Original Article

A lipid peroxidation product 9-oxononanoic acid induces phospholipase A_2 activity and thromboxane A_2 production in human blood

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Lipid peroxidation products are known to cause toxicity by reacting with biologically significant proteins, but the inducing role of peroxidation products has been not noted to produce degenerative disease-related eicosanoids. Here, 9-oxononanoic acid (9-ONA), one of the major products of peroxidized fatty acids, was found to stimulate the activity of phospholipase A_2 (PLA_2), the key enzyme to initiate arachidonate cascade and eicosanoid production. An exposure of fresh human blood to the atmosphere at 37°C accumulated 9-ONA, increasing peroxide value and thiobarbituric acid reactive substances in the blood. The lipid peroxidation was accompanied by significant increases of PLA_2 activity and thromboxane B_2 (TxB_2) production, which is a stable metabolite of thromboxane A_2 (TxA_2) and a potent agonist of platelet aggregation. These events were abolished by standing the blood under nitrogen. The addition of organically synthesized 9-ONA resumed the activity of PLA_2 and the production of TxB_2. Also, 9-ONA induced platelet aggregation dose-dependently. These results indicated that 9-ONA is the primary inducer of PLA_2 activity and TxA_2 production, and is probably followed by the development of diseases such as thrombus formation. This is the first report to find that a lipid peroxidation product, 9-ONA, stimulates the activity of PLA_2.

***Key Words*:** **9-oxononanoic acid, lipid peroxidation, phospholipase A_2, thromboxane A_2, arachidonate cascade**

Lipids with polyunsaturated fatty acids (PUFAs) are primary targets for attack by reactive oxygen species (ROS).[1] The oxidation of endogenous lipids is closely associated with various diseases, such as atherosclerosis and cancer, and neurodegenerative disorders including Alzheimer's disease and Parkinson's disease. For example, in atherosclerosis, a number of lipid peroxidation products have been detected in atheroma, and oxidation of low-density lipoprotein (LDL) accompanies the disease process.[2–4] Many early studies have demonstrated that peroxidation products cause various degenerative diseases directly, while several studies have suggested that they play an inducing role in degenerative disease-related chemical mediators.[5–9] The former toxicity of peroxidation products has been almost completely clarified, but various questions remain about the latter inducing role of the products.[10–12] In the present study, we were interested in the inducing role of lipid peroxidation products.

In signal transduction, phospholipids in cellular membranes play an important role in the control of cellular activities.[13] Hydrolysis of membranous phospholipids by phospholipase A_2 (PLA_2) releases free fatty acid, including arachidonic acid from the *sn*-2 position of phospholipids, which is the start of the arachidonate cascade and produces a precursor of essential cell-signaling eicosanoids. Arachidonic acid is converted by cytosolic prostaglandin G/H syntheses and tissue-specific isomerases through unstable intermediate prostaglandin H_2 to multiple eicosanoids such as thromboxane A_2 (TxA_2) and prostacyclin (PGI_2).[14] TxA_2 is a potent platelet agonist and vasoconstrictor and activates resting platelets, leading to the formation of a thrombus, while PGI_2 is a potent vasodilator and inhibitor of platelet aggregation.[15] Numerous studies have shown the important roles of TxA_2 and PGI_2 in preserving the dynamic balance among thrombosis, hemostasis and fluidity of blood. TxA_2 is high in patients with atherosclerotic disease and pulmonary hypertension, and the production of PGI_2 is depressed in these patients.[16,17] Evidence that elevated PLA_2 activity is associated with cardiovascular events has also been found.[18,19] Thus, the release of arachidonic acid by PLA_2 from membranous phospholipids is a rate-limiting step in eicosanoid synthesis; however, the inducing factors to activate the key enzyme PLA_2 are unknown.[20] The aim of the present study was therefore to identify a candidate of the inducer chemicals to activate PLA_2.

Linoleic acid, a PUFA, has been reported to comprise around 25% of the fatty acids of membranous phospholipids in human plasma.[21] The *sn*-2 position in membranous phopholipid is usually occupied by PUFA.[22] Thus, linoleic acid is the most abundant PUFA in the *sn*-2 position of membranous phospholipids in human plasma, and the major target of ROS is linoleic acid. ROS attack forms 9 or 13-hydroperoxide and is followed by the production of various decomposed aldehydes. As shown in Fig. 1, 9-oxononanoic acid (9-ONA) is a major aldehyde.[23–26] Previous studies showed that 9-ONA dosed orally significantly elevated the level of lipid peroxides, accompanied by a marked reduction of lipogenesis in rat liver.[8,27] Furthermore, the stimulation of lipid peroxidation by 9-ONA was inhibited significantly by 71% with the addition of a cyclooxygenase inhibitor, indomethacin or mepacrine, in rat hepatic microsomes.[9] This information indicated the possibility that 9-ONA is an inducer of the arachidonate cascade through the activation of PLA_2. In the present study, we investigated the inducing role of 9-ONA in PLA_2 activity followed by eicosanoid production and platelet aggregation in human blood.

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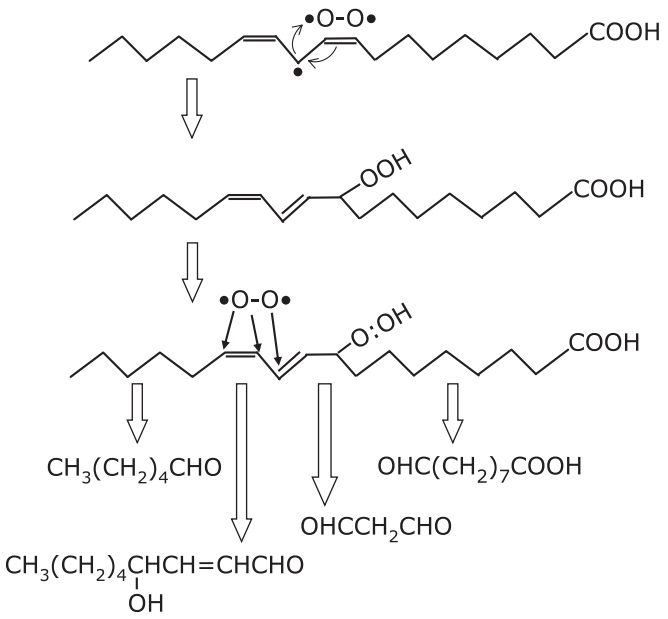

Fig. 1. Formation of linoleic acid 9-hydroperoxide and its homolytic decomposition. Abstraction of hydrogen at 11 position accompanies the addition of oxygen to 9 or 13 position.[23] Resulting 9-hydoperoxide decomposes to various aldehydes after attack by another oxygen at one of the arrow positions; hexanal, 4-hydroxy nonenal, malonaldehyde, and 9-oxononanoic acid from the left side in the figure.[25]

Materials and Methods

Materials. 9-ONA was synthesized according to published methods.[28] Briefly, oleic acid was oxidized to dihydroxystearic acid using potassium permanganate and was oxidized further by potassium periodate to pelargon aldehyde and 9-ONA. To separate 9-ONA from pelargon aldehyde, the reaction products were submitted to silica gel column chromatography, and 9-ONA was eluted with a solvent of 60% diethyl ether in hexane after eluting pelargon aldehyde by 20% diethyl ether in hexane. The 9-ONA was recrystallized from water 3 times until the purity was >95% on HPLC, and was dissolved in ethanol just before use. The other reagents used were of the highest grade available from commercial sources.

Human blood collection. This study was approved by the ethics committee of Kobe University Graduate School of Medicine (permission no. 615). Blood was carefully drawn from the forearm by fresh venipuncture through a 21-gauge butterfly cannula into a plastic syringe without anticoagulants. The fresh blood was immediately applied to the following experiment.

Autoxidation of human fresh blood. Fresh blood 5 ml was placed in four open centrifugation tubes and incubated for 0, 10, 20, and 40 min at 37°C, and then centrifuged at 3000 × *g* for 3 min at 4°C. Four samples of 50 µl serum layer were submitted to the following determinations: peroxide value (PV), thiobarbituric acid reactive substances (TBARS), PLA_2 activity, and TxB_2 level. The remaining serum and plasma were submitted to extraction for the following 9-ONA determination.

Alternatively, fresh blood was placed in the centrifugation tube and closed after filling up with nitrogen gas, and then submitting to the determination of PLA_2 activity and the TxB_2 level after 10- or 40-min incubation with the same treatment as above.

Determination of lipid peroxidation in blood. PV in the above serum was determined according to a published method.[29] Briefly, 50 µl serum was added to a 25 ml volumetric flask and mixed with 5 ml chloroform and 10 ml acetic acid, and then de-aerated by nitrogen gas purging for 1 min. The serum was mixed with 1 ml of 50% (w/v) KI aqueous solution and de-aerated for another 1 min, shaken, and allowed to stand in the dark for 5 min. The reaction mixture was diluted with 2% aqueous cadmium acetate solution and shaken, and then placed in the dark until the mixture had divided clearly into two layers. PV was calculated with the absorbance of the aqueous layer at 410 nm using the following equation: PV (meq/L) = [(A-B) × 60.14 + 0.69]/(0.008 × V). Where A is the absorbance of the sample, B is the absorbance of the blank, and V is the sample (ml).

TBARS was determined by fluorescent spectrophotometry according to the published method with slight modifications.[30] Briefly, the above 50 µl serum was mixed with 20 µl of 8.1% SDS, 0.15 ml of 20% acetic acid, and 0.15 ml of 0.8% TBA, and then vortexed and incubated at 95°C for 60 min. After cooling, the reaction mixture was combined with 0.5 ml butanol/pyridine (15:1) and centrifuged at 5000 rpm for 5 min. The upper butanol/pyridine phase was submitted to fluorescence analysis with an excitation wavelength at 520 nm and emission wavelength at 553 nm. TBARS was expressed as the level of malondialdehyde after calculation with a standard curve constructed with tetraethoxypropane.

Determination of PLA_2 activity in serum. PLA_2 activity in the above 50 µl serum was measured by a colorimetric method with a commercial kit (Cayman Chemical, Ann Arbor MI) according to the published method.[31] The reactions were initiated by the addition of substrate diheptanoyl thio-phosphorylcholine, and changes in absorbance were read at 405 nm after 60-min incubation at 37°C. Enzymatic activity was calculated using the manufacturer-provided formula and is expressed as µM/min/ml.

Determination of TxA_2 level in serum. TxA_2 has been well recognized to be immediately metabolized to thromboxane B_2 (TxB_2) after secretion and has been generally determined as equally produced TxB_2.[16] The present study also adopted the general method, in which the above 50 µl serum was measured with an enzyme immunoassay kit (Cayman Chemical) according to the published method.[32]

Determination of 9-ONA on HPLC. The above remaining serum and plasma in the autoxidation of fresh human blood were submitted to extraction with 5 ml chloroform/methanol (2:1, v/v) three times according to the Folch method.[33] The chloroform layer was dried under a nitrogen gas stream and the residue was dissolved in 500 µl methanol, and then a 20 µl aliquot was analyzed by HPLC, which was equipped with an L-7420 detector monitoring at a wavelength of 230 nm. The column (250 mm × 4.6 mm internal diameter) and guard column (10 × 4.0 mm internal diameter) were filled with Capcell Pak C18 UG80 (Shiseido, Tokyo, Japan) and maintained at 35°C. The mobile solvent was methanol/water (1:1, v/v), and the flow rate was 0.5 ml/min. 9-ONA was determined by calculation of the peak area on HPLC from the calibration curve constructed with the standard 9-ONA.

Effect of 9-ONA on platelet aggregation in human blood. Human blood in 3.2% of tri-sodium citrate was centrifuged at 200 × *g* for 15 min and separated into platelet-rich plasma (PRP). After confirming normal platelet numbers to be 2–4 × 10^8 platelets, the PRP was placed in the wells of 96-well plates at a volume of 100 µl and mixed with various concentrations of 9-ONA or platelet agonist 1 µM/L, arachidonic acid, and then vigorously shaken at 37°C on a microplate reader (SH-9000Lab; Corona Electric Co., Ibaragi, Japan). Changes in absorbance at 595 nm were monitored every 15 s for 15 min. Alternatively, a small part of the PRP was submitted to centrifugation at 15,000 × *g* for 5 min and separated to platelet-poor plasma (PPP). The PPP was treated similarly to the PRP and was referred as to a control as shown in the published method.[34]

Statistical Analysis. The data are reported as the mean ± SD.

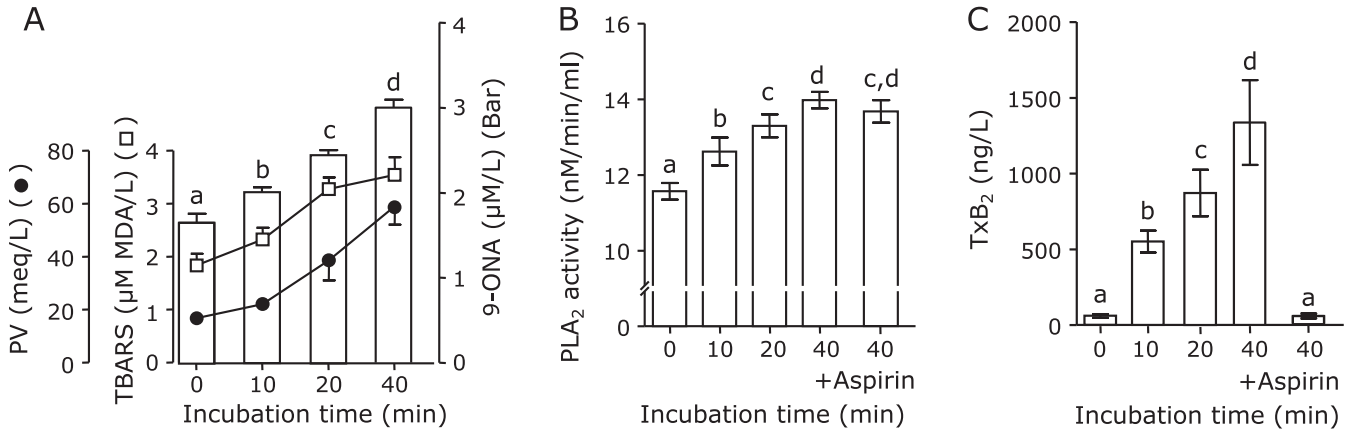

Fig. 2. 9-ONA production in autoxidation of blood (A), and its accompanying changes in PLA_2 activity (B) and TxB_2 level (C). Human fresh blood was exposed to the atmosphere with or without 100 μM/L aspirin at 37°C. After the indicated time, PV, TBARS, PLA_2 activity, and TxB_2 level in serum, and also 9-ONA amount in whole blood were measured as shown in Materials and Methods. Value are the mean ± SD, $n = 5$. Different letters indicate a significantly difference, $p<0.05$.

Statistical analysis was performed using Dunnett's post hoc test. Probability of <0.05 was considered significant.

Results

Production of 9-ONA in blood with autoxidation. PV and thiobarbituric acid (TBA) have been used generally for the evaluation of lipid peroxidation. PV specifically detects peroxides that are a primary product in lipid peroxidation, and TBA detects aldehydic products generated from the decomposition of lipid peroxides.[29,35] Fig. 2A shows that PV and TBARS increased with time when fresh human blood was exposed to atmospheric air. Thus, atmospheric oxygen induced lipid peroxidation in blood formed lipid peroxide, and produced its decomposed products, aldehydes. The fresh blood gave various peaks on HPLC (line b in Fig. 3), and one of the peaks increased after exposure to the atmosphere for 40 min (line c in Fig. 3). The increased peak at a retention time of 11.31 min coincided with authentic 9-ONA (line a in Fig. 3). Blood combined with authentic 9-ONA showed that the peak at 11.31 min of retention time was enlarged and symmetrical with no shoulders (line d in Fig. 3), similar to the peaks in HPLC a and c. These results clearly indicated that the lipid peroxidation of blood produced 9-ONA in the blood. 9-ONA has been recognized to be one of the major aldehyde products in lipid peroxidation, and Fig. 2A also shows that the level of 9-ONA increased in parallel with the increase of TBARS.[25,36]

Fig. 2B shows an increase in PLA_2 activity with the exposure time of blood to the atmosphere, and Fig. 2C shows the increase in TxB2. Thus, PLA_2 activity and TxB_2 production increased following lipid peroxidation in blood.

Interestingly, addition of aspirin to the blood clearly suppressed TxB_2 production after 40-min exposure to the atmosphere (Fig. 2C) without affecting PLA_2 activity (Fig. 2B).

Stimulating effect of 9-ONA on arachidonate cascade in blood. In the above results, the increases of PLA_2 activity and TxB_2 production followed by lipid peroxidation, suggested that one of the peroxidation products was a factor stimulating the activity of PLA_2, the key enzyme of the arachidonate cascade, and induced TxB_2 production. One of the major products of lipid peroxidation, 9-ONA, was added to fresh blood, and PLA_2 activity

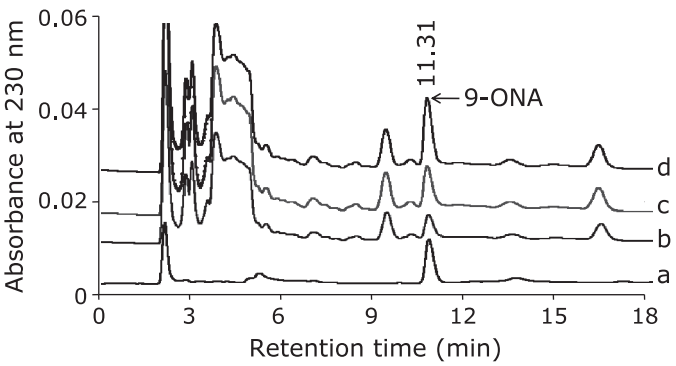

Fig. 3. Identification of 9-ONA in blood on HPLC. HPLC a: 10 μl of 17 μM/L of standard 9-ONA in methanol was applied to HPLC. HPLC b: blood exposed to the atmosphere for 0 min in Fig. 2 was extracted with 5 ml chloroform/methanol (2:1, v/v) as described in Materials and Methods, the chloroform layer was dried under nitrogen gas, and the residue was dissolved in 500 μl methanol, and then a 20 μl aliquot was applied to HPLC. HPLC c: blood exposed to the atmosphere for 40 min in Fig. 2 was extracted and analyzed. HPLC d: standard 9-ONA for HPLC a was added to the blood extract of HPLC c and then analyzed.

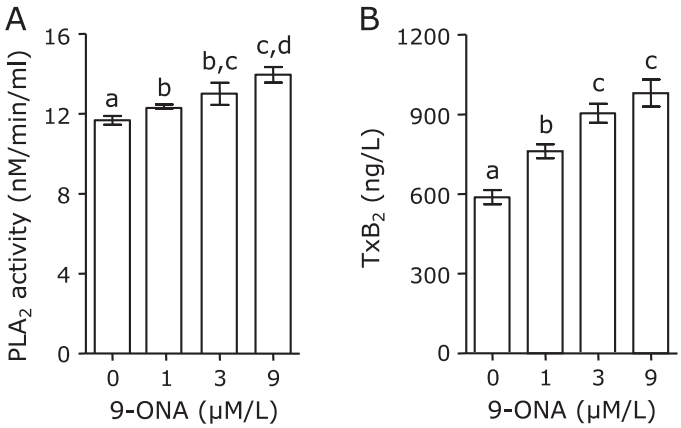

Fig. 4. Effect of organically synthesized 9-ONA on PLA_2 activity (A) and TxB_2 levels (B). Fresh human blood 1 ml was mixed with various concentrations of 9-ONA, incubated at 37°C for 10 min, and centrifuged at 3000 × *g* for 3 min at 4°C. The serum part was submitted to analyses of PLA_2 activity and TxB_2 levels with the same methods as in Fig. 2. Value are the mean ± SD, $n = 5$. Different letters indicate a significantly difference, $p<0.05$.

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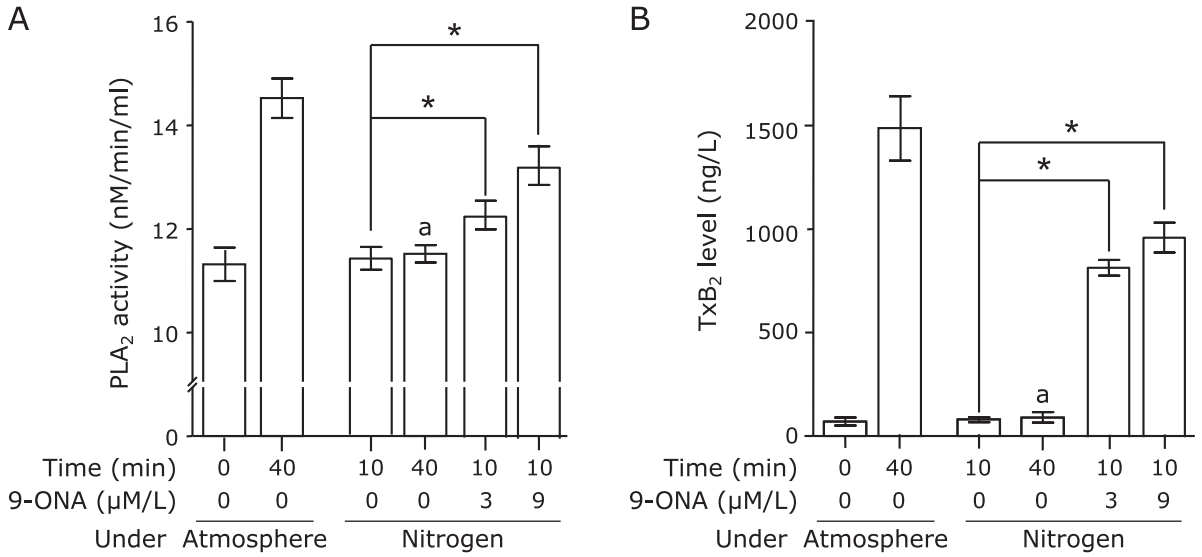

Fig. 5. PLA_2 activity (A) and TxB_2 level (B) in serum under nitrogen. Human fresh blood was left to stand in the atmosphere or under nitrogen with or without 9-ONA at 37°C. After the indicated time, PLA_2 activity and TxB_2 level in serum were measured, similarly to in Fig. 2. Value are the mean ± SD, $n = 5$. Asterisks show a significant difference from the results without 9-ONA, and letter a shows a significant difference from the results under an atmosphere for 40 min, $p<0.05$.

and the TxB_2 level determined in blood (Fig. 4). 9-ONA significantly increased PLA_2 activity (Fig. 4A) and the TxB_2 level (Fig. 4B) in a dose-dependent manner. The addition of 3 μM 9-ONA, a similar amount to that in blood when exposed to the atmosphere for 40 min, increased PLA_2 activity by around 10% (from 11.6 ± 0.2 nM/min/ml of the control without 9-ONA to 12.9 ± 0.6 nM/min/ml) and TxB_2 by around 50% (from 588 ± 27 ng/L of control to 904 ± 36 ng/L). These results clearly showed that 9-ONA activated PLA_2 and induced the production of TxB_2.

Fresh blood was placed under nitrogen and compared in PLA_2 activity and TxB_2 production to those in blood under the atmosphere (Fig. 5). PLA_2 activity in serum under nitrogen was similar to the activity at 0 time (Fig. 5A). TxB_2 production did not increase even after 40-min incubation at 37°C under nitrogen (Fig. 5B). The addition of 3 or 9 μM of 9-ONA to the blood under nitrogen revived the PLA_2 activity to 12.23 ± 0.28 or 13.14 ± 0.42 M/min/ml from 11.43 ± 0.22 M/min/ml, respectively, and TxB_2 production to 822 ± 38 or 967 ± 72 ng/L from 61 ± 18 ng/L, respectively. These results strongly indicated that 9-ONA was an inducer to stimulate PLA_2 activity and then increase TxB_2 production.

TxA_2 is known to be an inducer of blood clotting, and production of TxA_2 in platelets showed a linear relationship with platelet aggregation *in vitro*.[34] TxB_2 is a stable metabolite of TxA_2 after playing a signaling role in platelet aggregation.[16] Fig. 6 shows that the addition of 3 μM/L 9-ONA produced similar aggregation activity in blood with 1 μM/L arachidonic acid which is a potent platelet agonist.[14] This result together with the results in Fig. 5 indicates that 9-ONA induces platelet aggregation by producing TxA_2 in the blood.

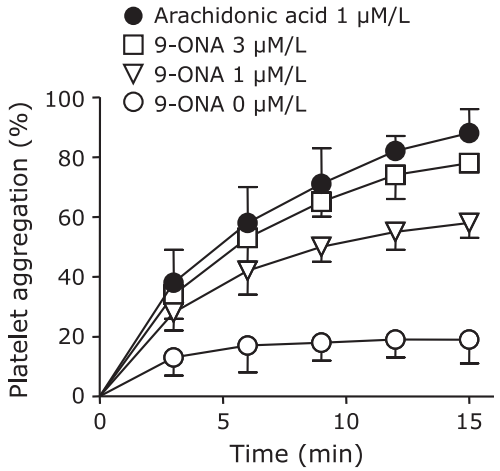

Fig. 6. Effect of 9-ONA on aggregation of human platelets. PRP 100 μl in a 96-well plate was treated with the indicated concentrations of 9-ONA or platelet agonist 1 μM arachidonic acid. Aggregation was measured on a plate reader by the changing absorbance at 595 nm every 15 s for 15 min, shaking vigorously at 37°C. All figures are $n = 5$.

Discussion

The association of lipid peroxidation has been well recognized in numerous disorders, such as inflammation, atherosclerosis and other degenerative diseases.[2–5,37] Lipid peroxidation is generally known to be initiated by ROS such as superoxide anion, which is generated in several systems; neutrophil phagocytosis, oxidation of xenobiotics by cytochrome P450, and xanthine oxidase, or under clinical conditions; shock, ischemia, and organ preservation.[38–43] The primary targets of ROS attack are PUFAs, which usually occupy the *sn*-2 position in membrenous phospholipids.[1,22] The peroxidation of PUFAs forms the primary product, lipid peroxides, and its decomposition forms secondary products including various aldehydes. Among aldehydic products, α, β-unsaturated carbonyls such as 4-hydroxynonenal, malonaldehyde and decadienal are highly unstable and easily form protein adducts.[26,44–46] Many previous studies have reported that the α, β-unsaturated carbonyls attack the biological components and produce toxicity.[6,7,26,44–46] Thus, peroxidation products directly cause degenerative diseases. On the other hand, involvement in the induction of diseases has not been noted in lipid peroxidation products.

In human plasma, linoleic acid is most abundant at the *sn*-2

position in membranous phospholipids.[21] The peroxidation of linoleic acid produces 9-ONA simultaneously with α, β-unsaturated carbonyls (Fig. 1).[23–26] 9-ONA is relatively stable while α, β-unsaturated carbonyls are highly unstable and easily form protein adducts. Thus, stable 9-ONA is assumed to occur in human plasma when ROS is generated in the blood and to exhibit some biological activity. The present study found that 9-ONA plays an initiating role in the arachidonate cascade and the production of eicosanoids, and then induces platelet aggregation.

In the present study, the accumulation of 9-ONA was detected in peroxidized blood with increased of lipid peroxidation (Fig. 2A and 3). Peroxidation stimulated PLA_2 activity and TxB_2 production, and addition of aspirin suppressed the production of TxB_2, indicating that the arachidonate cascade was involved (Fig. 2B and 2C).[16] Then, organically synthesized 9-ONA was added to fresh blood, and this addition caused an increase of PLA_2 activity and TxB_2 production (Fig. 4). Additionally, standing the blood under nitrogen invalidated the increases of PLA_2 activity and TxB_2 production, and the addition of 9-ONA restored both the activity and production (Fig. 5). The 9-ONA induced platelet aggregation dose-dependently (Fig. 6). These results clearly show that 9-ONA makes produces signaling of eicosanoid TxA_2 for platelet aggregation inducing the arachidonate cascade by stimulating key enzyme PLA_2 activity. Thus, the lipid peroxidation product 9-ONA is an inducer of platelet aggregation, which is closely associated with atherothrombosis.[15,47]

9-ONA stimulated the arachidonate cascade, which should produce not only TxA_2 but also PGI_2. PGI_2 is a potent vasodilator and inhibitor of platelet aggregation.[15] PGI_2 was produced and released from the macrovascular endothelium while TxB_2 was released from platelets.[48] In the present study, human blood was used but human endothelium was not. In order to examine PGI_2 production induced by 9-ONA, a human trial would be necessary in a future study.

A remaining question is how 9-ONA stimulates PLA_2 activity. PLA_2 activity was increased in minutes (Fig. 2B). The short-time induction indicates that the stimulation does not involve the gene expression, and it is likely that 9-ONA activated PLA_2 enzyme via a post-translational mechanism. van Rossum GS *et al.*[49] reported that hydrogen peroxide can stimulate PLA_2 activity through a $p42/44^{MARK}$-dependent mechanism. We have also found that a dietary fiber, fucoidan, causes the secretion of hydrogen peroxide into blood by stimulating NADPH oxidase 1 in intestinal epithelial cells and increases PGI_2 in the blood.[50] An interesting point is that hydrogen peroxide is an inducer of PGI_2 production but 9-ONA is an inducer of TxA_2, although both are produced in the cyclooxygenase pathway of the arachidonate cascade. The stimulating mechanism for the key enzyme of the arachidonate cascade PLA_2 should be examined in a future study.

On the basis of the present results, the following speculation may be made: lipid peroxidation arises mainly in the *sn*-2 position of membranous phospholipids of blood cells when any ROS is generated in the blood circulating system.[1,21] Peroxidation produces various aldehydic products. Among them, α, β-unsaturated carbonyls easily form adducts with biological components,[6,7,26,44–46] and then relatively stable aldehydes accumulate in the blood. One of the major accumulated products is 9-ONA, which initiates the arachidonate cascade by stimulating its key enzyme PLA_2 activity. The arachidonate cascade produces TxA_2, which induces platelet aggregation and causes thrombus formation, and then develops diseases such as atherosclerosis. Thus, the present study provided new insight that lipid peroxidation product 9-ONA could play an inducing role in key enzyme PLA_2 activity in the arachidonate cascade.

Acknowledgments

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Abbreviations

9-ONA	9-oxononanoic acid
PGI_2	prostacyclin
PLA_2	phospholipase A_2
PUFA	polyunsaturated fatty acid
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2

Conflict of Interest

No potential conflicts of interest were disclosed.

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