Docosahexaenoic acid suppresses angiotensin II-induced A7r5 vascular smooth muscle cell proliferation and migration under pulsatile pressure stress

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ABSTRACT
Elevated mechanical stress applied to vascular walls is well known to modulate vascular remodeling and plays a part in the pathogenesis of atherosclerosis. On the other hand, docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid, has been shown to protect against several types of cardiovascular diseases including atherosclerosis and hypertension. The aim of this study was to clarify the effect of pulsatile pressure stress and DHA on angiotensin II-induced proliferation and migration in A7r5 vascular smooth muscle cells (VSMCs). Pulsatile pressure of between 80 and 160 mmHg was repeatedly applied to VSMCs at a frequency of 4 cycles per min using an apparatus that we developed. Cell proliferation and migration were evaluated using a live cell movie analyzer. Application of pulsatile pressure stress for 24 h significantly increased cell proliferation. Angiotensin II also significantly increased cell proliferation in the presence or absence of pressure stress. DHA significantly inhibited angiotensin II-induced cell proliferation regardless of the pressure load. Angiotensin II significantly induced cell migration regardless of the pulsatile pressure load. Pulsatile pressure stress alone slightly, but not significantly, induced cell migration. DHA inhibited angiotensin II-induced VSMC proliferation and migration under abnormal pressure conditions. Pressure stress tended to induce extracellular signal-regulated kinase (ERK) phosphorylation in the absence of angiotensin II, whereas it significantly induced ERK phosphorylation in the presence of angiotensin II. However, the pressure-induced ERK phosphorylation was not observed in the DHA-treated VSMCs. Our findings may contribute to the understanding of the beneficial effect of DHA on various cardiovascular disorders.
brief, culture dishes were placed in the pressure-loading apparatus, and VSMCs were then exposed to pulsatile atmospheric pressure between 80 and 160 mmHg for the appropriate time at a rate of 4 cycles/min. Angiotensin II and/or DHA was added to the culture medium at the beginning of pressurization.

Cell count. On day 5 after seeding, the medium was changed to fresh medium without reagents (control) or with reagents (angiotensin II and/or DHA) in the presence or absence of pressure stress for 24 h. Cells were then washed 3 times with phosphate-buffered saline (pH 7.4), harvested with 0.25% trypsin-EDTA, and counted using a live cell movie analyzer (JuLi™ Br; NanoEnTek, Seoul, Korea).

Cell migration assay. Cell migration was evaluated using culture-inserts according to the manufacturer’s protocol (ibidi, Martinsried, Germany). When confluence was reached, the insert created a 500-μm cell-free gap. At the same time, the medium was changed to fresh medium containing 2% FCS and mitomycin C (20 μM) without reagents (control) or with reagents (angiotensin II and/or DHA) in the presence or absence of pressure stress for 4 h. The cell-free zone was captured using JuLi™ Br. The cell re-colonization rate was evaluated by measuring the denuded surface area using Image J software (version 2.0.0; NIH, Bethesda, MD, USA).

Western blot analysis. Phosphorylation of ERK was evaluated by Western blot analysis as described previously (22). The resultant blots were incubated for 2 h with primary antibody, and were subsequently incubated with horseradish peroxidase-conjugated secondary antibody and visualized by using an enhanced chemiluminescence kit. All bands were analyzed by densitometry in ImageJ software (NIH).

Statistical analysis. Results were expressed as the mean ± standard error. Statistical analysis of the results was performed with Student’s t test or one way analysis of variance followed by Tukey’s multiple comparisons test; p-values less than 0.05 were considered significant.
Effect of DHA on VSMC function

II-induced proliferation was further potentiated. Regardless of the presence or absence of the pulsatile pressure stress, DHA (30 μM) almost completely inhibited angiotensin II-induced cell proliferation (Fig. 2A and 2B).

We next evaluated the effect of pulsatile pressure stress and angiotensin II on the migration of A7r5 cells by using a wound healing assay. Pulsatile pressure alone slightly, but not significantly, induced migration (Fig. 3). Regardless of the presence or absence of pulsatile pressure stress, angiotensin II significantly enhanced the closure of the linear injury line as compared with control cells. DHA almost completely inhibited angiotensin II-induced cell migration in the presence or absence of pressure stress.

Activation of mitogen-activated protein kinase (MAPK), including ERK and p38 MAPK, has been shown to play a critical role for cell proliferation and migration (8). We next investigated the effect of pulsatile pressure stress and angiotensin II on ERK phosphorylation. As shown in Fig. 4A, the pressure stress alone tended to activate ERK phosphorylation, reaching a maximum after 1–3 h. Angiotensin II also rapidly activated ERK phosphorylation within 1 h (Fig. 4B). As summarized in Fig. 4C and D, pressure stress tended to activate ERK phosphorylation in the absence of angiotensin II ($P < 0.1$), and it significantly activated ERK phosphorylation in the presence of angiotensin II. Regardless of the presence or absence of angiotensin II, the pressure stress-induced ERK phosphorylation was not observed in the DHA-treated VSMCs (Fig. 5).

Fig. 1  Pulsatile pressure stress and angiotensin II promote VSMC proliferation. Cells were treated without (control) or with angiotensin II (100 nM) in the presence or absence of pressure stress for 24 h. Cell numbers were counted using a live cell movie analyzer, JuLi™ Br. Open columns: non-pressureized; hatched columns: pressurized. Each column represents the mean ± standard error of the mean ($n = 9$). *$P < 0.05$ versus untreated control.

Fig. 2A  DHA inhibits angiotensin II-induced cell proliferation in the absence (A) or presence (B) of pressure stress. Cells were treated without (control) or with DHA (30 μM) in the presence or absence of angiotensin II for 24 h. Cell numbers were counted using a live cell movie analyzer, JuLi™ Br. Open columns: non-pressureized; hatched columns: pressurized. Each column represents the mean ± standard error of the mean ($n = 5$). **$P < 0.01$ versus untreated control; ††$P < 0.01$ versus angiotensin II.

DISCUSSION

Hypertension is a major risk factor for atherosclerosis, which has a role in the pathogenesis of cardiovascular and cerebrovascular diseases. VSMCs play a crucial role in the local regulation of vascular tone. Because pressure stress rather than shear stress acts on VSMCs, we have been focusing to clarify the effect of pressure stress on VSMC function. We have developed a novel pulsatile pressure-loading apparatus and reported that this apparatus is useful as an in vitro model of hypertension (13, 20). Using the pulsatile pressure-loading apparatus, we have re-
ation and migration of VSMCs play critical roles in the pathogenesis of atherosclerosis (30). Angiotensin II stimulates atherosclerosis development and vascular restenosis through various deleterious effects such as endothelial dysfunction and inflammation. It was reported that pulsatile pressure stress induces cell proliferation and promotes ACE expression in human aortic smooth muscle cells (SMCs) (13) and inhibits interleukin-1β (IL-1β)-induced inducible nitric oxide expression in rat primary VSMCs (21). The proliferation and migration of VSMCs play critical roles in the pathogenesis of atherosclerosis (30). Angiotensin II stimulates atherosclerosis development and vascular restenosis through various deleterious effects such as endothelial dysfunction and inflammation.

Fig. 3 DHA inhibits angiotensin II-induced cell migration in the absence or presence of pressure stress. A: Representative result. B: Wound closure rate. Cells were treated without (control) or with DHA (30 μM) in the presence or absence of angiotensin II and pressure stress for 4 h. The cell re-colonization rate was evaluated using Image J software (version 2.0.0, NIH). Open columns: non-pressureized; hatched columns: pressurized. Each column represents the mean ± standard error of the mean (n = 4). *P < 0.05, **P < 0.01 versus untreated control; †P < 0.05, ††P < 0.01 versus angiotensin II.
Effect of DHA on VSMC function

In fact, angiotensin II causes VSMC proliferation and migration, an increase in matrix production of fibronectin and collagen, and induction of oxidative stress (2, 31, 35).

In this study, pulsatile pressure stress increased the proliferation of A7r5 VSMCs, a rat cell line. The combination of pressure and angiotensin II additively increased proliferation. These results were consistent with our previous findings using human aortic SMCs (13). Therefore, it was shown that the

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**Fig. 4** Pulsatile pressure stress promotes ERK phosphorylation. A: Cells were cultured in the presence or absence of pressure stress for indicated time periods. Representative Western blot results. B: Cells were treated without (control) or with angiotensin II (100 nM) in the presence or absence of pressure stress for indicated time periods. Representative Western blot results. C and D: Cells were treated without (C) or with angiotensin II (D) in the presence or absence of pressure stress for 3 h. The values are expressed as the ratio of p-ERK/t-ERK, with the non-pressurized control as 1. Open columns: non-pressurized; hatched columns: pressurized. Bars are the mean ± standard error of the mean (n = 9). *P < 0.05 versus untreated control.
pressure stress promotes the proliferation of VSMCs both in rats and humans. We also found in the present study that the pressure stress alone tended to slightly promote cell migration. Therefore, it may be possible that increased cell proliferation and migration by pressure stress may synergistically exacerbate the vascular wall environment in vivo. Activation of MAPK, including extracellular signal-regulated kinase (ERK) and p38 MAPK, has been shown to play a critical role for cell proliferation and migration (18). We previously reported that pulsatile pressure stress phosphorylates ERK in human aortic SMCs (13), which was consistent with our present study using that rat cell line. The pressure-induced proliferation is completely inhibited by PD 98059, an inhibitor of ERK activation in human aortic SMCs (28). Therefore, ERK is a key enzyme for pressure-induced proliferation and migration. Further studies are required to clarify the role of p38 MAPK in pressure-induced VSMCs function.

DHA significantly inhibited angiotensin II-induced cell proliferation and migration in the presence or absence of pressure stress. Furthermore, pressure-induced ERK phosphorylation was not observed in the DHA-treated VSMCs. Therefore, inhibition of ERK phosphorylation by DHA may have an important role for the inhibitory effect of cell proliferation and migration. It has been reported that DHA inhibits angiotensin II-induced migration in cardiac fibroblasts by inducing reversion-inducing cysteine-rich protein with Kazal motifs (RECK) (33). Since angiotensin II inhibits RECK expression and RECK inhibits release and activation of metalloendopeptidase MMP2 (32), which triggers migration, reversal of angiotensin II-induced RECK suppression by DHA seems to be a key mechanism. DHA has also been reported to inhibit IL-1β-induced migration of rat VSMCs and suppress IL-1β-induced MMP2 as well as MMP9 via mechanisms involving DHA-induced Notch signaling pathways (4). Further studies are required to clarify the precise mechanisms.

Previously, we reported that a diet containing DHA in young stroke-prone spontaneously hypertensive rats (SHRs) for 14 weeks remarkably inhibited the development of hypertension (15). Furthermore, when DHA was continuously administered to stroke-prone SHRs from the age of 6 weeks until death, the life-span of DHA-treated animals increased compared with that of the non-treated group (24). The concentration (30 μM) of DHA used in this study is close to that found in the plasma of stroke-prone SHRs fed a diet containing 5% DHA (25). The amounts of angiotensin II (27) as well as renin (1)

Fig. 5 DHA cancels pressure-induced ERK phosphorylation. A: Cells were cultured with DHA (30 μM) in the presence or absence of pressure stress for 3 h. B: Cells were cultured with DHA (30 μM) and angiotensin II (100 nM) in the presence or absence of pressure stress for 3 h. Upper: Representative Western blot results. Lower: The values are expressed as the ratio of p-ERK/t-ERK, with the non-pressurized control as 1. Open columns: non-pressureized; hatched columns: pressurized. Bars are the mean ± standard error of the mean (n = 6).
in the vascular wall isolated from SHRs are reported to be higher than that isolated from normotensive Wistar-Kyoto rats. Furthermore, the generation of angiotensin II in VSMCs from SHRs is considered to contribute to abnormal basal growth in an autocrine–paracrine manner by stimulating the angiotensin II AT₁ receptor (8). In fact, an AT₁ receptor antagonist, CV-11974, and an ACE inhibitor, delapril, reduced basal DNA synthesis in VSMCs from SHRs, but not in those from Wistar-Kyoto rats (16). ACE expression in VSMCs from SHRs is significantly higher than that in VSMCs from Wistar-Kyoto rats (8). Therefore, the finding of an inhibitory effect of angiotensin II-induced proliferation and migration by DHA under abnormal pressure stress may contribute to the understanding of the basic mechanisms underlying the therapeutic potential of DHA for cardiovascular diseases.

In conclusion, abnormal pulsatile pressure stress induced VSMC proliferation. DHA inhibited angiotensin II-induced cell proliferation and migration under abnormal pressurized conditions. These findings may contribute to understanding the beneficial effects of DHA on various cardiovascular diseases.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES


