cell Technology Inc (Mountain View, CA), Caspase-Glo® 9 luminescent assay kit was purchased from Promega (Madison, WI), and Nuclear-ID™ Green Chromatin Condensation Detection Kit was purchased from Enzo Life Sciences (Plymouth Meeting, PA).

Syntheses of veskamide, enferamide, becatamide, and oretamide

Chemical synthesis was performed as previously described (Park 2005a,b). Briefly, benzoic acid, protocatechuic acid, vanillic acid, or syringic acid were dissolved in dichloromethane (DCM) and converted to the symmetrical anhydride with 1,3-diisopropylcarbodiimide (DIC). Tyramine was added to the reaction mixture and incubated with a gentle stirring for 12 h. The synthesized products were recovered and purified by HPLC (Waters, Milford, MA) (Park 2005a).

NMR analyses

For NMR experiments, the samples were prepared by dissolving veskamide, enferamide, becatamide or oretamide (20 mg) in d6-DMSO (0.75 ml). ^1H and ^{13}C spectra were acquired at ambient temperature on the JEOL BCX-400 NMR spectrometer operating 400 MHz for ^1H and 100 MHz for ^{13}C. Chemical shifts were referenced to DMSO (2.50 ppm for ^1H, 39.5 ppm for ^{13}C).

Cell culture and treatment

PC-12 cells were grown in Ham's F12K supplemented with 15% (v/v) heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. Experiments were carried out 24 h after cells were seeded.

Measurement of mitochondrial membrane depolarization

Mitochondrial membrane depolarization was monitored by measuring mitochondrial membrane potential (MMP) using the Mito Flow fluorescent dye (Cell Technology Inc), a cell permeable cationic dye to emit a strong fluorescent signal in mitochondria with the highly negative membrane potential. Veskamide, enferamide, becatamide, and oretamide were prepared in ethanol and added to samples less than 0.1% (v/v). The PC-12 cells were pre-incubated with indicated concentrations of the amides for 20 min, prior to the addition of H_2O_2 (200 μM) to the cells. After 3 h, the cells were collected, centrifuged at 1200 × *g* for 5 min, and resuspended in DMEM. Mito Flow fluorescent dye was added to the cells at the final concentration of 1 mM. After 1 h incubation at 37 °C, the intracellular fluorescence intensity associated with the dye was measured using a flow cytometry with excitation at 480 and 530 nm long-pass filter.

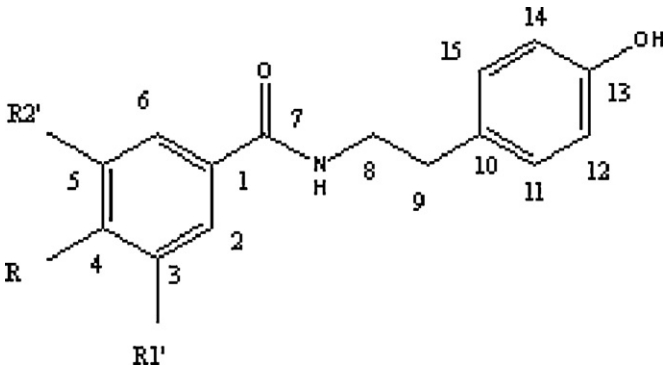

Fig. 1. Chemical structures of veskamide, enferamide, becatamide, and oretamide. Veskamide (R = OH, R1′ = OCH_3, R2′ = OCH_3), enferamide (R = OH, R1′ = OCH_3, R2′ = H), becatamide (R = OH, R1′ = OH, R2′ = H) and oretamide (R = OH, R1′ = H, R2′ = H).

Caspase-9 assay

Caspase-9 activity was measured using a Caspase-Glo® 9 luminescent assay kit (Promega), according to the manufacturer's protocol. The PC-12 cells were seeded in 96 well-culture dish and pre-incubated with indicated concentrations of the amides for 20 min prior to the addition of H_2O_2 (200 μM) to the medium. Becatamide was prepared in ethanol and added at less than 0.1% (v/v). After 3, 6, and 9 h incubation, the cells were collected and relative light units (RLU) output was measured to determine caspase-9 activity.

Chromatin condensation

Chromatin condensation was measured using Nuclear-ID™ Green Chromatin Condensation Detection Kit (Enzo Life Sciences), according to the manufacturer's protocol. The green fluorescent dye enables monitoring of the dynamics of nucleolar changes in intracellular distribution, trafficking, and localization coincident with cell death. For the experiment, the PC-12 cells were seeded in a 6 well-culture dish, pre-incubated with becatamide for 20 min, followed by the addition of H_2O_2 (200 μM). The cells were collected after 24 h and chromatin condensation was measured by calculating the fluorescence intensity of the dye using a flow cytometry.

Results and discussion

Syntheses and NMR analyses of veskamide, enferamide, becatamide, and oretamide

Veskamide (*N*-syringoyltyramine), enferamide (*N*-vanilloyltyramine), becatamide (*N*-protocatechuoyltyramine) and oretamide (*N*-benzoyltyramine) are *N*-phenylethylbenzoylamides, whose analogues were found in several plants such as *Aniba riparia*, *Begonia nantoensis*, *Haplophyllum tuberculatum*, *Hortia regia* and *Houttuynia cordata*. Years ago, we synthesized and named the four amides as veskamide, enferamide, becatamide, and oretamide, because the amides are most likely to have important biological activities as well as to be present in numerous plant sources. To attest our assumption, becatamide was subsequently isolated from *Houttuynia cordata* and its structure (houttuynamide A) was published during our study (Chou et al. 2009). However, there is currently not much information about their biological effects. Therefore, in this study, veskamide, enferamide, becatamide, and oretamide (Fig. 1) were prepared via a chemical synthesis method

using tyramine and the acids (syringic acid, vanillic acid, protocatechuic acid, and benzoic acid), according to the method described in "Experimental" section (Park 2005a, 2009). The syntheses were relatively simple, and their yields were greater than 43%. Each synthesized product was purified by HPLC, and analyzed using NMR spectroscopic methods described in "Experimental" section. In the following are the NMR data for veskamide (1): ^{1}H NMR (d6-DMSO, 400 MHz) d: 7.27 (1H, d, J = 8.4 Hz, H-2), 7.30 (1H, dd, J = 8.4 Hz, H-6), 7.01 (1H, d, J = 8.6 Hz H-11, -15), 6.71 (1H, d, J = 8.6 Hz, H-12, -14), 5.00 (1H, br s, OH), 3.51 (2H, t, J = 7.5 Hz, CH_2-8), 2.79 (2H, t, J = 7.5 Hz, CH_2-9); ^{13}C NMR (d6-DMSO, 100 MHz) d: 169.3 (C-7), 156.3 (C-13), 146.5 (C-4), 150.1 (C-3), 131.7 (C-10), 130.5 (C-11, -15), 128.1 (C-1), 108.8 (C-6), 116.1 (C-12, -14), 150.2 (C-5), 108.7 (C-2), 56.2 (C-1′), 56.1 (C-2′), 43.9 (C-8), 36 (C-9); Thus, the structure of the synthesized product was determined as being 4-hydroxy-N-[2-(4-hydroxy-phenyl)-ethyl]-3,5-dimethoxy-benzamide (veskamide). In the following are the NMR data for enferamide (2): ^{1}H NMR (d6-DMSO, 400 MHz) d: 7.27 (1H, d, J = 8.4 Hz, H-2), 7.30 (1H, dd, J = 8.4 Hz, H-6), 7.01 (2H, d, J = 8.6 Hz H-11, -15), 6.78 (1H, d, J = 1.7 Hz, H-5), 6.71 (2H, d, J = 8.6 Hz, H-12, -14), 5.00 (1H, br s, OH), 3.51 (2H, t, J = 7.5 Hz, CH_2-8), 2.79 (2H, t, J = 7.5 Hz, CH_2-9); ^{13}C NMR (d6-DMSO, 100 MHz) d: 169.3 (C-7), 156.3 (C-13), 146.5 (C-4), 148.7 (C-3), 131.7 (C-10), 130.5 (C-11, -15), 127.1 (C-1), 120.1 (C-6), 116.1 (C-12, -14), 115.7 (C-5), 114.7 (C-2), 56.1 (C-1′), 43.9 (C-8), 36.8 (C-9); Thus, the structure of the synthesized product was determined as being 4-Hydroxy-N-[2-(4-hydroxy-phenyl)-ethyl]-3-methoxy-benzamide (enferamide). In the following are the NMR data for becatamide (3): ^{1}H NMR (d6-DMSO, 400 MHz) d: 7.24 (1H, d, J = 2.0 Hz, H-2), 7.31 (1H, dd, J = 8.4 Hz, H-6), 7.01 (2H, d, J = 8.6 Hz H-11, -15), 6.78 (1H, d, J = 1.9 Hz, H-5), 6.71 (2H, d, J = 8.6 Hz, H-12, -14), 5.00 (1H, br s, OH), 3.51 (2H, t, J = 7.5 Hz, CH_2-8), 2.79 (2H, t, J = 7.5 Hz, CH_2-9); ^{13}C NMR (d6-DMSO, 100 MHz) d: 169.3 (C-7), 156.3 (C-13), 149.3 (C-4), 146.1 (C-3), 131.7 (C-10), 130.5 (C-11, -15), 127.2 (C-1), 120.5 (C-6), 116.1 (C-12, -14), 115.9 (C-5), 115.7 (C-2), 43.9 (C-8), 36.8 (C-9); Thus, the structure of the synthesized product was determined as being 3,4-Dihydroxy-N-[2-(4-hydroxy-phenyl)-ethyl]-benzamide (becatamide). In the following are the NMR data for oretamide (4): ^{1}H NMR (d6-DMSO, 400 MHz) d: 7.24 (1H, d, J = 2.0 Hz, H-2), 7.31 (1H, dd, J = 8.4 Hz, H-6), 7.01 (2H, d, J = 8.6 Hz H-11, -15), 6.78 (1H, d, J = 1.9 Hz, H-5), 6.71 (2H, d, J = 8.6 Hz, H-12, -14), 5.00 (1H, br s, OH), 3.51 (2H, t, J = 7.5 Hz, CH_2-8), 2.79 (2H, t, J = 7.5 Hz, CH_2-9); ^{13}C NMR (d6-DMSO, 100 MHz) d: 169.3 (C-7), 156.3 (C-13), 149.3 (C-4), 146.1 (C-10), 130.5 (C-11, -15), 127.2 (C-1), 120.5 (C-6), 116.1 (C-12, -14), 115.9 (C-3), 115.7 (C-5), 115.7 (C-2), 43.9 (C-8), 36.8 (C-9); Thus, the structure of the synthesized product was determined as being 4-Hydroxy-N-[2-(4-hydroxy-phenyl)-ethyl]-benzamide (oretamide).

Effect of veskamide, enferamide, becatamide, and oretamide on H_2O_2-induced mitochondrial membrane depolarization

Mitochondrial membrane depolarization (MMP) was determined using a cell permeable cationic fluorescent dye (Mito Flow, Cell Technology Inc). Depolarization of mitochondria membrane potential induced by the H_2O_2-induced damage of the outer membrane resulted in the loss of the dye from the mitochondria and a decrease in intracellular fluorescence (Lemasters et al. 1998). PC-12 cells were prepared in 6 groups: control (A), H_2O_2 (B), veskamide (C), enferamide (D), becatamide (E), and oretamide (F) groups. The cells in C-F groups were treated with veskamide, enferamide, becatamide or oretamide (10 μM) for 20 min, then treated with H_2O_2 (200 μM) for 3 h. The cells in the B group were only treated with H_2O_2 (200 μM) for 3 h and the control A group

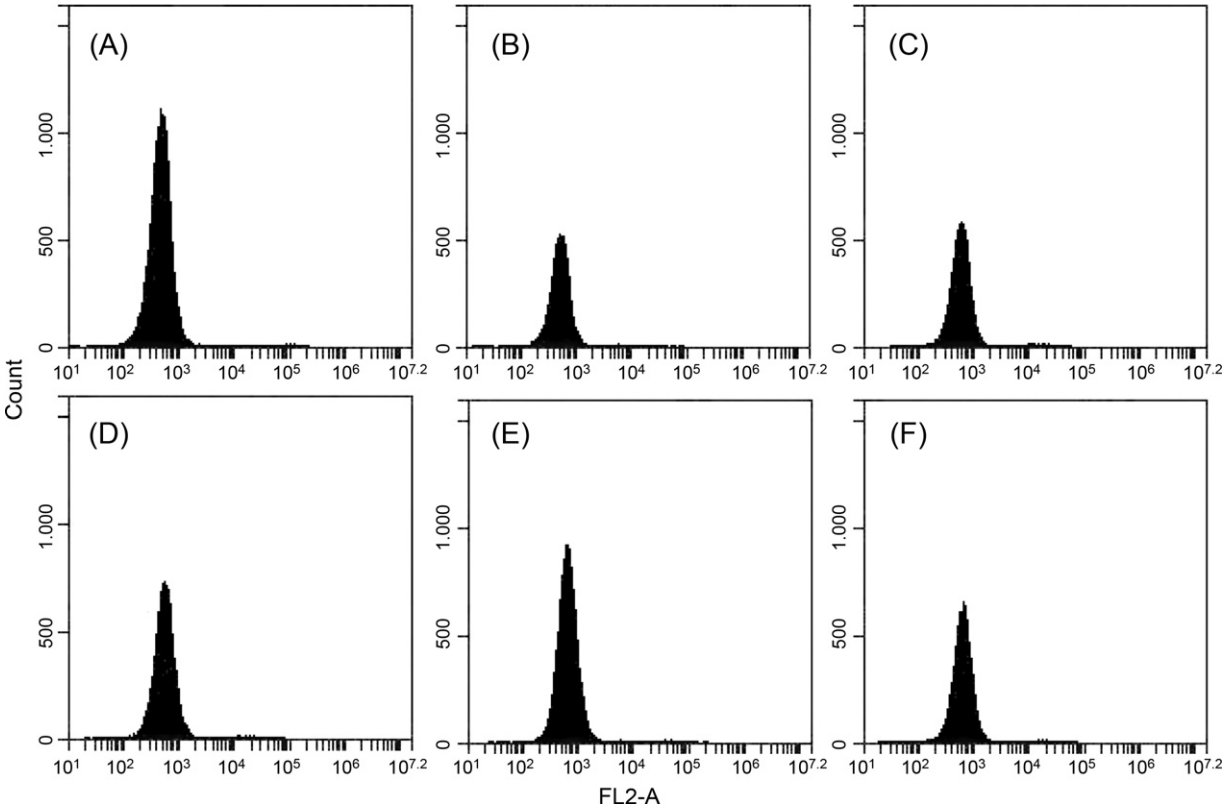

Fig. 2. Effects of veskamide, enferamide, becatamide, and oretamide on mitochondrial membrane depolarization. Mitochondria depolarization was determined using Mito Flow dye, a cell permeable cationic dye that has a strong fluorescent signal and exhibits strong mitochondria membrane potential (MMP) dependent binding. PC-12 cells were treated with veskamide (C), enferamide (D), becatamide (E), and oretamide (F) (all at 10 μM), followed by the treatment of H_2O_2 (200 μM) except control (A). After 3 h, the cells were collected, centrifuged, re-suspended, and analyzed using flowcytometer as described in "Experimental" section.

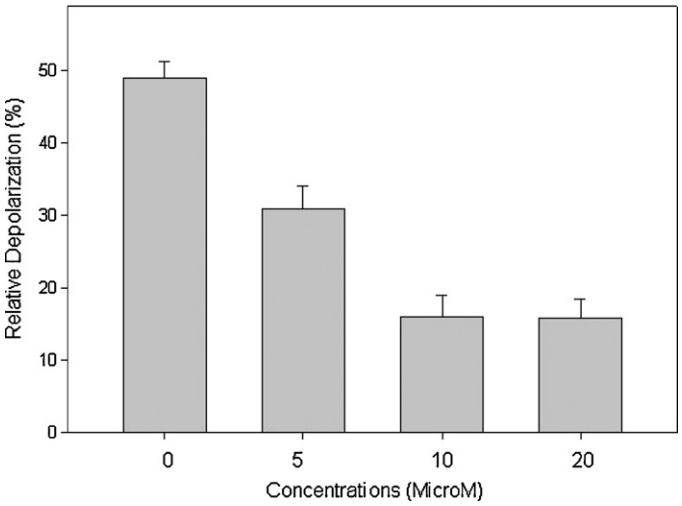

Fig. 3. Effects of becatamide on mitochondrial membrane depolarization. Mitochondria integrity was determined as described in Fig. 2. PC-12 cells were pre-treated with becatamide 5, 10 and 20 μM for 20 min and then treated with 200 μM H_2O_2 for 3 h. The cells were collected and analyzed using flowcytometer.

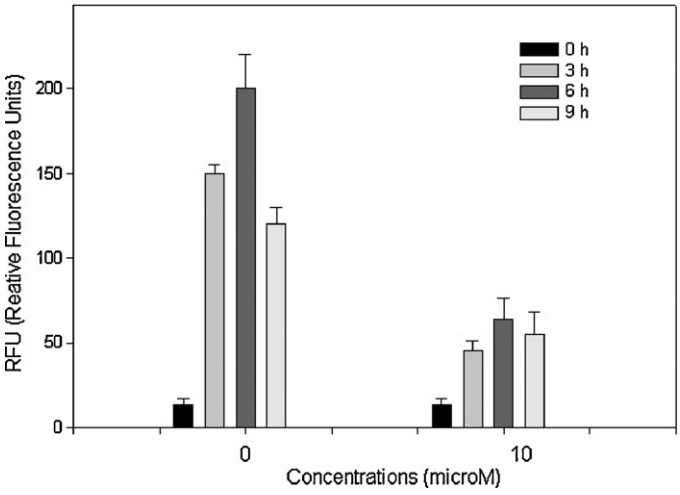

Fig. 4. The inhibition of hydrogen-peroxide induced caspase 9 activation by becatamide: PC-12 cells were pre-treated with becatamide at the concentration of 10 μM for 20 min, then treated with 200 μM H_2O_2. The capase-9 activation was measured by the Caspase-Glo® 9 Assay kit (Promega) at 0, 3, 6, and 9 h. Data points represent the mean ± SD of more than 3 samples. Becatamide pre-treated cells produced significant inhibition of H_2O_2-induced caspase-9 activation at all time periods measured compared to cells treated only with H_2O_2 ($P < 0.05$, ANOVA, Holm-Sidak method).

received no treatment. After the treatment, PC12 cells were collected and analyzed using a flowcytometer. As shown in Fig. 2, the pre-exposure of H_2O_2-treated cells with becatamide (Fig. 2E) significantly maintained mitochondrial membrane polarization (83%), based on the quantization of intracellular fluorescence compared to non-treated cells ($P < 0.001$). In contrast, H_2O_2-treated cells maintained the polarization at only 49% of the control cells (Fig. 2B). Since becatamide (10 μM) exhibited significant inhibition of H_2O_2-induced mitochondrial membrane depolarization, we investigated the dose-dependent protective effects of becatamide (5 and 20 μM). As shown in Fig. 3, at the lower concentration (5 μM), the mitochondrial membrane polarization was maintained at 69%, but the effect was not increased further at the higher concentration (20 μM), suggesting that the highest protective effect can be achieved at a range of less than 20 μM. That seemed very reasonable because mitochondrial membrane polarization was generally around 89% in the control cells without the H_2O_2 treatment (data not shown here). We also tested the protective effects of the precursors (e.g., protocatechuic acid and tyramine) and other well-known phenolic and anti-oxidant compounds (e.g., caffeic acid, rosmarinic acid, catechin, resveratrol, quercetin, vitamin-C). But, the same level protective effect of becatamide could not be achieved in the tested concentrations (data not shown here). Together, these data indicated that among the four amides, becatamide was the most potent compound able to maintain mitochondrial membrane polarization, probably via protecting mitochondrial membrane integrity from H_2O_2.

Effect of becatamide on caspase 9 activity in H_2O_2-treated PC12 cells

Caspases (cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases) are a family of cysteine proteases that play essential roles in apoptosis (programmed cell death) (Vaux and Strasser 1996; Alnemri 1997). Caspase-9 is an initiator caspase linked to the mitochondrial death pathway and activated during programmed cell death (apoptosis) (Caroppi et al. 2009). After treatment of cells with apoptotic agents including H_2O_2, cytochrome c is released from mitochondrial intermembrane space and binds to the apoptosis protease activation factor (APAf-1) to forms an apoptosome complex. This complex activates caspase 9. Once initiated, caspase-9 cleaves procaspase-3 as well as procaspase-7, which are responsible for several cellular apoptosis processes including poly ADP ribose polymerase (Garrido et al. 2006). Since becatamide was a most potent inhibitor of mitochondrial membrane depolarization, the effect of this amide on caspase 9 activity in H_2O_2-treated PC12 cells was investigated. As shown in Fig. 4, the cells pre-treated with becatamide exhibited significant suppression of H_2O_2-induced caspase-9 activation, compared to H_2O_2-treated cells ($P < 0.05$). The data suggest that becatamide may inhibit activation of caspase-9 via protecting mitochondrial membrane integrity from H_2O_2 and suppressing cytochrome c release from the mitochondrial inter-membrane space.

Effect of becatamide on H_2O_2-induced chromatin condensation

Since there are also caspase-independent apoptotic events, we investigated the effects of becatamide on caspase-independent chromatin condensation. The treatment of PC-12 cells with H_2O_2 makes the outer mitochondrial membrane more permissible, thereby triggering the release of apoptosis-inducing factor (AIF) that is normally located in the mitochondrial inter-membrane space. Upon the release, AIF translocates to the nucleus, binds to DNA, and invokes caspase-independent chromatin condensation. During this process, chromatin undergoes a phase change from a heterogeneous, genetically active network to an inert highly condensed form that is subsequently fragmented and packaged into apoptotic bodies. Recent studies suggested that AIF was a major factor determining caspase-independent neuronal death, emphasizing the central role of mitochondria in the control of physiological and pathological cell death (Candé et al. 2002). Therefore, caspase-independent chromatin condensation was measured in H_2O_2-treated PC-12 cells with and without the pre-treatment with becatamide (Fig. 5). Chromatin condensation was evident in cells treated with H_2O_2 for 24 h (Fig. 5B), but the chromatin condensation was significantly attenuated by 73% ($P < 0.05$) in becatamide-pre-treated PC-12 cells (Fig. 5C). This level of the protective effect could not be demonstrated in the PC-12 cells pre-treated with veskamide, enferamide, oretamide or their precursors (data not shown here). In summary, becatamide may be able to suppress caspase-independent chromatin condensation as well as

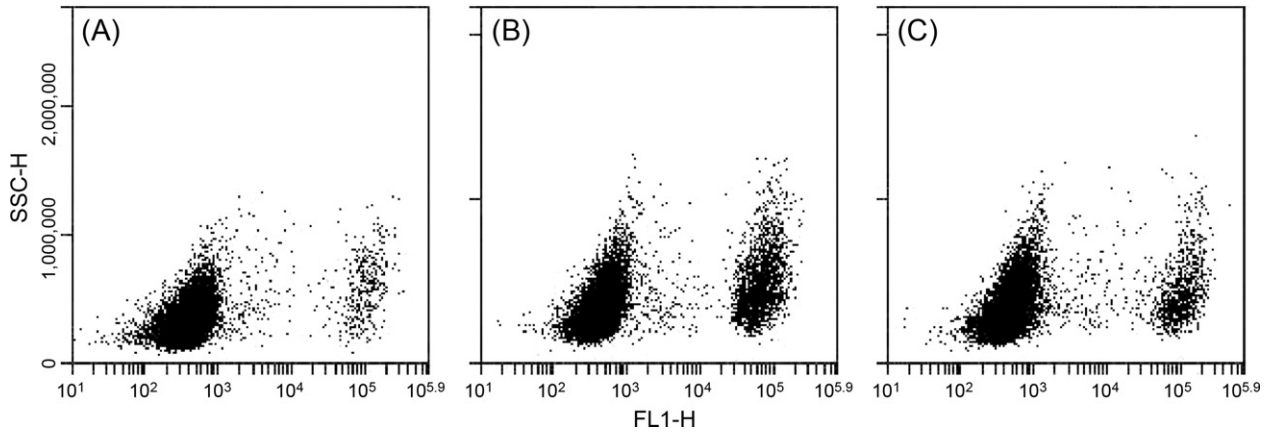

Fig. 5. The protection of hydrogen-peroxide induced DNA condensation by becatamide. Chromatin condensation was measured using Nuclear-ID™ Green Chromatin Condensation Detection Kit (normal chromatin in the left and chromatin condensation in the right). Chromatin condensation was evident in cells treated with H_2O_2 (200 μM) for 24 h (B) compared to control untreated PC-12 cells (A). In PC-12 cells pre-treated with becatamide (C), chromatin condensation was significantly attenuated ($P < 0.05$) compared to that in H_2O_2 only-treated cells (B).

caspase-9 activation, probably via protecting mitochondrial membrane integrity from H_2O_2-induced damage.

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