Intake of dissolved organic matter from deep seawater inhibits atherosclerosis progression

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Abstract:

Dissolved organic matter (DOM) in seawater can be defined as the fraction of organic matter that passes through a filter of sub micron pore size. In this study, we have examined the effect of DOM of deep seawater (DSW) from Pacific Ocean on platelet aggregation and atherosclerosis progression. DSW was passed through a series of filters and then through an Octadecyl C18 filter; the retained substance in ethanol was designated as C18 extractable DOM (C18-DOM). Our studies showed that C18-DOM treatment inhibited platelet aggregation, P-selectin expression and activity of COX-1 significantly. C18-DOM increased the expression of anti-atherogenic molecule namely heme oxygenase-1 in endothelial cells and all these data showed that C18-DOM is exhibiting aspirin-like effects. Moreover our *in vivo* studies showed that C18-DOM feeding slowed remarkably the progression of atherosclerosis. Our study demonstrated a novel biological effect of oceanic DOM, which has several important implications, including a possible therapeutic strategy for atherosclerosis.

Keywords:

Deep seawater; Dissolved organic matter; Atherosclerosis; Cyclooxygenase-1

Atherosclerosis is a progressive disease of arteries, characterized by accumulation of lipids and fibrous elements and is responsible for up to 50% of deaths in the modern society [1].

In the history of drug discovery, so far terrestrial resources have been targeted which led to the creation of various medicines. Recently, the trend has been switched on to the oceans, which is considered as a potential source for the next generation of drugs. Chemical compounds extracted from marine organisms such as fungi, sponges have been reported to possess anti-cancer, anti-fungal effect, etc [2], [3], [4] and [5].

In this study we have focussed on the dissolved organic matter (DOM) from deep seawater (DSW). DSW was collected from depths beyond 374 meters off (mesopelagic zone) the Cape of Muroto, facing the north-east Pacific Ocean, (Latitude 33.5º N and Longitude 134.10º E) using a DSW-drawing system, where intense up welling of DSW occurs due to the movement of ocean currents. Surface seawater was also collected from depth 5 meters off at the same location. The purpose of this research is to examine the effect and the mechanism of action of DOM in DSW on the progression of atherosclerosis.

Materials and methods

Preparation of dissolved organic matter. We isolated DOM using a solid phase extraction method in which 1500 l of original SW was passed through a series of filters of size 50, 5 and 0.2 μm pore sizes, respectively (Y. S. Filter Japan) and then through an 3M Empore^{TM} Octadecyl (C18) FF disc (90 mm) to retain the organic substance. The filtered DOM was dissolved in 25 ml of ethanol and was designated as C18-DOM. To prepare desalinated DSW, original DSW was passed through 50, 5μm filters, and then desalinated twice by a reverse osmosis membrane module using HR3155PI (Element Configuration: Hollow fiber, single open-ended) in a desalination plant. 1% DSW (v/v) was prepared by diluting original DSW with desalinated DSW to a concentration approximately 1% of that of original concentration. 1% DSW (v/v) was passed through 3M Empore^{TM} Octadecyl (C18) FF disc (90 mm) to remove remnants of organic matter and the filtrate is designated Inorganic deep seawater (Inorg-DSW).

Rabbit balloon injury model. Japanese Male White Rabbits (JMWRs) weighing 3-3.2 kg (Japan SLC, Inc., Shizuoka, Japan) were used in this study. All animal experimental protocols were followed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23. revised 1996) and the guidelines of our university. Anesthesia was achieved

by inhalation of 1.39% isofluorane with ketamine hydrochloride (20 mg/kg) and Pentobarbital Sodium (50 mg/kg) as pre-anesthetic medication. Balloon injury was performed in RCCA as described [13]. Following which the rabbits were divided into 5 groups. Group a, b, c, d were fed with 50 ml of either distilled water, surface seawater, Inorg-DSW, 1% DSW (v/v) per day respectively. The C18-DOM group (e) were fed 200 µl of C18-DOM dissolved in 50 ml of distilled water once a week. All the groups were fed a 1% high cholesterol diet for 4 weeks. After 4 weeks the animals were sacrificed, right common carotid artery (RCCA) was harvested, and Van Gieson elastic staining was performed in paraffin sections to evaluate the neointimal thickness.

Platelet aggregation. JMWRs were divided into 3 groups. In Group a, rabbits were fed with 200 µl of C18-DOM dissolved in 50 ml of distilled water/week. In Group b & c were fed with 50 ml/day of 1% DSW (v/v) & 50 ml/day of Inorg DSW respectively. Platelets were stimulated by 10 or 40 µM of ADP and 0.2 µg/ml of collagen. Platelet aggregations were measured as a change in light transmittance at the start of the experiment, 1-week, 2 weeks after feeding [14].

Cholesterol measurement. JMWRs were divided into 3 groups. In Control gp. rabbits

were fed with 50 ml/day of distilled water. In 1% DSW (v/v) gp., rabbits were fed with 50 ml/day of 1% DSW (v/v). In C18-DOM gp., rabbits were fed with 200 µl of C18-DOM dissolved in 50 ml of distilled water/week. Rabbits were fed with 1% high cholesterol diet for 3 months. Serum samples were collected at the start and end of the experiment.

Flow cytometry. To quantify the P-selectin (platelet adhesion molecule) expression, platelets were isolated from human blood by centrifugation method as described in literature [15]. Ethanol in C18-DOM was evaporated in an automatic environmental speed vac system (Savant, Instruments, Inc. Farmingdale, NY) then reconstituted in an equal volume of distilled water containing 5% of ethanol. $2x10^5$ platelets in 200 µL of buffer were pre-incubated either with 1 mM of aspirin or 50, 500, 5000 times dilution of C18-DOM for 15 mins. Then followed by stimulation with 20 µM of ADP for 10 mins at 37°C. The platelets were suspended in FAC staining buffer (1x PBS containing 0.05% sodium azide and 3% BSA) and then reacted with FITC-labelled mouse anti-human CD62P at recommended dilution (BD Pharmingen) for 30 mins in dark. The platelets were then washed, and analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, CA.). Non-specific fluorescence was assessed by substitution of the mouse IgG1 k

Isotype control.

Cyclooxygenase-1 assay. Inhibitory activity of C18-DOM on COX-1 was assayed by the use of colorimetric COX (ovine) Inhibitor Screening Assay Kit (Cayman, No. 560131) according to the protocol recommended by the supplier [16]. The assay was performed in triplicate and repeated at three times. 100 μM of aspirin was used as a reference. Ethanol in 100 μl of C18-DOM was evaporated in an automatic environmental speed vac system (Savant, Instruments, Inc. Farmingdale, NY) then reconstituted in an equal volume of distilled water. C18-DOM was added to the reaction mixture with various dilutions of 50, 500, 5000 times that of original volume of C18-DOM.

Western blotting. Ethanol in C18-DOM was evaporated in automatic environmental speed vac system (Savant, Instruments, Inc. Farmingdale, NY) then reconstituted in equal volume of cell culture medium. Human umbilical vein endothelial cells (HUVECs) obtained from ATCC, Manassas (VA, USA) (3×10^6) were incubated with 5 or 10 μl of C18-DOM and with 0.3mM of aspirin as control for 12 h [17]. Expression of HO-1 was measured by western blotting with anti-HO-1 mouse monoclonal Ab (1:2000,

Stressgen Biotechnology).

NMR spectroscopy. C18-DOM was subjected to ^{1}H, ^{13}C-NMR spectroscopy (Bruker Biospin Avance 400, Ehime Techno Research Center). The ethanol in C18-DOM was evaporated to dryness and reconstituted in MeOD, at 25ºC. The reference for ^{1}H-NMR was tetra methyl silane (4.699 ppm, 400.13MHz), and for ^{13}C-NMR tetra methyl silane (100.1MHz) was used.

ESI/ TOF/ Mass spectrometry (MS). C18-DOM was subjected to ESI-TOF-MS (Applied Biosystem Q-STAR XL). The eluant used is H2O/methanol (1/1) + 0.1% formic acid (positive), H2O / methanol (1/1) + 5 mM acetic acid ammonium (negative). The references used were reserpine, dimethylacetamide (positive) and taurocholic acid (negative).

Preparation of the biosensor chip. Cyclooxygenase-1 (Cayman Chemical, MI, USA) was immobilized (yielding approximately 9000 relative response [RU]) on the surface of a CM5 sensor chip by using an amine coupling kit, following the manufacturer's instructions [18]. The sensor chip surface was washed repeatedly with 50 mM NaOH

for 1 min at a flow rate of 60 μl/min.

BIACORE for C18-DOM. Mixing of all the reagents, injection, and washing were performed manually by the Biacore J. The running buffer was HBS-N buffer (10mM HEPES/0.15M NaCl, pH7.4) at a flow rate of 20 μl/min. Ethanol in C18-DOM was evaporated, then reconstituted in equal volume of HBS-N buffer and filtered through 0.2 μm Millex filter. The mixture was injected for 3 min over the immobilized surface and the reference (non-immobilized) surface. The surface was regenerated with 25 mM NaOH for 1 min at a flow rate of 60 μl/min. The total run time between the samples was about 2 min. RUs were recorded on each sensor gram 15 s after the injection. All RU values were corrected by subtraction of the RUs from the reference surfaces.

Statistical analysis. Data were expressed as mean ± S.E.M. Differences between means were examined by paired Students t-test and $p < 0.05$ were regarded as being statistically significant.

Results

C18-DOM inhibits the progression of neointimal hyperplasia

In balloon-injured RCCAs, the ratio of neointima versus media is significantly reduced in C18-DOM treated group (0.08 ± 0.05 versus 1.86 ± 0.50, C18-DOM fed versus controls, $p < 0.05$) and also in 1% DSW (v/v) group (0.32 ± 0.14 versus 1.86 ± 0.50, 1% DSW fed versus controls, $p < 0.05$) (Fig.1A and B). In contrast there is no significant reduction in the ratio of neointima versus media in Inorg-DSW fed group, which suggests that the anti-atherosclerotic effect is derived specifically from the organic components of seawater. Moreover our results that the surface water does not have anti-atherosclerotic effect, implies that only DOM existing below the surface ocean may have the effects.

C18-DOM inhibits platelet aggregation

In C18-DOM fed group showed significant decrease in platelet aggregation value in 2 weeks compared to pre value when stimulated by 40 μM of ADP (32.7 ± 5.0 versus 46.4 ± 3.3, 2 week value versus pre value, $p < 0.05$). Moreover, C18-DOM fed group showed significant decrease in platelet aggregation value in 2 weeks compared to pre value when stimulated by 0.2μg / ml of collagen (45.2 ± 3.3 versus 69.2 ± 3.0, 2 week value versus pre value, $p < 0.01$) (Fig. 2a). 1% DSW (v/v) fed group showed significant decrease in platelet aggregation value (% of change in light transmittance) in 2 weeks

compared to pre value when stimulated by 10 μM of ADP (27.0 ± 4.3 versus 38.5 ± 3.0, 2 week value versus pre value, $p < 0.05$) (Fig. 2b). In contrast, Inorg-DSW has no effect on the platelet aggregation (Fig. 2c).

Cholesterol profile in rabbits

Although C18-DOM inhibited the development of neointimal thickness, platelet aggregation, total cholesterol levels were not reduced. After 4 weeks, the total cholesterol level increased from 32.67±14.92 mg/dl to 424.74 ± 36.24 mg/dl in the control group and from 50.76 ± 9.16 mg/dl to 445.40 ± 87.28 mg/dl in the C18-DOM group. The differences between the 2 groups at both the starting ($p = 0.17$ vs. control) and end point ($p = 0.41$ vs. control) were not significantly different (Table.1)

Aspirin like effects of C18-DOM

In addition to our *in vivo* studies, we analysed the effect of C18-DOM on the expression of platelet adhesion molecule named P-selectin upon stimulation by 20 μM of ADP. C18-DOM markedly attenuated the P-selectin expression (Fig.3A). To investigate the effect of C18-DOM, we performed COX inhibitor screening assay with aspirin as reference and measured the production of $PGF_{2\alpha}$. C18-DOM inhibited the activity of

COX-1 significantly when compared to vehicle group in a dose dependent manner (Fig. 3B). Our western blot analyses showed that C18-DOM induces the expression of HO-1 in human umbilical vein endothelial cells (HUVECs) in a dose dependent manner with a feature similar to that of aspirin (Fig. 3C).

Further, we conducted chemical characterization of C18-DOM using NMR technique. The results obtained were consistent with Hertkorn, et al (Supplemental Data 1) [19]. Moreover our ESI/ TOF/ Mass Spectrometry (MS) analysis showed that C18-DOM is composed of molecules with molecular weight ranging from 220 to 1000 amu with peak occurring around 400 amu (data not shown).

Binding pattern of C18-DOM to immobilized COX-1 on sensor chip

COX-1 was immobilized onto the surface of a CM5 sensor chip. Significant difference in response unit (RU = 9000) was observed in the sensor gram prior to immobilization and post deactivation, reflecting a successful immobilization of COX-1 onto the sensor surface. The Resonance Unit (RU) indicated the bound mass on sensor surface with time. The functionalised CM5 chip was evaluated for its ability to detect the binding capacity of molecules in C18-DOM with COX-1. C18-DOM binds to the COX-1

yielding a maximum strength at flow rates of 100 µl/min with an effect similar to aspirin (Fig.4).

Discussion

Deep seawater (DSW) designates the water, which flows below 1000 meters from the surface of the sea [20]. Our previous studies in healthy human volunteers showed that desalted DSW intake inhibited the platelet aggregation significantly with $p < 0.01$ (data not shown). However, the knowledge about the biologically active substances, which is responsible for the above-mentioned effect, is still unclear.

Dissolved organic matter (DOM) is the largest reservoir of reduced carbon in the oceans. The amount of carbon in oceanic DOM (~700 x 10^{15} g) is similar to that of atmospheric carbon, which has raised interest in the DOM pool in global carbon cycle research [21]. The production of DOM includes extra cellular release by phytoplankton, grazer mediated release, excretion release via cell lyses (both viral and bacterial) & solubilization of particles and bacterial transformation and release [22], [23], [24], [25] and [26]. In contrast with surface DOM, much of the DOM in the deep ocean is quite resistant to microbial degradation, photo oxidation and has an average radiocarbon age

of several millennia [27] and [28]. Efforts to improve methods for isolation and characterization continued as a major research area in the 1980s, with an emphasis on NMR methods [29]. DOM has various functions and plays important roles in chemical, biological and even physical oceanography [30].

Recently, activated platelets have not only been implicated in thrombosis but also in inflammatory reactions, immune responses, and in distinct aspects of atherosclrerosis. Importantly, an intermittent injection of activated platelets has been shown to exacerbate the formation of native atherosclerotic lesions, a process involving platelet surface receptors that facilitate mononuclear cell recruitment. By similar mechanisms, platelet adhesion to subendothelial smooth muscle cells has been shown to increase MCP-1 secretion by SMCs, as well as their migratory properties, which appears to be particularly relevant in the context of arterial injury and restenosis. Activated platelets exacerbate atherosclerosis in apo$E^{-/-}$mice in a P-selectin dependent manner [6], [7], [8], [9], [10], [11] &[12].

Aspirin is a cyclooxygenase-1 (COX-1) inhibitor, thus inhibiting the prostaglandin release, which is a mediator of inflammation, platelet aggregation, etc. Previous studies showed that high dose of aspirin (12mg/kg/day) significantly inhibited the

neointima/media ratio in balloon injured iliac artery compared to control group, although it has no effect on cholesterol level of rabbits. But the most important side effect of high dose is aspirin resistance in the treatment of atherosclerosis. These side effects limit administration of aspirin in the treatment of atherosclerosis [31].

It has been reported that heme oxygenase-1 (HO-1) exerts anti-atherosclerotic effects in different animal models. Hence HO-1 is considered to be a novel therapeutic approach to treat atherosclerosis [32], [33] and [34]. Previous reports showed that aspirin induces the expression of HO-1in endothelial cells by NO dependent pathway [17].

This is the first study showing the anti-atherosclerotic effects of DOM from DSW. DOM attenuated the neointimal hyperplasia in balloon injured RCCAs, platelet aggregation, P-selectin expression and COX-1 activity. Considering the results of NMR analysis, ESI/TOF/MS analysis and that of the animal and other in vitro experiments, we could hypothesize that some compound of marine origin possessing active functional groups similar to aspirin might be present in C18-DOM, moreover

C18-DOM consists of many different molecules further analysis should be done to differentiate the antiatherosclerotic activity from that of aspirin.

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References

[1] A.J. Lusis. Atherosclerosis. *Nature* **407** (2000) 233-241.

[2] T.F. Molinski and P.A. Searle. Phorboxazoles A and B: Potent cytostatic macrolides from marine sponge phorbas sp, *J. Am. Chem. Soc.* **117** (1995) 8126-8131.

[3] D.R. Williams, A.A. Kiryanov, U. Emde, M.P. Clark, M.A. Berliner and J.T. Reeves. Studies of stereocontrolled allylation reactions for the total synthesis of phorboxazole A, *Proceedings of the National Academy of Sciences* **101** (2004) 12058-12063.

[4] J. Antonio and T.F. Molinski. Screening of marine invertebrates for the presence of ergosterol-sensitive antifungal compounds. *J. Nat. Prod.* **56** (1993) 54-61.

[5] J. Gafni, J.A. Munsch, T.H. Lam, M.C. Catlin, L.G. Costa, T.F. Molinski and I.N. Pessah. Xestospongins: Potent membrane permeable blockers of the inositol 1, 4, 5-triphosphate receptor. *Neuron* **19** (1997) 723-733.

[6] W. Christian. Platelets and chemokines in atherosclerosis: Partners in crime. *Circ. Res.* **96** (2005) 612-616.

[7] S. Willoughby, A. Holmes, J. Loscalzo. Platelets and cardiovascular disease. Eur. J Cardiovasc Nur. **1** (2002) 273-288.

[8] Z.M. Dong, S.M. Chapman, A.A. Brown, P.S. Frenette, R.O. Hynes and D.D. Wagner. The combined role of P- and E- selectins in atherosclerosis. J. Clin. Invest. **102** (1998) 145-152.

[9] G. Davi, C. Patrono. Platelet activation and atherothrombosis. *N. Engl. J. Med.* **357** (2007) 2482-2494.

[10] O. Belton, Fitzgerald DJ. Cyclooxygenase isoforms and atherosclerosis. *Expert Reviews in molecular Medicine* **5** (2003) 1-18.

[11] G. Gaetano, M.B. Donati, C. Cerletti. Prevention of thrombosis and vascular inflammation: benefits and limitations of selective or combined COX-1, COX-2 and 5-LOX inhibitors. *Trends in Pharmacological Sciences* **24** (2003) 245-252.

[12] F. Catella Lawson. Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. *N. Engl. J. Med.* **346** (2002) 1589-1590.

[13] Q. Xu. Mouse models of arteriosclerosis. *Am. J. Pathol.* **165** (2004) 1-10.

[14] F.J. Rosenberg, P.E. Gimber-Philips, G.E. Groblewski, C. Davison, D.K. Philips, S.J. Goralnick, Cahill ED. Acetyl salicylic acid: Inhibition of platelet aggregation in the rabbit. *J. Pharmacol. Exper. Thera.* **179** (1971) 410-418.

[15] K. Daub, H. Langer, Seizer P, Stellos K, May A, Goyal P, Bigalke B, Schonberger T, Geisor T, Seigel-Axel D, Oostendorp RJ, Linderman S, Gawaz M. Platelets induce differentiation of human CD34+ progenitor cells into foam cells and endothelial cells. *The FASEB Journal* **20** (2006) E1935-1944.

[16] T. Noguchi, R. Shimazawa, K. Nagasawa, Y. Hashimoto. Thalidomide and its analogues as cyclooxygenase inhibitors. *Bioorganic & Medicinal Chemistry Letters* **12** (2002) 1043-1046.

[17] N. Grosser, A. Abate, Oberle S, Vreman HJ, Dennery PA, Becker JC, Pohle T, Seidman DS, Schroder H. Heme oxygenase-1 induction may explain the antioxidant profile of aspirin. *Biochem Biophysic Res Commun.* 308 (2003) 956-960.

[18] T. Tsutsumi, N. Miyoshi, Sasaki K, Maitani T. Biosensor immunoassay for the screening of dioxin-like polychlorinated biphenyls in retail fish. *Analytica. Chimica. Acta.* **617** (2008) 177-183.

[19] N. Hertkorn, R. Benner, M. Frommberger, P. Schmitt-Kopplin, M. Witt, Kaiser K, Kettrup A, Hedges JI. Characterization of a major refractory component of marine dissolved organic matter. *Geochim. Cosmochim. Acta.* **70 (**2006) 2990-3010.

[20] W.S. Broecker. The great ocean conveyor. *Oceanography* **4 (**1991) 79-89.

[21] R. Benner, J. D Pakulski, M. McCarthy, Hedges JI, Hatcher. PG. Bulk chemical characteristics of dissolved organic matter in the ocean. *Science* **255(**1992) 1561-1564.

[22] M. McCarthy, J.I. Hedges, R. Benner. Major bacterial contribution to marine dissolved organic nitrogen. *Science* **281** (1998) 231-234.

[23] E. Tanoue, S. Nishiyama, M. Kamo, A. Tsugita. Bacterial membranes: Possible source of a major dissolved protein in seawater. *Geochim. Cosmochim. Acta.* **59** (1995) 2643-2648.

[24] C.A. Carlson. Biogeochemistry of Marine Dissolved Organic Matter (ed, Hansell, D. A & Carlson, C. A.) 91-151. (Academic press, London, UK, 2002)

[25] M.A. Wilson, A.H. Gilliam, P.J. Collins. Analysis of the structure of dissolved marine humic substances and their phytoplanktonic precursors by ^{1}H and ^{13}C nuclear magnetic resonance. *Chem. Geol*. **40** (1983) 187-201.

[26] W.C. Chin, M.V. Orellana, P. Verdugo. Spontaneous assembly of marine dissolved organic matter into polymer gels. *Nature* **391**(1998) 568-570.

[27] H. Ogawa, Y. Amagai, I. Koike, K. Kaiser, R. Benner. Production of refractory dissolved organic matter by bacteria. *Science* **292** (2001) 917-920.

[28] R.T. Barber. Dissolved organic carbon from deep waters resists microbial oxidation. *Nature* **220** (1968) 274-275.

[29] D.F. Gruber, J-P Simjouw, S.P. Seitzinger, G.L. Taghon. Dynamics and characterization of refractory dissolved organic matter produced by a pure

bacterial culture in an experimental predator-prey system. *Appl. Environ. Microbiol.* **72** (2006) 4184-4191.

[30] H. Ogawa, E. Tanoue. Dissolved organic matter in oceanic waters. *J. Oceanogr.* **59** (2003) 129-147.

[31] M. Li, Y Zhang, H Ren, Y Zhang, X Zhu. Effect of clopidogrel on the inflammatory progression of early atherosclerosis in rabbit model. *Atherosclerosis* **194** (2007) 348-356.

[32] T. Morita. Heme oxygenase and atherosclerosis. *Arterioscler. Thromb. Vas. Biol.* 25 (2005) 1786-1795.

[33] K. Ishikawa, D. Sugawara, J. Goto, Y. Watanabe, K. Kawamura, M. Shiomi, H. Itabe, Y Maruyama. Heme oxygenase-1 inhibits atherogenesis in watanabe heritable hyperlipidemic rabbits. *Circulation* **104** (2001) 1831-1836.

[34] R.C. Siow M, H. Sato, G.E. Mann. Heme oxygenase-Carbon monoxide signaling pathway in atherosclerosis: anti-atherogenic actions of bilirubin and carbon monoxide? *Cardiovas. Res.* **41** (1999) 385-394.

Figure Legends

Fig. 1. C18-DOM attenuates the neointimal hyperplasia in balloon injured common carotid arteries of JMWRs. (A) depicts the photomicrographs of Von Gieson elastic lamina staining of balloon injured RCCAs in distilled water (a), surface SW (b), Inorg DSW (c), 1% DSW (v/v) (d) and C18-DOM (e) fed groups'. Scale bar, 200 μm (n = 6). (B) the graph represents the ratio of neointima vs. media in various groups. Data are presented as an average for each group. Error bars indicate standard error of the mean ± SEM.

Fig. 2. (a) Shows that C18-DOM significantly inhibited collagen-induced (0.2 μg/ml) PA with $^{**}p < 0.01$. (b) 1% DSW (v/v) significantly inhibited the ADP-induced (20 μM) platelet aggregation with $^{*}p < 0.05$. (c) In contrast, Inorg-DSW had no effect on the platelet aggregation of rabbit. Data are presented as an average of percentage of change in light transmittance (n = 6) for each group. Error bars indicate standard error of the mean ± SEM. Black shaded, striped, and dotted bars represent the pre, 1, 2 weeks platelet aggregation values respectively.

Fig. 3. **(**A) C18-DOM inhibited the expression of P-selectin significantly with $^{**}p <$ 0.01 when compared to vehicle group. Data are presented as mean ± SEM (error bars) (n = 3) of the percentage of mean fluorescence intensity compared with ADP stimulated

platelets. (a) Vehicle group; (b) aspirin group (1mM); (c), (d) & (e), C18-DOM group treated platelets at 50, 500, 5000 times dilutions. Data are expressed as percentages, arbitrary values of 100% being attributed to the vehicle group.

(B) C18-DOM attenuated the enzyme activity of COX-1 with $^{**}p < 0.05$ when compared to vehicle group (unpaired t-test). Data are presented as mean ± SEM (error bars) ($n = 3$) of enzyme activity for each individual experiments. (a) Positive control (b) vehicle (5% ethanol), (c) 100 μM of aspirin, (d, e, & f) 50, 500, 5000 times dilution of C18-DOM. PG: prostaglandin.

(C) Western blotting analysis shows the HO-1 expression in HUVECs. Panels a & b show the expression of HO-1 by 5 or 10 μl of C18-DOM. Panel c shows the expression of HO-1 by 0.3 mM of aspirin.

Fig. 4. Evaluation of C18-DOM on immobilized COX-1.

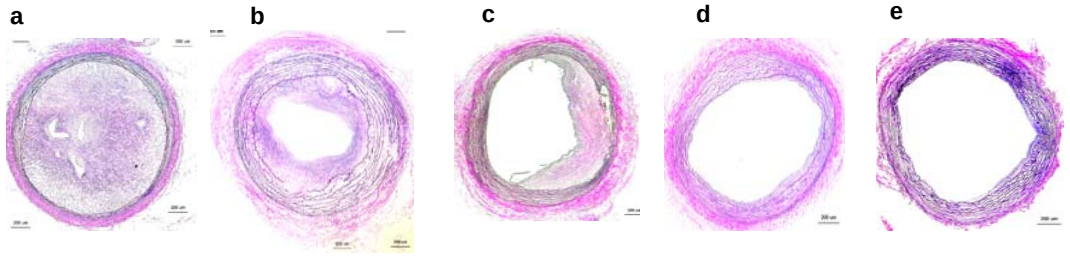
(A) Shows coupling of COX-1 enzyme on CM5 sensor chip. (B) Evaluation of binding of aspirin (5 mM) on immobilized COX-1. (C) Evaluation of binding of C18-DOM on immobilized COX-1 on the CM5 sensor chip. C18-DOM binding with COX-1 was observed an effect similar to 5mM of aspirin.

Table 1

Table shows the total and LDL cholesterol levels in 3 groups both at the start of the experiment and after 4 weeks. After 4 weeks, the total cholesterol level increased from 32.67±14.92 mg/dl to 424.74 ± 36.24 mg/dl in the control group and from 50.76 ± 9.16 mg/dl to 445.40 ± 87.28 mg/dl in the C18-DOM group. The differences between the 2 groups at both the starting (p = 0.17 vs. control) and end point (p = 0.41 vs. control) were not significantly different.

	T-CHO (mg/dl)		LDL-CHO (mg/dl)	
Groups (Gp)	Pre	Post	Pre	Post
Control Gp	32.6 ± 14.9	424.7 ± 36.2	15.1 ± 8.6	93.8 ± 3.6
1% DSW Gp	44.0 ± 13.3	945.6 ± 107.5	22.9 ± 7.9	187.0 ± 23.0
C18-DOM Gp	50.7 ± 9.1	445.4 ± 87.2	28.8 ± 6.9	76.2 ± 14.7

A



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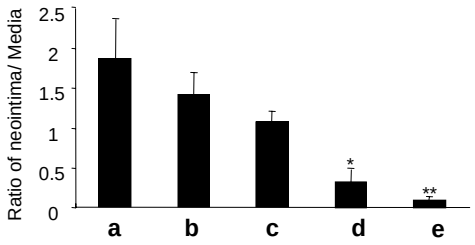

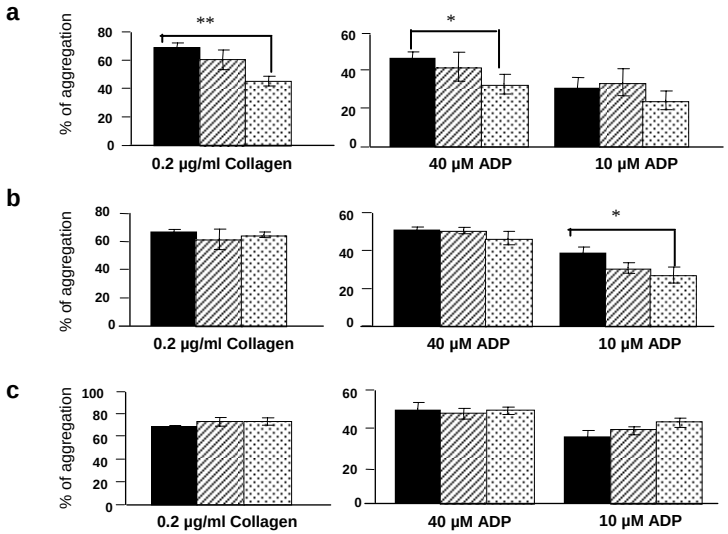
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0.2 µg/ml Collagen
40 µM ADP
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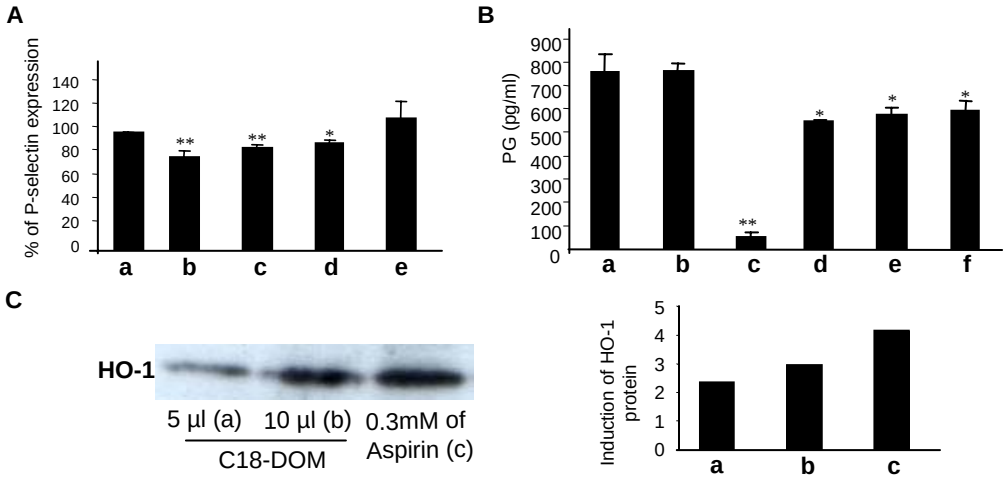
A
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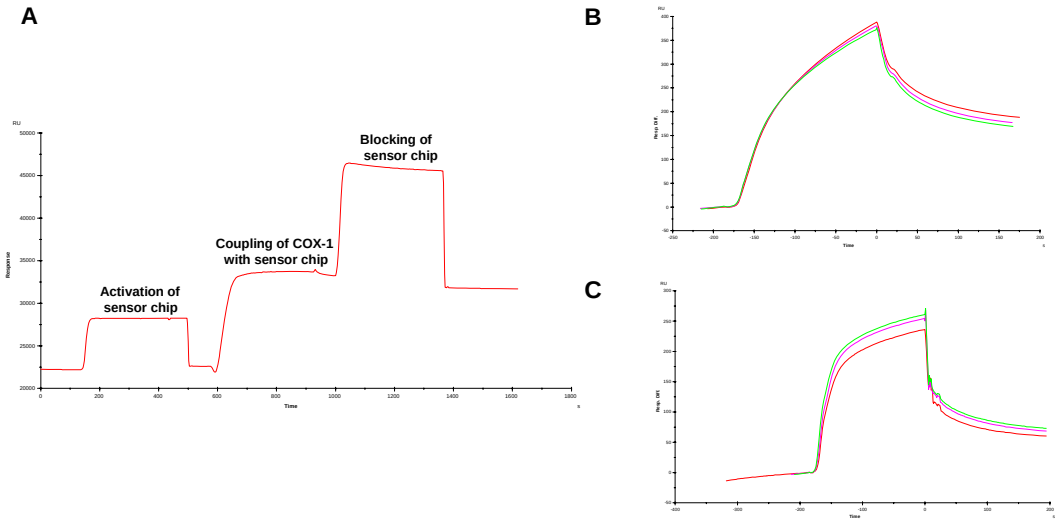
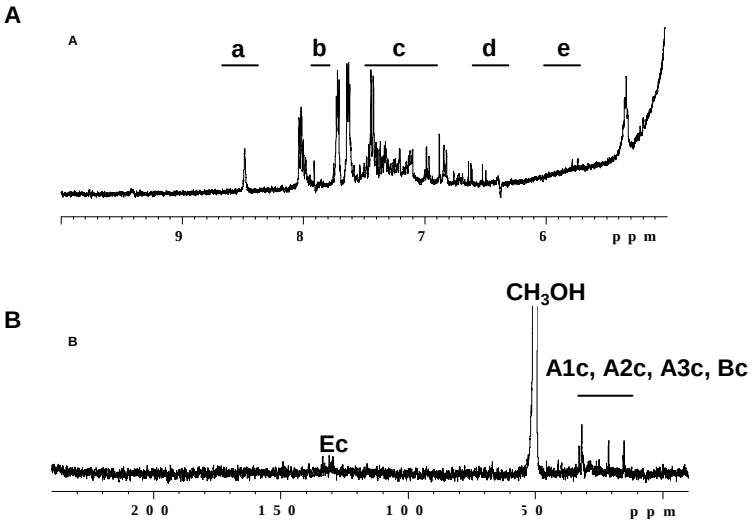
B
PG (pg/ml)
900
800
700
600
500
400
300
200
100
0
**
*
*
*
a
b
c
d
e
f
C
HO-1
5 µl (a)
10 µl (b)
C18-DOM
0.3mM of Aspirin (c)
Induction of HO-1 protein
5
4
3
2
1
0
a
b
c


A
B
C
RU
Response
Time
s
Activation of
sensor chip
Coupling of COX-1
with sensor chip
Blocking of
sensor chip


A
A
a
b
c
d
e
9
8
7
6
p p m
B
B
CH3OH
A1c, A2c, A3c, Bc
Ec
200
150
100
50
p p m