Docosahexaenoic Acid, a Peroxisome Proliferator–Activated Receptor-α Ligand, Induces Apoptosis in Vascular Smooth Muscle Cells by Stimulation of p38 Mitogen-Activated Protein Kinase

Quy N. Diep, Rhian M. Touyz, Ernesto L. Schiffrin

Abstract—Omega-3 fatty acids (n-3 FAs) have been shown to exert a blood pressure–lowering effect in hypertension, possibly in part by influencing vascular structure. We previously demonstrated that n-3 FAs induce vascular smooth muscle cell (VSMC) apoptosis, which could exert an effect on the structure of blood vessels. In the present study, we investigated signaling pathways through which n-3 FAs mediate apoptosis in VSMCs. Cultured mesenteric VSMCs from Sprague-Dawley rats were stimulated with docosahexaenoic acid (DHA), a representative n-3 FA. Morphological changes in apoptosis and DNA fragmentation were examined with phase-contrast microscopy and fluorescence microscopy with Hoechst 33342 staining. To clarify possible pathways of apoptosis, we evaluated the expression of phosphorylated p38 mitogen-activated protein kinases, bax, bcl-2, cytochrome c, and peroxisome proliferator-activated receptor-α (PPAR-α) with Western blot analysis. DHA treatment induced cell shrinkage, cell membrane blebbing, and apoptotic bodies in VSMCs. DHA time-dependently activated p38 mitogen-activated protein kinases, bax, PPAR-α, and cytochrome c, with maximal effects obtained after 5 and 30 minutes and 1 and 3 hours, respectively. SB-203580 and SB-202190, selective p38 inhibitors, reduced DHA-elicited apoptosis and expression of PPAR-α but had no effect on the expression of bax or cytochrome c. The present results indicate that DHA induces apoptosis in VSMCs through ≥2 distinct mechanisms: (1) a p38-dependent pathway that regulates PPAR-α and (2) a p38-independent pathway via dissipation of mitochondrial membrane potential and cytochrome c release. The death-signaling pathway stimulated by DHA may involve an integration of these multiple pathways. By triggering VSMC apoptosis, DHA may play a pathophysiological role in vascular remodeling in cardiovascular disease. (Hypertension. 2000;36:851-855.)

Key Words: fatty acids ■ docosahexaenoic acid ■ apoptosis ■ cells ■ muscle, smooth, vascular ■ protein kinases

Abnormal fatty acid (FA) metabolism has been described in hypertension.1 Dietary saturated FAs are associated with hypertensive effects, whereas polyunsaturated FAs may be hypotensive.2,3 Thus, omega-3 FAs (n-3 FAs), which are polyunsaturated FAs, may play a role in blood pressure regulation. Dietary n-3 FAs in the form of fish oil have been shown to exert an antihypertensive effect in hypertensive subjects,4–6 spontaneously hypertensive rats (SHR),7–9 and stroke-prone SHR.10–12 These effects may be mediated via n-3 FA actions on vascular smooth muscle cell (VSMC) growth, which could influence vascular structure. n-3 FAs cause apoptosis in nonvascular cells, including cardiomyocytes.13–15 We recently reported that docosahexaenoic acid (DHA), a representative n-3 FA, induces apoptosis in VSMCs.16 Apoptosis or programmed cell death is a fundamental biological process whereby individual cells die through the activation of a genetically programmed cell suicide mechanism.17 Apoptosis is characterized by morphological changes such as cell shrinkage, cell membrane blebbing, compaction of cytoplasmic organelles, dilatation of the endoplasmic reticulum, and alterations to the plasma membrane that result in the condensation of chromatin and nuclear fragments into membrane-enclosed apoptotic bodies. These morphological changes are accompanied by biochemical changes, including internucleosomal DNA fragmentation. Recent studies reported that apoptosis may play an important role in cardiovascular disorders by influencing cardiac and vascular remodeling.18,19

Signaling mechanisms whereby DHA induces apoptosis in VSMCs are unclear. We previously demonstrated that DHA induced apoptosis through translocation of plasma membrane phosphatidylserine and disruption of mitochondrial transmembrane potential, followed by increased bax expression and caspase 3 activation.16 It is also possible that mitogen-activated protein kinases (MAPKs) play a role. The MAPK family of kinases are regulators of cell growth and apoptosis20.
and include extracellular signal–regulated kinases (ERKs), p38 MAPKs, and c-Jun N-terminal kinase/stress-activated protein kinases. Although ERK activation is characteristically associated with growth signaling pathways, the activation of p38 MAPK and c-Jun N-terminal kinase/stress-activated protein kinase has been associated with apoptosis and could be involved in DHA-induced apoptosis in VSMCs.

Previous studies have reported a relationship between peroxisome proliferator-activated receptors (PPARs), particularly PPAR-γ, and the MAPK signaling pathway. PPARs (α, β, and γ) belong to the superfamily of nuclear hormone receptors that are ligand-activated transcription factors. PPAR-α is a target for various long-chain FAs, including n-3 FAs, and is found predominantly in tissues that exhibit high catabolic rates of FAs (liver, heart, kidney, and muscle), whereas PPAR-γ is found primarily in adipose tissue, where it plays a critical role in the differentiation of preadipocytes into adipocytes. Recently, it was shown that both isoforms of PPARs may induce apoptosis in macrophages. Even though the transcriptional activity of PPAR-γ has been shown to be negatively regulated by phosphorylation mediated by the MAPK signaling pathway, little is known about the links between MAPK and PPAR-α. The aims of the present study were therefore to determine whether DHA-induced apoptosis is mediated via activation of p38 MAPK and to assess the role of PPAR-α in this signaling event.

Methods

Chemicals
DHA and Hoechst 33342 were purchased from Sigma-Aldrich Ltd. The highly specific p-38 MAPK inhibitors SB-203580 (HCl form) and SB-202190 (HCl form) were obtained from Calbiochem-Novabiochem Corp. Antibody for specific phosphorylated p38 was from CN Corp. Antibodies for bax, bcl-2, cytochrome c, and PPAR-α were from Santa Cruz Biotechnology.

Cell Culture
Mesenteric VSMCs from Sprague-Dawley rats were isolated as previously described and cultured until subconfluency in DMEM that contained 10% FBS. Cells were rendered quiescent in serum-free DMEM for 24 to 36 hours before stimulation with vehicle (ethanol) or DHA in the absence or presence of p38 inhibitors. DHA was dissolved in ethanol (maximum final concentration of ethanol 0.01% v/v).

Morphological Assessment of Apoptosis
The morphological features of apoptosis were assessed with phase-contrast microscopy. Visualization and quantification of nuclei condensation and DNA fragments were also evaluated with Hoechst 33342 staining as previously described. The number of condensed nuclei with DNA fragmentation was quantified with an image analyzer. At least 400 cells from randomly selected fields were counted in each experiment.

Western Blot Analysis of Phosphorylated p38, bax, bcl-2, Cytochrome c, and PPAR-α
Cultured VSMCs were treated with vehicle or DHA for various time periods. After stimulation, cells were harvested and protein was extracted as previously described. Protein (15 μg) was separated through electrophoresis on a 15% polyacrylamide gel at 100 V for 1 hour and transferred onto a PVDF membrane in a cooling system at 100 V for 1 hour. Membranes were incubated with specific antibody-
overnight exposure to DHA (17 and 24 hours), cell shrinkage, cell membrane blebbing, and cytoplasmic condensation became clearly evident (Figure 1A).

**p38 Inhibitors SB203580 and SB202190 Protect VSMCs From DHA-Induced Apoptosis**

As shown in Figure 1B, when the cells were preincubated with SB203580 or SB202190, specific p38 inhibitors, the increase in apoptosis induced by DHA was significantly reduced. However, the specific inhibitor of the ERK pathway, PD98509, had no effect on cell death mediated by DHA (data not shown).

**Activation of p38 in VSMCs by DHA**

Results from Western blots demonstrated that DHA (40 μmol/L) increased p38 phosphorylation after 1 minute and reached maximal effects after 5 minutes (Figure 2). As shown in Figure 2, both SB 203580 and SB 202190 inhibited p38 MAPK activation induced by DHA in a time-dependent manner when added to the culture medium 1 hour before DHA treatment.

**Effect of p38 Inhibitors on DHA-Induced bax, bcl-2, and Cytochrome c**

Western blotting was performed to quantify expression of the proapoptotic protein bax, the antiapoptotic protein bcl-2, and cytochrome c in the presence and absence of p38 inhibitors. As shown in Figures 3A and 3B, the bax signal was greater in DHA-treated cells than in control cells after 15 minutes of stimulation. However, no significant changes were found in expression of bcl-2 between the 2 groups. The time course of changes in the bax/bcl2 ratio is shown in Figure 4: it increased significantly at 30 minutes. Expression of cytochrome c was increased by DHA (40 μmol/L), with maximal effect obtained 3 hours after stimulation (Figure 5). p38 inhibition did not alter bax, bcl2, or cytochrome c expression in DHA-stimulated cells.

**Effect of p38 Inhibitors on PPAR-α Expression**

To assess whether p38 could be an upstream regulator of PPAR-α in DHA-induced apoptosis, we measured PPAR-α expression after DHA stimulation in the presence and absence of p38 inhibitors. As shown in Figure 6, PPAR-α expression was increased after 30 minutes of DHA stimulation, and this increase was normalized to control levels when cells were preincubated with either SB203580 or SB202190.

**Discussion**

The major findings of the present study are the signaling mechanisms that underlie DHA-induced apoptosis in VSMCs. This may have importance in the regulation of blood pressure, because DHA has been shown to influence blood pressure, which may occur via effects on vascular remodeling. One of the mechanisms involved in these effects may be the reported inhibition of smooth muscle cell proliferation. Another mechanism that we previously reported is DHA-
induced apoptosis. The latter appears to occur through the translocation of plasma membrane phosphatidylserine and disruption of mitochondrial transmembrane potential ($\Delta$cm), followed by an increase in bax and caspase 3 activation. We now explored further the signaling pathways that underlie DHA-induced apoptosis and demonstrate that p38 activation, release of cytochrome c, and PPAR-$\alpha$ expression play important roles in proapoptotic events in response to DHA.

The present data provide the first evidence that DHA, an n-3 FA, activates the p38 signaling pathway, which could be an upstream regulator of other proteins involved in the cell death signaling pathway, including PPAR-$\alpha$, because 2 specific inhibitors of p38, SB203580 and SB202190, blocked both apoptosis and activation of p38 and PPAR-$\alpha$. There is evidence that DHA is a PPAR-$\alpha$ activator. To our knowledge, this is the first study that shows PPAR-$\alpha$ is expressed in rat VSMCs isolated from small (mesenteric) arteries that contribute to peripheral resistance and consequently to blood pressure regulation. PPAR-$\alpha$ activation has been shown to induce macrophage apoptosis through negative interference with the antiapoptotic NFkB signaling pathway. In this study, we show that DHA induced apoptosis through the activation of PPAR-$\alpha$ in a p38-dependent manner.

Both bax and bcl2 are predominantly mitochondrial proteins. Mitochondrial dysfunction is one of the initial steps in the death program, and a loss in $\Delta$cm is a specific marker to detect early apoptotic changes. We previously reported that DHA induced apoptosis in VSMCs through dissipation of $\Delta$cm. In the present study, DHA did not affect bcl-2. However, an overexpression of bax occurred, resulting in an increase in cytochrome c release. In agreement with our data, bax overexpression has been shown to be associated with dissipation of $\Delta$cm. Increased bax and loss of $\Delta$cm resulted in a release of cytochrome c from mitochondria, which is a key signal that initiates the irreversible death sequence. The release of cytochrome c may result in stimulated superoxide production. Whether DHA subsequently stimulates the production of superoxide in VSMCs requires further investigation. Although a close collaborative interaction between bcl-2 family proteins and loss of $\Delta$cm has been demonstrated, we could not find any relation between them. The apoptotic process in VSMCs may be bcl-2 independent, as we showed in an in vivo study and confirmed in this in vitro system. p38 did not have any effect on the increase in bax and cytochrome c induced by DHA, indicating that the disruption of mitochondria is p38 independent.

In conclusion, DHA, a PPAR-$\alpha$ activator, induced apoptosis in VSMCs through 2 distinct mechanisms: (1) a p38-dependent pathway and (2) a p38-independent pathway via...
dissipation of ΔΨm. The death-signaling pathway induced by DHA may involve integration of these multiple signaling pathways. These effects that involve PPAR-α may contribute to vascular remodeling in hypertension and other cardiovascular diseases.

Acknowledgments

This work was supported by a group grant from the Medical Research Council of Canada (MRC) to the Multidisciplinary Research Group on Hypertension. Dr Diep is the recipient of an MRC postdoctoral fellowship. The authors are grateful to André Turgeon for his excellent technical assistance.

References

Docosahexaenoic Acid, a Peroxisome Proliferator–Activated Receptor-α Ligand, Induces Apoptosis in Vascular Smooth Muscle Cells by Stimulation of p38 Mitogen-Activated Protein Kinase

Quy N. Diep, Rhian M. Touyz and Ernesto L. Schiffrin