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The Benefit of Docosahexanoic Acid on the Migration of Vascular Smooth Muscle Cells Is Partially Dependent on Notch Regulation of MMP-2/-9

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The Notch pathway is involved in the regulation of the migratory/proliferative phenotype acquired by vascular smooth muscle cells (VSMCs) in the pro-inflammatory context of vascular diseases. Here, we investigated whether docosahexaenoic acid (DHA), a polyunsaturated, ω-3 fatty acid, could reduce fibrinolytic/matrix-metalloproteinase (MMP) activity and whether this reduction occurs through the modulation of Notch signaling. Rat VSMCs were transdifferentiated with interleukin-1β and then treated with DHA. Migration/proliferation was determined by performing a wound healing assay and measuring MMP-2/-9 activity, type 1 plasminogen activator inhibitor levels, and the expression of these proteins. The involvement of Notch in regulating the fibrinolytic/MMP system was evidenced using Notch pathway inhibitors and the forced expression of Notch1 and Notch3 intracellular domains. DHA significantly decreased VSMC migration/proliferation induced by interleukin-1β as well as fibrinolytic/MMP activity. Prevention of Notch1 target gene transcription enhanced the interleukin-1β effects on MMPs and on migration, whereas Notch3 intracellular domain overexpression reduced these effects. Finally, DHA increased Notch3 expression, Hes-1 transcription (a Notch target gene), and enhanced γ-secretase complex activity. These results suggest that inhibition of the Notch pathway participates in the transition of VSMCs toward a migratory phenotype. These results also suggest that the beneficial inhibitory effects of DHA on fibrinolytic/MMP activity are related in part to the effects of DHA on the expression of Notch pathway components, providing new insight into the mechanisms by which ω-3 fatty acids prevent cardiovascular diseases. ***(Am J Pathol 2008, 172:1430–1440; DOI: 10.2353/ajpath.2008.070951)***

Epidemiological studies showed that regular consumption of ω3 polyunsaturated fatty acids [such as eicosapentaenoic acid and docosahexaenoic acid (DHA)] lowers incidence and death from cardiovascular diseases.[1] In addition to their hypolipidemic effect, the benefit of ω3 on blood pressure, cardiac rhythm, and inflammation are also related to their direct effects on endothelial and smooth muscle cells [vascular smooth muscle cell (VSMC) membrane enrichment with eicosapentaenoic acid and/or DHA reduced the transition toward an inflammatory state[2,3]]. Indeed, such a treatment inhibited the synthesis of the inflammatory prostaglandin E2 through the inhibition of the enzymes involved in the lipid mediator biosynthesis pathway (cyclooxygenase 2 and type II-a secreted phospholipase A2)[4,5] and the vascular cellular adhesion-molecule-1, monocyte chemoattractant protein-1 synthesis.[6,7] They also modulate the VSMC cycle by inhibiting proliferation and migration and/or stimulating apoptosis.[8,9] Although the mechanisms by which ω3 prevents vascular pathologies are partially elucidated, those underlying inhibition of VSMC transition toward a migrative/proliferative phenotype are poorly documented. Taking into account the importance of this step in the development of most vascular pathologies, the major aim of this study was to investigate whether and how DHA reduces the VSMC migration/proliferation.

VSMC migration and proliferation observed during restenosis and atherosclerosis involve the degradation of the extracellular matrix and require the secretion of matrix-metalloproteinases (MMPs).[10] Indeed, up-regulation

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of MMP-2 and induction of MMP-9 rapidly occur in response to mechanical injury and inflammatory cytokines allowing cells to migrate.[11,12] Regulation of MMP activity occurs at several levels, including activation of latent zymogen, which can be achieved as follows: circulating plasminogen is cleaved into plasmin by t-plasminogen activator (t-PA) or urokinase, leading to an increase of MMP activity; both of these activators are controlled by type 1 plasminogen activator inhibitor (PAI-1).[10] Here, we show that the DHA inhibitory effect on VSMC migration/proliferation is associated with a down-regulation of MMP-2/-9 activities and t-PA expression, as well as an increase of PAI-1 secretion.

Recent data demonstrated that the Notch pathway regulates some of the new features acquired by VSMCs in physiological or pathological vessels[13–15] In particular, it has been previously shown that two of the four subtypes of Notch receptors characterized in mammals, Notch1 and 3, regulate the decision of VSMC to grow and migrate or, on the contrary, to follow a cell death program in response to growth factors in the context of vascular injury.[16,17] Thus, we have evaluated the role of Notch1 and 3 in the regulation of the MMPs activity and fibrinolytic system and showed that preventing Notch target gene transcription enhanced the effect of interleukin (IL-1β) on MMPs and migration. Conversely, the overexpression of Notch intracellular domain (NICD) 3 reduced it. Finally, DHA increased the transcription of Notch3 and Hes-1, one of its target genes, as well as the γ-secretase complex activity, which is responsible for the Notch active form generation.[18] This provides evidence that the beneficial effects of DHA on the fibrinolytic system leading to migration inhibition could be related, at least in part, to its influence on the expression of the Notch signaling pathway components.

Materials and Methods

Cell Culture

VSMCs were isolated from the aorta of male Wistar rat aged of 8 weeks, after removing fat tissue and extruding the media mechanically from the adventitia, by enzymatic digestion, and cultured as previously described.[19] All experiments were performed on cells at passages 3 up to 7. Immunochemistry experiments visualizing α-actin show that, among rat aorta vascular cell types, plated cells always display a well organized network of actin stress fibers in control, untreated conditions. This characteristic demonstrates first, cell provenance, the smooth muscle, and second, the maintenance of their differentiated state (data not shown).

Twenty-four hours before any treatment, confluent cells were starved and all experiments were continued in serum-free medium. Conditions of cell treatment are indicated in the figure legends. DHA (SPI BIO, Montigny le Bretonneux, France) and *N*-(*N*-(3,5-difluorophenacetyl)-L-alanyl)-*S*-phenylglycine *t*-butyl ester (DAPT; Sigma-Aldrich, Saint Quentin Fallavier, France), a γ-secretase complex inhibitor, were dissolved, respectively, in absolute ethanol and dimethyl sulfoxide. Final concentrations of vehicles were, respectively, 0.1% and 0.001%. All procedures were performed in accordance with European Community Standards on the care and use of laboratory animals (authorization 4270), and conform to NIH Guidelines.

Transfection by Electroporation

VSMCs were transfected with each plasmid by electroporation in the Amaxa (Gaithersburg, Germany) electroporation device using the D-33 program. Briefly, 10^6 cells were suspended in 100 μl of Amaxa electroporation transfection solution containing 2 to 4 μg of expression vectors encoding for mouse NICD1 or 3, human recombination signal binding protein for immunoglobulin kappa J region, dominant-negative (RBP-Jκ DN) and ΔE Notch. After electroporation, transfected cells were transferred in 2 wells of a 6-well plate containing 1 ml of complete culture medium. Cells were starved 24 hours after plasmid transfection. The next day, cells were treated as indicated in figure legends. Culture medium was collected, and RNA or protein was extracted as described below. The mouse NICD3 expression vector was a gift from Dr U. Lendahl (Karolinska Institutet, Stockholm, Sweden). The mouse NICD1 expression vector was obtained as described by Gupta-Rossi et al.[20] The expression vector encoding human RBP-Jκ DN was a gift from Dr A. Sergeant (Institut Pasteur, Paris France). ΔE Notch1, a ligand-independent Notch construct constitutively cleaved by the γ-secretase complex,[21] containing the transmembrane and intracellular domains of mouse Notch 1 terminated at amino acid 2185 with a hexameric Myc epitope, was a gift from Dr. J. Nye (Northwestern University, Chicago, IL). The efficacy of transfection was evaluated by real-time reverse transcription PCR (RT-PCR) and Western blot as described below.

Wound Healing Assay

VSMCs were cultured in a 6-well culture plate. When confluence was reached, cells were incubated with or without mitomycin C (20 μmol/L, dissolved in culture medium), a potent inhibitor of cell proliferation, for 2 hours. After this incubation, cells were scraped with a 200-μl pipette tip. After washing with phosphate-buffered saline, cells were treated as described in figure legends. The scraped zone was captured with an inverted microscope, right after the wound and 24/48 hours later. The cell re-colonization rate was evaluated by measuring the denuded surface area, using Image J (1.36b, NIH) software (*http://rsb.info.nih.gov./ij*). To ensure the capture of the same zone throughout experiments a perpendicular line to the scrapped zone was drawn to serve as a benchmark.

Proliferation Assay

The proliferative capacity of VSMCs was determined using the Cell Proliferation Biotrak enzyme-linked immunosorbent assay system (Amersham Biosciences, Buckinghamshire, UK). Briefly, VSMCs were seeded in a

Table 1. List of Primers

	Forward	Reverse
HPRT	5′-AGGACCTCTCGAAGTGT-3′	5′-ATCCCTGAAGTGCTCATTATAG-3′
MMP-2	5′-ATCGCCCATCATCAAGTTC-3′	5′-CATGGTCTCGATGGTGTT-3′
MMP-9	5′-AGAATCTCTACACGGAT-3′	5′-GGACAGAAGCCATACAGCTTA-3′
TIMP-1	5′-ACTGGAGAGTGACAGTCATT-3′	5′-GGAAGGCTTCGGGTCATC-3′
TIMP-2	5′-GACCTGACAAGGACATCGAA-3′	5′-TCCCAGGGCACAATAAAGT-3′
t-PA	5′-CACAGCTTTACCACATCC-3′	5′-CAGATAGCACCCAGCAGG-3′
PAI-1	5′-CTCTCGAAGAGGAAAGAGTCTAAC-3′	5′-CTTTGCTGAGTGAAGGCG-3′
Hes1	5′-GACGGCCAATTTGCTTTC-3′	GACACTGCGTTAGGACC-3′
HRT1	5′-GAAGCGCCGACGAGACCGAATCAA-3′	CAGGGCGTGCGCGTCAAAATAACC-3′
HRT2	5′-CGACGTGGGGAGCGAGAACAAT-3′	GGCAAGAGCATGGGCATCAAAGTA-3′
Mouse Notch1-ICD	5′-CTGTCATCTCTGACTTCATCTACC-3′	GCTTTGCAGCATCTGAACG-3′
Mouse Notch3-ICD	5′-GAACAACGTGGAGGCTAC-3′	CTCATAGCTGCCCTCCC-3′
Rat Notch 3	5′-AGCGAGCATCCTTATTTGAC-3′	5′-TTGCTGGACTAGGCGTT-3′
Human RBP-Jκ	5′-CACAGATAAGGCAGAGTATACATTT-3′	5′-ACCGCCATTCAATGAAG-3′

The primers used in the study were designed using LightCycler Probe design software 2.0 (Roche Diagnostics, Veylan, France).

96-well microtiter plate at a density of 10^4 or 10^5 cells/well, and incubated with or without IL-1β, in the presence or absence of DHA or DAPT for 48 hours. After incubation, 5-bromo-2′deoxyuridine (BrdU) 10 μmol/L was added to the medium overnight. After removing the culture medium, the cells were fixed and the DNA was denatured by the addition of a fixative. After a 30-minute incubation at room temperature, the fixative was removed. Subsequently, peroxidase-labeled anti-BrdU monoclonal antibody was added and the plate was incubated at room temperature for 90 minutes. The BrdU-antibody complexes were detected by a colorimetric reaction with the substrate (3,3′,5,5′-tetramethylbenzidine), with the optical density read at 450 nm.

Determination of MMP-2/-9 Activity and PAI-1 Level

Zymography was performed as previously described[22] on conditioned media. Briefly, an equal volume of each treatment condition was loaded onto a 7.5% polyacrylamide gel containing 1% of type 1 gelatin. After electrophoresis, gels were washed in a 2.5% Triton X-100 solution (2 × 30 minutes), incubated for 20 hours in buffer containing 50 mmol/L Tris and 2.5 mmol/L $CaCl_2$, and stained with Coomassie Blue. When (aminomethyl)phosphinic acid (Sigma-Aldrich) was used to identify the band corresponding to the active form of MMP-2, conditioned media were incubated with (aminomethyl)phosphinic acid 1 μmol/L for 3 hours at 37°C prior to zymography. MMP isoform identity was controlled by immunoprecipitation using a selective antibody of each MMP-(2 or 9) (Tebu-Bio, Le Perray en Yvelynes, France) and conditioned media derived from a 24-hour incubation of rat lung in serum free medium. Briefly: the conditioned media were pre-cleared with A/G agarose beads (Tebu-Bio). The pre-cleared media (2 mg of protein) were incubated with 8 μg of rabbit anti-MMP-2 or goat anti-MMP-9 overnight at 4°C. The A/G agarose beads were added and incubation continued for 2 hours at room temperature. As a negative control, naïve rabbit or goat Igs (Tebu-Bio) were used. The resin was pelleted, washed 3 times with serum-free medium, resuspended in 30 μl of a buffer containing 8.5M urea, 2% CHAPS, and 0.1% bromphenol blue, before zymography.

The surfaces of lysis areas were quantified using Scion Image software (NIH Image). Concentrations of PAI-1 were determined in the same samples using the Zymutest Rat PAI-1 Antigen assay from Hyphen BioMed (Neuville sur Oise, France).

Real-Time RT-PCR Amplification

Total RNA was isolated from VSMCs using the mini RNeasy mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Reverse transcription and PCR amplification were performed as previously described.[19] The single-stranded oligodeoxynucleotide primers used to amplify each transcript encoding for the different genes are described in Table 1. Results are expressed as relative induction (versus control) after normalization to the level of hypoxanthine phosphoribosyl transferase (HPRT) mRNA.

Western Blot

Total protein was extracted in 50 mmol/L Tris, pH7.9, 2 mmol/L $NaVO_3$, 10 mmol/L Na pyrophosphate, 20 mmol/L NaF, 400 mmol/L NaCl, 1% NP40, and a cocktail of proteases inhibitors. Protein concentration of each sample was determined using DC Protein Assay (Bio-Rad, Marnes-La-Coquette, France). Thirty μg or 60 μg of protein were loaded on a 4% to 12% NuPAGE gel (Invitrogen, Cergy Pontoise, France) and transferred to nitrocellulose membrane (Hybond; Amersham Biosciences). After blocking in 5% nonfat dried milk, membranes were incubated with respective primary antibodies (mouse anti c-*myc*, 1/1000 (Sigma-Aldrich); rabbit anti-Notch1 Val 1744, 1/1000 (Ozyme, Saint Quentin en Yvelynes, France); anti-Flag, 1/1000 (Sigma-Aldrich); and appropriate secondary antibodies conjugated to horseradish peroxidase (P.A.R.I.S Antibody, Compiegne, France) were used. Detection was performed by using enhanced chemiluminescence reagents (Amersham Biosciences).

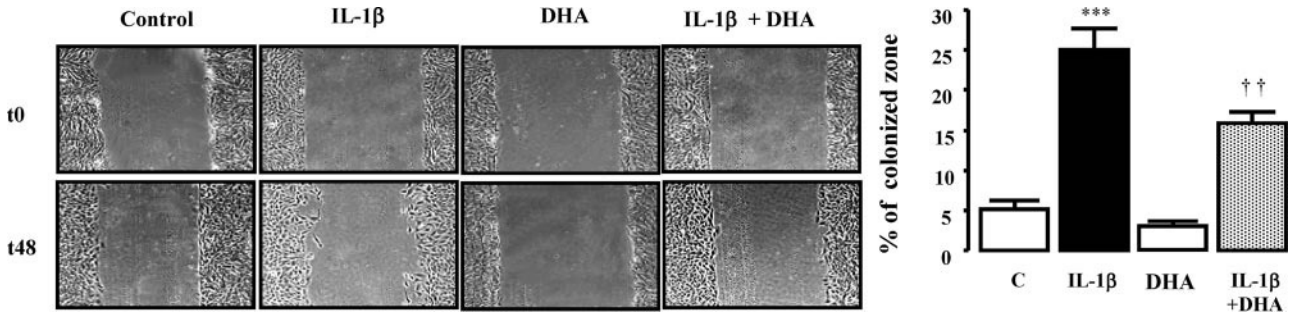

Figure 1. DHA inhibition of VSMC wound healing capacity. After 24 hours of serum starvation a linear wound was made in the center of culture dish and cells were treated by IL-1β (10 ng/ml) and/or DHA (50 μmol/L) during 48 hours (t48). The denuded area was visualized by using an inverted microscope ($\times$40) and captured at t48. The surface of the colonized zone was calculated as described in Materials and Methods. For each condition, the results are expressed as % of colonized zone at t0 and represent mean $\pm$ SE for four independent experiments (shown to the right). $^{***}P < 0.0001$, vs control, and $^{\dagger\dagger}P < 0.001$ IL-1β+DHA vs IL-1β.

Statistical Analysis

Results are expressed as mean $\pm$ SEM of at least four independent experiments. Data were analyzed by one-way analysis of variances (ANOVA), for each interaction using Statview software. When a statistically significant difference was obtained, further analysis was conducted using Bonferroni/Dunn post hoc test. For all comparisons, statistical significance was assumed when $P < 0.05$.

Results

DHA Partially Prevents the Transition of VSMC Toward Migrative/Proliferative Phenotype Acting on the Fibrinolytic System

We first evaluated the effect of DHA on the migration and/or proliferation capacity of IL-1β-induced cells by performing a wound healing assay. In this study, DHA was preferred to eicosapentaenoic acid because of its more accurate beneficial effect on arrhythmia, and hypertension.[9] As expected, IL-1β (10 ng/ml) applied for 24 (data not shown) or 48 hours (Figure 1, left) enhanced the closure of the linear injury line (of $\sim$15% and 25%, respectively), as compared with control cells. Consistent with the literature,[9,23] this migratory/proliferative effect of IL-1β was significantly prevented by co-incubating cells with DHA (50 μmol/L) during 48 hours (Figure 1, left). Indeed, the denuded area was always (ie, regardless of the experiment and the zone analyzed) appreciably larger when cells were treated with both compounds, as compared with that re-colonized by cells treated with IL-1β alone (Figure 1, quantification to the right). In serum-free medium, no significant growth and/or migration was observed for DHA-treated cells (data not shown) or control cells, even when treated with DHA vehicle (ethanol 0.1%). This inhibitory impact of DHA on the wound healing resulted from an effect on both cell responses: migration and proliferation. Indeed, after having inhibited cell proliferation with mitomycin C, DHA still reduced in a significant manner the recolonized zone; DHA blocked BrdU incorporation in IL-1β-induced cells (Supplemental Figure S1, A and B, see *http://ajp.amjpathol.org*).

Part of the DHA impact on the IL-1β-induced VSMC migration/proliferation resulted from its effect on the constitutively expressed MMP-2 and the pro-inflammatory cytokine-induced MMP-9 expression.[24,25] Indeed, pro-MMP-2 and MMP-2 activity visualized by gelatin zymography (Figure 2A, left panel) was significantly lower in conditioned media issued from co-treated VSMCs than that obtained from the conditioned media of VSMCs treated with IL-1β alone (Table 2). The identity of the zymographic bands was controlled by performing an immunoprecipitation using an anti-MMP-2 antibody and naïve Ig on conditioned media derived from rat lung incubated in serum-free medium. The active form of MMP-2 was identified as the lowest band by performing (aminomethyl)phosphnic acid activation of a control sample before zymography. As evidenced by real-time RT-PCR (Figure 2A, right panel), DHA modulation of the IL-1β-induced MMP-2 probably resulted in part from a down-regulation of its own expression, because the mRNA was significantly decreased by $\sim$ 63%. It also resulted from a decrease of its proteolytic activity, because the percentage of active form (expressed as the ratio of active MMP-2 on total MMP-2) is significantly reduced by DHA (Table 2). To note, the expression of the tissue inhibitor of metalloproteinase-2, although diminished by IL-1β, was not restored by DHA (Supplemental Figure S2, see *http://ajp.amjpathol.org*), clearing it from a possible contribution to the DHA-induced inhibition of MMP-2 activity. Similar results were obtained when MMP-9 activity was measured, except that the active form of MMP-9 was undetectable under these experimental conditions, as evidenced by pretreatment (Figure 2B, left panel; Table 2). Here again, the identity of the zymographic band was controlled by performing an immunoprecipitation experiment. Of note, the upper band was unidentified but was always present whatever antibody used. Surprisingly, the tissue inhibitor of metalloproteinase-1 expression (Supplemental Figure S2, see *http://ajp.amjpathol.org*), one of the MMP-9 inhibitors, was induced by IL-1β and decreased by DHA. The post-translational regulation of MMP-2 most probably resulted from the influence of DHA on two of the major components regulating the fibrinolytic system the t-PA and PAI-1. Indeed, the expression of transcripts (Figure 2C, lower panel) encoding t-PA induced by IL-1β, was

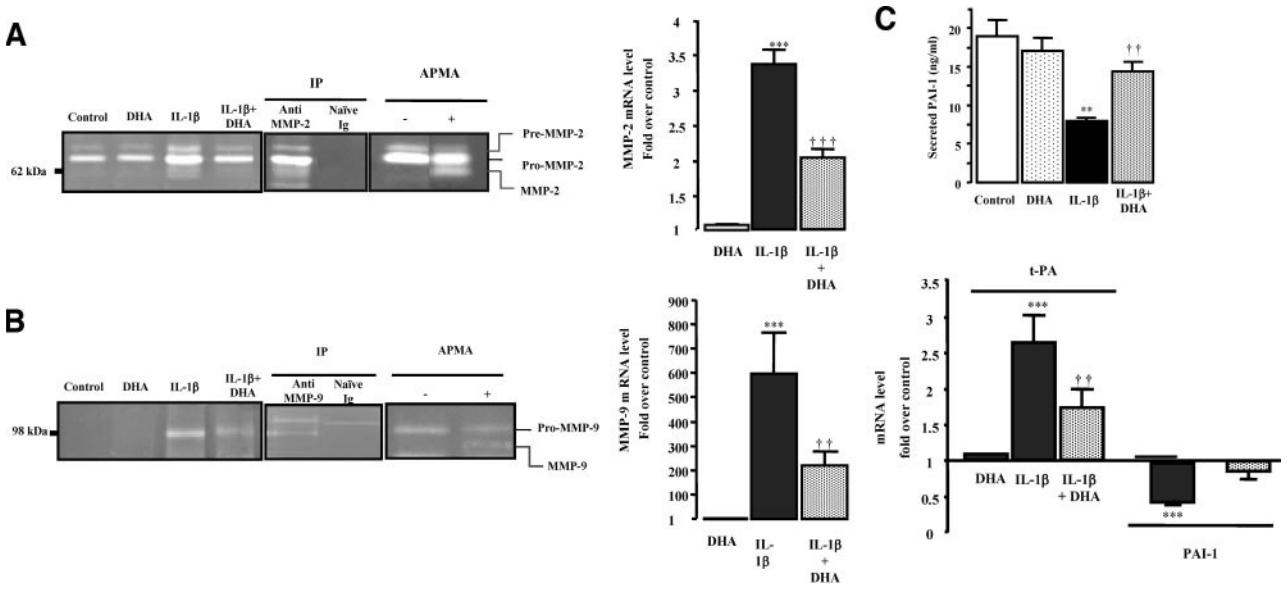

Figure 2. DHA effect on the fibrinolytic system. After 48 hours of IL-1β (10 ng/ml) and/or DHA (50 μmol/L) treatments, the culture medium was recovered for determination of MMP activity, PAI-1 secretion. mRNA were extracted as described in Materials and Methods. The lysis bands corresponding to the pro- or active form of MMP-2 and the pro-MMP-9 were visualized after Coomassie Blue coloration (**A** and **B**, respectively, **left panels**). The mRNA level of MMP-2 and -9 (**A** and **B**, respectively, **right panels**) were determined by real-time RT-PCR (LightCycler; Roche Diagnostics). The concentration of secreted PAI-1 (ng/ml of medium) (**C upper panel**) and mRNA level of t-PA and PAI-1 (**C lower panel**) were also determined. Amounts of mRNA expression were systematically normalized to HPRT transcripts level. Results are expressed as percentage of mRNA level of control cells. $^{**}P < 0.001$, $^{***}P < 0.0001$ vs control $^{\dagger\dagger}P < 0.001$, $^{\dagger\dagger\dagger}P < 0.0001$ vs IL-1β.

statistically decreased by DHA co-treatment. By contrast, the expression of PAI-1, which was largely decreased by IL-1β treatment, as compared with that of control cells, was partially restored by DHA, acting on the transcript level, and this resulted in an increase of PAI-1 secretion (Figure 2C, upper panel). Neither DHA alone nor its vehicle (ethanol 0.1%) had any effect whatever the parameters considered.

Taken together, these data suggest that DHA could limit the transition of VSMC toward a migratory/proliferative phenotype by modulating the activity and/or expression of the molecular entities constituting part of the VSMC fibrinolytic system. Taking into account recent data, demonstrating the role of the Notch pathway in regulating VSMC migrative and proliferative capacity,[17] we thought that ω3 could influence this signaling to modulate both of these capacities.

VSMC Proliferation/Migration and MMP-2/-9 Up-regulation by IL-1β Occur through Notch Pathway Inhibition

First, we determined whether the Notch signaling pathway (which is modulated by IL-1β[15]) interfered with IL-1β-induced VSMC migration and or growth. To do so, we first treated control and IL-1β (10 ng/ml)-stimulated VSMCs with the γ-secretase complex inhibitor DAPT (0.5 μmol/L), or transfected cells with an expression plasmid encoding constitutively active NICD1 or 3 and assessed the wound healing assay. Of note, the transfection efficiency of mouse NICD1 or 3 cDNA was evidenced by Western blot in control cells (Supplemental Figure S3A, see *http://ajp.amjpathol.org*); the functionality of both Notch intracellular domains was attested by the fact that

Table 2. Effect of DHA on the Secreted MMP-2 and MMP-9 Activity

	Pro MMP-2 (AU)	Active MMP-2 (AU)	% of active MMP-2
Control	8913 ± 665	1633 ± 105	15.7 ± 1.4
DHA	7987 ± 818	1364 ± 268	14.5 ± 1.9
IL-1β	42,089 ± 3,644***	17,078 ± 3037***	28.4 ± 2.3***
IL-1β + DHA	18,642 ± 3162††	4599 ± 482†††	19.9 ± 1.9†††
	Pro MMP-9 (AU)	Active MMP-9 (AU)	% of active MMP-9
Control	151 ± 30	ND	
DHA	137 ± 26	ND	
IL-1β	1151 ± 59***	ND	
IL-1β + DHA	212 ± 50††	ND	

The lysis bands corresponding to the pro- or active form of MMP-2 and the pro-MMP-9 were visualized after Coomassie Blue coloration. Their surfaces were quantified by measuring optic density as described in Materials and Methods and expressed in arbitrary units (AU). The percentage of active MMP-2 was calculated using the formula (OD MMP-2)/(OD MMP-2 + OD pro-MMP-2) × 100. $^{***}P < 0.0001$ vs control; $^{\dagger\dagger}P < 0.001$ and $^{\dagger\dagger\dagger}P < 0.0001$ IL-1β + DHA vs IL-1β. ND, not detectable.

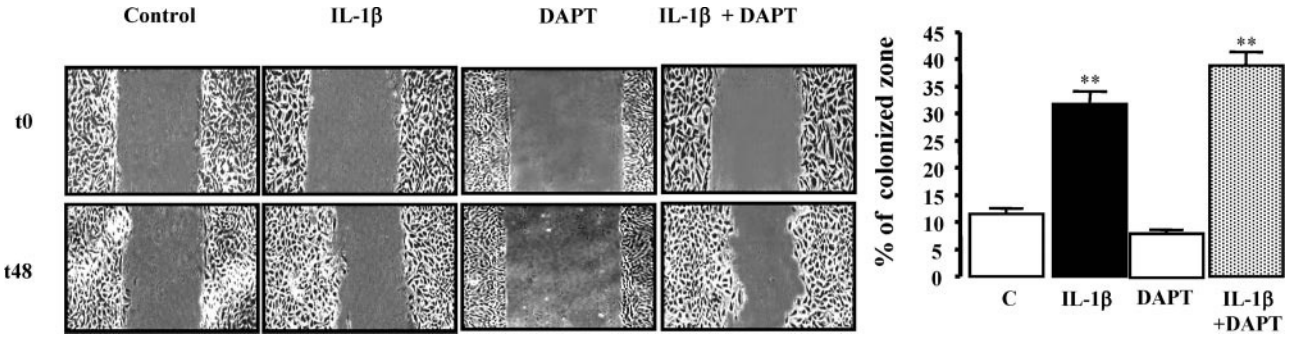

Figure 3. γ-Secretase inhibition potentiates the effect of IL-1β on VSMC proliferation/migration. After serum starvation, a linear wound was made in the center of culture dish and cells were treated by IL-1β (10 ng/ml) and/or DAPT (0.5 μmol/L) during 48 (t48) hours. The denuded area was visualized and captured as described in Figure 1 legend. The percentage of the colonized zone is shown to the right. $^{**}P < 0.01$, vs control.

Notch downstream effector transcripts (Hes1 for Hairy/enhancer of split and HRT1 for Hairy Related Transcription factor[26]) were up-regulated by approximately two- to fivefold, as compared with cells transfected with control plasmids, referred to as mock (Supplemental Figure S3B, see *http://ajp.amjpathol.org*).

The effect of IL-1β on wound healing was significantly potentiated by the co-incubation with DAPT because, after 48 hours, the denuded zone was smaller than that observed in the presence of IL-1β alone, whether these experiments were done with (Supplemental Figure S4A, see *http://ajp.amjpathol.org*) or without (Figure 3) mitomycin C pre-incubation. Considering that DAPT did not affect BrdU incorporation in IL-1β-treated cells [even seeded at a lower density (10^4 cells per well); Supplemental Figure S4B, see *http://ajp.amjpathol.org*], one may

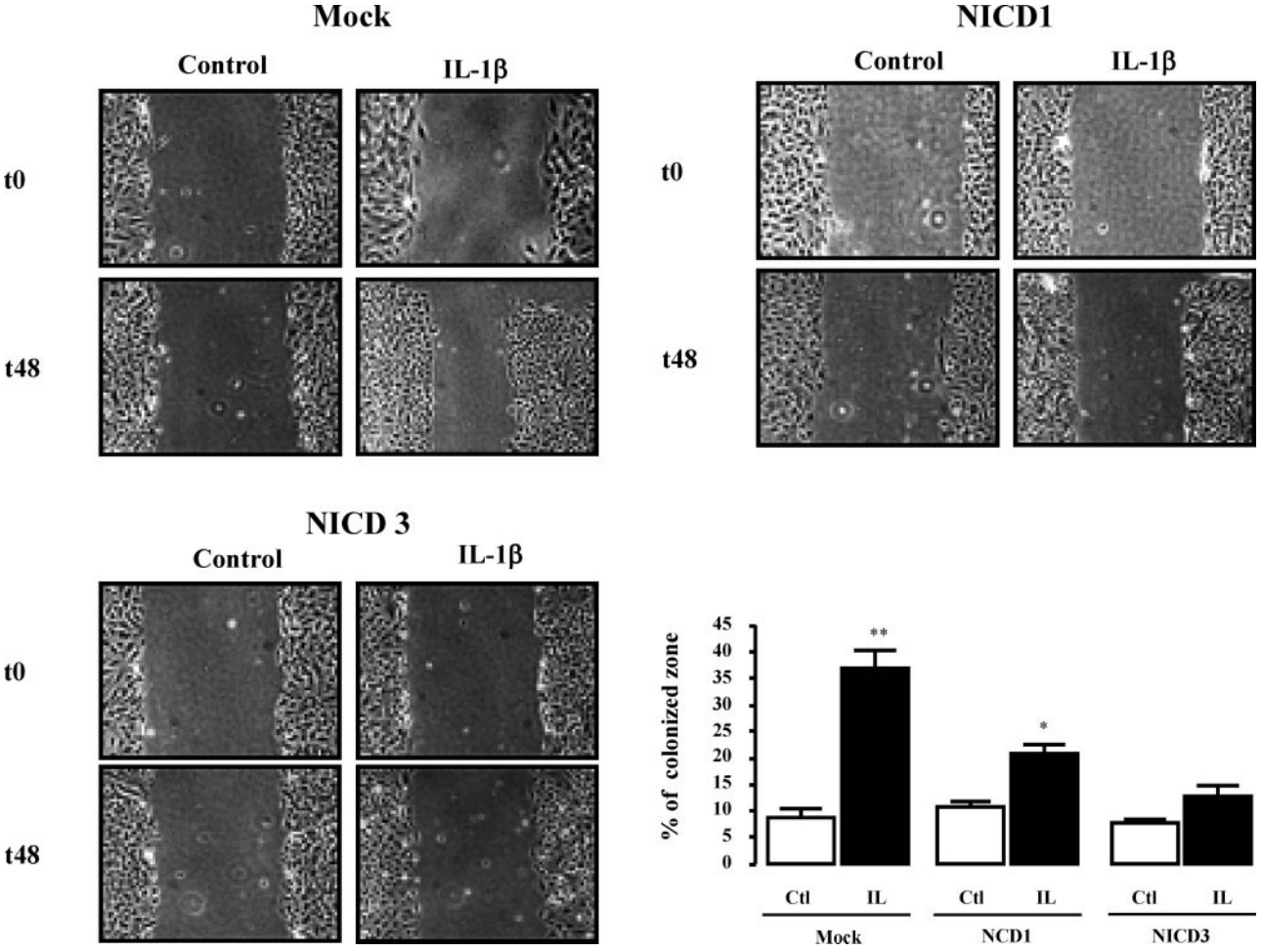

Figure 4. Effect of NICD1 and NICD3 overexpression on VSMC migration/proliferation. VSMC were transfected as described in Materials and Methods with the constructs encoding NICD1, NICD3, or Mock. Twenty-four hours after, cells were starved. After serum starvation, a linear wound was made in the center of culture dish and cells were treated or not with IL-1β (10 ng/ml) during 48 (t48) hours. The denuded area was visualized and captured as described in Figure 1 legend. $^{**}P < 0.01$, vs control.$^{*}P < 0.05$, $^{**}P < 0.01$, vs untreated cells.

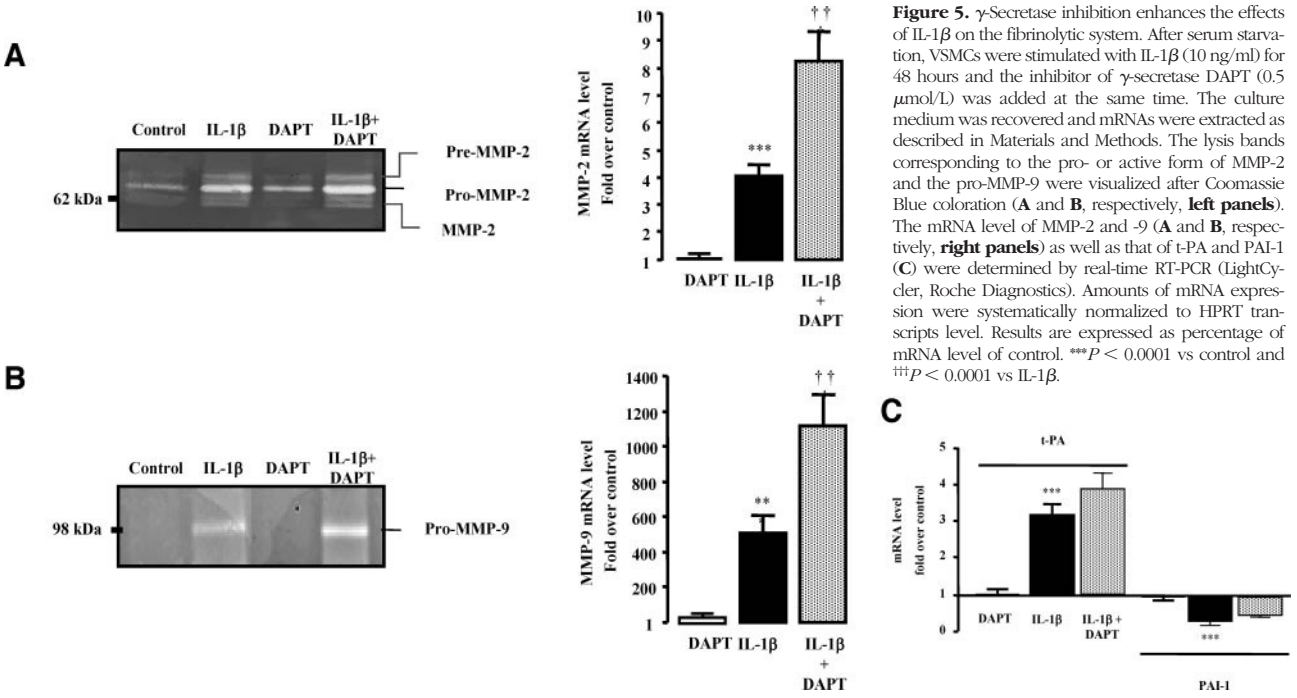

Figure 5. γ-Secretase inhibition enhances the effects of IL-1β on the fibrinolytic system. After serum starvation, VSMCs were stimulated with IL-1β (10 ng/ml) for 48 hours and the inhibitor of γ-secretase DAPT (0.5 μmol/L) was added at the same time. The culture medium was recovered and mRNAs were extracted as described in Materials and Methods. The lysis bands corresponding to the pro- or active form of MMP-2 and the pro-MMP-9 were visualized after Coomassie Blue coloration (**A** and **B**, respectively, **left panels**). The mRNA level of MMP-2 and -9 (**A** and **B**, respectively, **right panels**) as well as that of t-PA and PAI-1 (**C**) were determined by real-time RT-PCR (LightCycler, Roche Diagnostics). Amounts of mRNA expression were systematically normalized to HPRT transcripts level. Results are expressed as percentage of mRNA level of control. $^{***}P < 0.0001$ vs control and $^{\dagger\dagger\dagger}P < 0.0001$ vs IL-1β.

conclude that potentiation of the IL-1β-induced healing (induced by DAPT) was not to be attributed to VSMC proliferation but only to their migration. Consistent with a Notch implication in the negative regulation of this cell response, the overexpression of NICD1 or 3 prevented it (Figure 4). Of note, although the Notch pathway was activated in untreated cells, as shown by DAPT-induced down-regulation of Notch target genes expression (data not shown), DAPT alone was able to trigger migration of VSMCs.

DAPT (0.5 μmol/L) increased the IL-1β-induced activity of MMP-2, as well as that of pro-MMP-9 (Figure 5A and B, left panel, Table 3). Furthermore, the inhibition of the γ-secretase complex translated into an over-expression of the MMP-2/-9 transcripts (Figure 5A and B, right panel). Considering that the percentage of active MMP-2 obtained from co-treated cells was very similar to that issued from IL-1β-treated cells (Table 3), one may conclude that the γ-secretase complex inhibition exclusively modulates MMPs activity, down-regulating their expression at the transcriptional level. Consistent with this, DAPT affected neither the IL-1β-induced expression of t-PA and PAI-1 (Figure 5C), nor the IL-1β-induced down-regulation of tissue inhibitor of metalloproteinase-2 expression (Supplemental Figure S5, see *http://ajp.amjpathol.org*). Here again, DAPT alone had no effect the parameters considered. Of note, because the IL-1β-induced tissue inhibitor of metalloproteinase-1 expression was decreased by DAPT treatment, this effect could also participate in the DAPT induced-potentiation of VSMC migration (Supplemental Figure S5, see *http://ajp.amjpathol.org*). The DAPT potentiation of the IL-1β-mediated transition toward the migratory state demonstrated here occurred through Notch target genes. Indeed, in cells treated with IL-1β, the forced expression of RBP-Jκ DN, preventing Notch target gene transcription[27] (as evidenced by RT-PCR, data not shown), significantly in-

Table 3. Effect of γ-Secretase Inhibition on the Secreted MMP-2 and MMP-9 Activity

	Pro MMP-2 (AU)	Active MMP-2 (AU)	% of active MMP-2
Control	13,891 ± 1349	782 ± 93	5.3 ± 0.3
DAPT	15,718 ± 2674	944 ± 124	6.6 ± 1.7
IL-1β	36,252 ± 6173**	6306 ± 917**	15.2 ± 2.1**
IL-1β + DAPT	60,700 ± 7868††	13,504 ± 1191††	18.4 ± 1.2**
	Pro MMP-9 (AU)	Active MMP-9 (AU)	% of active MMP-9
Control	201 ± 11	ND	
DAPT	203 ± 69	ND	
IL-1β	1234 ± 100**	ND	
IL-1β + DAPT	2247 ± 258†††	ND	

The lysis bands corresponding to the pro- or active form of MMP-2 and the pro-MMP-9 were visualized after Coomassie Blue coloration. Their surfaces were quantified by measuring optic density as described in Materials and Methods and expressed in arbitrary units (AU). The percentage of active MMP-2 was calculated using the formula (OD MMP-2)/(OD MMP-2 + OD pro-MMP-2) × 100. $^{**}P < 0.001$ vs control; $^{\dagger\dagger}P < 0.001$ and $^{\dagger\dagger\dagger}P < 0.0001$ IL-1β + DAPT versus IL-1β. ND, not detectable.

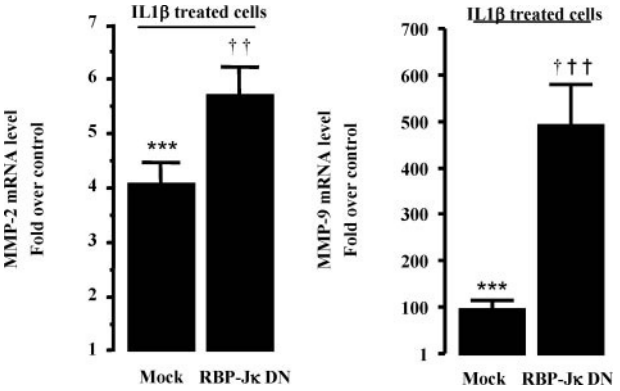

Figure 6. RBP-Jκ DN enhances the effects of IL-1β on MMP-2/-9 expression. VSMCs were transfected as described in Materials and Methods with the constructs encoding RBP-Jk DN or empty plasmid (mock). Twenty-four hours later, cells were starved and treated with IL-1β (10 ng/ml) for 48 hours. mRNA was extracted as described in Materials and Methods. mRNA levels of MMP-2 (**left panel**) and MMP-9 (**right panel**) were determined by real-time RT-PCR (LightCycler, Roche Diagnostics). Amounts of mRNA expression were systematically normalized to HPRT transcripts level. Results are expressed as percentage of mRNA level of control. $^{***}P < 0.0001$ vs untreated mock and $^{\dagger\dagger}P < 0.001$, $^{\dagger\dagger\dagger}P < 0.0001$ vs IL-1β.treated mock.

creased MMP-2 and MMP-9 expression (Figure 6 left and right panels, respectively).

Overexpression of both NICD1 and 3 significantly inhibited the effect of IL-1β on MMP activity (Figure 7A and B, left panels). Similar results were obtained evaluating the mRNA expression of MMP-2 and -9 (Figure 7A and B, respectively, right panels). Of note, the inhibitory effect induced by the over-expression of NICD1 on MMP-2, although significant, was always slightly lower as compared with that of NICD3. None of the overexpression affected the IL-1β-induced expression of t-PA and PAI-1 (data not shown). Taken together, this series of experiments demonstrated that the expression of Notch3 target genes prevents, at least in part, the phenotypic transition of VSMCs occurring in an inflammatory context that leads cells to migrate, inhibiting the fibrinolytic system activity.

DHA Treatment Improves the Activation of Notch Signaling Pathway

The beneficial effects of DHA on the fibrinolytic/MMP system activity could be related in part to its influence on the expression of the Notch signaling pathway components. In this regard, we first evaluated the impact of DHA (50 μmol/L) on the IL1β-induced down-regulation of Notch3 gene expression.[15] DHA could partially prevent the IL-1β effect, since a 48-hour DHA treatment increased the level of Notch3 approximately 3-fold (as compared with that of IL-1β stimulated cells) translating into a significant up-regulation of the transcription of Hes-1 (Figure 8A). Based on the literature,[28] we also put forward an assumption according to which the incorporation of DHA into the plasma membrane could modulate the activity of the γ-secretase complex. This assumption was evaluated in cells over-expressing Notch ΔE by following the production of Notch intracellular fragment in control and DHA-treated cells. As illustrated by a Western blot representative of four experiments (Figure 8B), DHA treatment increased the intensity of the band corresponding to NICD and decreased that of corresponding to the ΔE form. Altogether, this last set of experiments strongly suggests that DHA influences the signaling of Notch pathway that, in turn, would modulate at least one of the aspects of the IL-1β-induced *trans*-differentiation occurring in VSMCs.

Discussion

In this study, we demonstrated a beneficial inhibitory effect of DHA on MMP-2/-9 activity, two enzymes involved

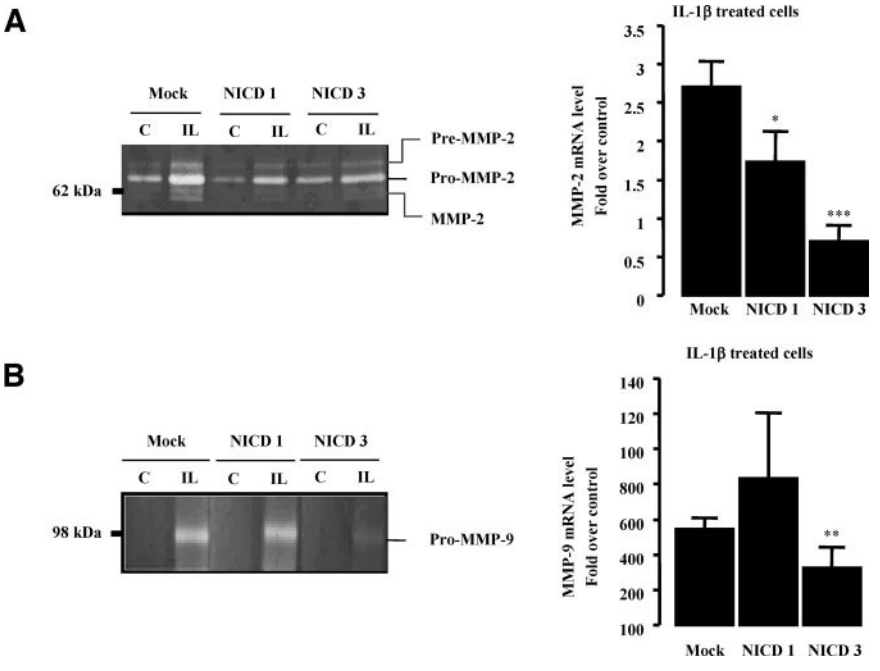

Figure 7. Effect of NICD1 and NICD3 over-expression on MMPs activity and expression. VSMCs were transfected as described in Mthods with the constructs encoding NICD1, NICD3,or Mock. Twenty-four hours later, cells were starved and treated with IL-1β (10 ng/ml) for 48 hours. The culture medium was then recovered and mRNA extracted as described in Materials and Methods. The lysis bands corresponding to the pro- or active form of MMP-2 (**A, left panel**) and the pro-MMP-9 (**B, right panel**) were visualized and their surfaces quantified as described in Materials and Methods and Figure 2 legend. The mRNA level of MMP-2 (**A, right panel**) and MMP-9 (**B, right panel**) were determined by real-time RT-PCR (LightCycler, Roche Diagnostics). Amounts of mRNA expression were systematically normalized to HPRT transcripts level. Results are expressed as percentage of mRNA level of control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Mock IL-1β.

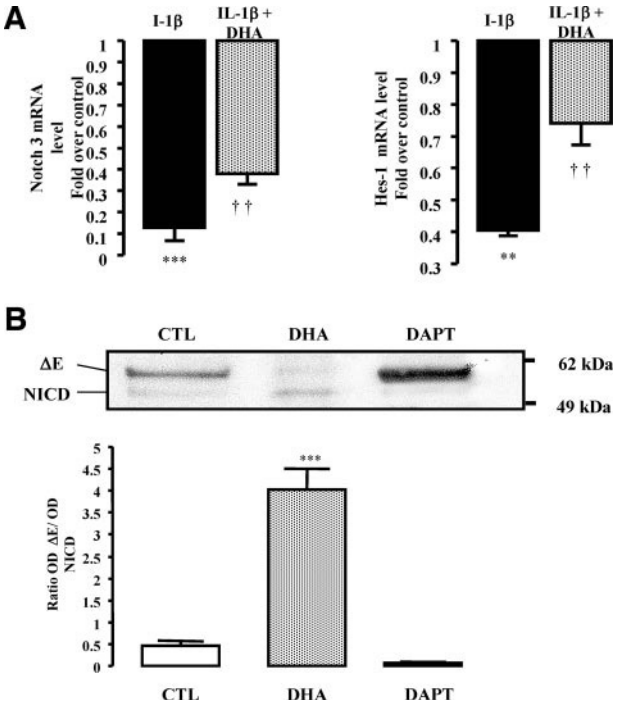

Figure 8. Effect of DHA treatment on Notch signaling pathway. After 48 hours of IL-1β (10 ng/ml) and/or DHA (50 μmol/L) treatments, mRNA or total protein were extracted as described in Materials and Methods. **A**: mRNA levels of Notch3 and Hes-1 were determined by real-time RT-PCR (LightCycler, Roche Diagnostics). Amounts of mRNA expression were systematically normalized to HPRT transcripts level. Results are expressed as percentage of mRNA level of control. **B**: Immuno-detections were performed as described in Materials and Methods. After blocking, membranes were probed with monoclonal mouse anti c-*myc* (1/1000). Apparent molecular mass of protein markers are given on the left side of the blot in kDa. The autoradiogram shown is representative of four independent experiments. The intensity of each band was quantified using Scion software (**lower panel**).

in extracellular matrix degradation known to play a critical role in the proliferation and/or migration of VSMCs occurring in pathological arteries.[29,30]

As evidenced here by different experimental approaches, this beneficial effect of DHA occurs, in part, through its capacity to regulate the expression of these proteins and probably that of some of the fibrinolytic system. According to the literature, this DHA mediated-regulation could result from its property to bind nuclear receptors, such as peroxisome proliferator-activated receptor-α or -γ,[8,31] and prevent the activity of transcription factors, such as nuclear factor-κB, adaptor-related protein-1, and signal transducer and activator of transcription-1, known to directly increase MMP transcription.[32] It could also come from several other indirect mechanisms including the decrease of reactive oxygen species production induced by the inhibition of NADPH oxidase expression.[33,34] Because DHA modulates the expression and/or secretion of two major regulatory components of the fibrinolytic system, t-PA and PAI-1, the DHA inhibition of the IL-1β-induced MMP-2 activity (evidenced by zymography) may also result from its effect on this system.

In a second set of experiments, we confirmed previous data obtained by Sweeney et al,[17] stating that activated Notch1 and 3 negatively regulate the migrative capacity of transdifferentiated VSMC. In addition, we demonstrate that this Notch effect induces a decreased expression of MMP-2/-9 and t-PA. As a corollary, the inhibition of the Notch signaling pathway (by DAPT and RBP-Jk DN overexpression) induces their potentiation. Because we demonstrated, under the same experimental conditions, that activated Notch3 also prevents the IL-1β down-regulation of contractility markers,[15] these results are in line with other studies stating a role of Notch in maintaining the differentiated state of VSMC like that of i) Domenga et al,[35] demonstrating with Notch3 knock-out mice, that VSMC do not complete their differentiation[35]; and, ii) Doi et al,[26] showing that Notch3 transforms fibroblasts into contractile cells and enhances human aortic smooth muscle cells differentiation.[26] They also extend our findings that demonstrate that Notch3 and IL-1β signals exert opposite effects on the transition of vascular smooth muscle cells toward an inflammatory/de-differentiated state.[15] Surprisingly, in our experimental conditions the Notch signaling pathway does not regulate the IL-1β-induced VSMC proliferation, because no effect on BrdU incorporation was observed on DAPT/IL-1β-treated cells. This contradicts the results of Sweeney et al[17] who showed that the overexpression of NICD1 or 3 in serum stimulated rat VSMCs results in a significant up-regulation of growth (evidenced by the increase of cell number and expression of proliferating cell nuclear antigen). Although this can translate the fact that Notch differently regulates IL-1β and serum-induced proliferation, the reasons for this discrepancy remain unknown.

Our work also showed that preventing Notch target gene transcription (by the use of a mutated form of the nuclear protein RBP-Jκ) up-regulates the MMP-2/-9 gene expression initially induced by IL-1β. In this context, the IL-1β-mediated down-regulation of the major components of the Notch pathway expressed in adult VSMCs[15] can also be regarded as one of the mechanisms allowing them to adopt a migrative phenotype. To establish a clear cause-and-effect relationship between these two molecular events, it would be of interest to analyze the expression of the MMP-2/-9 and fibrinolytic system in VSMCs from Notch3 knockout mice[36] after a balloon injury or inducing atherosclerosis. Nevertheless, supporting such a relationship, the IL-1β-induced down-regulation of Notch3 and Hes-1 gene expression occurs through NF-κB activation (because the use of a mutated, non-phosphorylatable form of IkappaB prevents it[15]) and this activation increases the expression of MMP-2/-9 in VSMCs (data not shown). If their expression and other transdifferentiation markers were enhanced in Notch3 knockout mice after a balloon injury or inducing atherosclerosis, it would reinforce the idea that IL-1β down-regulation of Notch3 (the major receptor in differentiated VSMC[37]), is critical for switching VSMC phenotype.

According to our present and previous data, we can suggest that outside of any inflammatory environment, at least some of the Notch target gene products are able to repress the genes encoding MMP-2/-9. Such a repressive effect of Notch pathway on MMP-2/-9 expression would occur as previously suggested for phospholipase A-2 and cyclooxygenase-2.[15] Consistent with this idea, an E- box is present in the promoter of the genes encoding MMP-2[38]; the Notch-Hes1 signal has been shown to

regulate transcriptionally genes interacting with the E response element.[39] In an inflammatory context, the decreased expression of Notch3 receptors and target genes[15] could leave free the E-box of MMPs promoters thus allowing the NF-κB-dependent transcription of these enzymes.

Because the IL1β-induced down-regulation of Notch 3 expression is NF-κB dependent[15] and DHA enhances IκB-α expression,[4] one may conclude that the reduced effect of IL-1β on Notch3 transcripts expression in presence of DHA is a direct consequence of the DHA effect on IκB expression. Since we demonstrate here that IL-1β induced MMP-2/-9 secretion is a Notch 3 regulated process, the benefit of DHA on the migration/proliferation of VSMC is to be partially dependent on the ω3 effect on IL-1β/NF-κB induced down-regulation of Notch 3 and Hes-1 genes expression. Interestingly enough, this study also revealed that the molecular mechanisms by which DHA limits VSMC transdifferentiation toward a migrative phenotype is probably, at least in part, related to its ability to increase Notch signaling (as evidenced by the enhanced release of Notch intracellular domain when plasma membrane fluidity is enhanced by incorporation of DHA, Figure 8). This phenomenon is related to the subcellular localization of the γ-secretase complex in plasma membrane[40] and is consistent with previous studies showing that incorporation of DHA or cholesterol depletion decreases β-secretase activity of neurons derived from the Alzheimer mouse model.[28,41]

In conclusion, here we show for the first time that the IL-1β-induced VSMC migrative/proliferative phenotype observed in vascular pathologies is associated to an increase of MMPs activation. As evidenced in this study, this phenomenon is down-regulated by DHA and part of this DHA beneficial effect, leading to the inhibition of VSMC migration, probably results from its influence on Notch signaling. Because several studies established that Notch regulates many other aspects of VSMC transdifferentiation,[17,42] it would be of interest to determine whether DHA modulates them through its effect on Notch pathway. As an initial approach, we could evaluate the impact of DHA, on mice susceptible to developing atherosclerosis, where the notch pathway is disrupted.

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