Lipid peroxidation products are known to cause toxicity by reacting with biologically significant proteins, but the inducing role of peroxidation products has been 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 A2 (PLA2), 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 PLA2 activity and thromboxane B2 (TxB2) production, which is a stable metabolite of thromboxane A2 (TxA2) 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 PLA2 and the production of TxB2. Also, 9-ONA-induced platelet aggregation dose-dependently. These results indicated that 9-ONA is the primary inducer of PLA2 activity and TxA2 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 PLA2.

Key Words: 9-oxononanoic acid, lipid peroxidation, phospholipase A2, thromboxane A2, arachidonate cascade

Lipids with polyunsaturated fatty acids (PUFAs) are primary targets for attack by reactive oxygen species (ROS). 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.

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. The former toxicity of peroxidation products has been almost completely clarified, but various questions remain about the latter inducing role of the products. 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. Hydrolysis of membranous phospholipids by phospholipase A2 (PLA2) 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 synthases and tissue-specific isomerases through unstable intermediate prostaglandin H2 to multiple eicosanoids such as thromboxane A2 (TxA2) and prostacyclin (PGI2). TxA2 is a potent platelet agonist and vasoconstrictor and activates resting platelets, leading to the formation of a thrombus, while PGI2 is a potent vasodilator and inhibitor of platelet aggregation. Numerous studies have shown the important roles of TxA2 and PGI2 in preserving the dynamic balance among thrombosis, hemostasis and fluidity of blood. TxA2 is high in patients with atherosclerotic disease and pulmonary hypertension, and the production of PGI2 is depressed in these patients. Evidence that elevated PLA2 activity is associated with cardiovascular events has also been found. Thus, the release of arachidonic acid by PLA2 from membranous phospholipids is a rate-limiting step in eicosanoid synthesis; however, the inducing factors to activate the key enzyme PLA2 are unknown. The aim of the present study was therefore to identify a candidate of the inducer chemicals to activate PLA2.

Linoleic acid, a PUFA, has been reported to comprise around 25% of the fatty acids of membranous phospholipids in human plasma. The sn-2 position in membranous phospholipids is usually occupied by PUFA. 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. 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. 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. This information indicated the possibility that 9-ONA is an inducer of the arachidonate cascade through the activation of PLA2. In the present study, we investigated the inducing role of 9-ONA in PLA2 activity followed by eicosanoid production and platelet aggregation in human blood.
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 sodium 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: $\text{PV (meq/L)} = ([A-B] \times 60.14 + 0.69) / (0.008 \times V)$. 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. 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 of 520 nm and emission wavelength at 535 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. 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$_2$. 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.

**Determination of 9-ONA on HPLC.** The above remaining serum and plasma in the autodissolution of fresh human blood were submitted to extraction with 5 ml chloroform/methanol (2:1, v/v) three times according to the Folch method. 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.

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

Fig. 2B shows an increase in PLA2 activity with the exposure time of blood to the atmosphere, and Fig. 2C shows the increase in TxB2. Thus, PLA2 activity and TxB2 production increased following lipid peroxidation in blood. Interestingly, addition of aspirin to the blood clearly suppressed TxB2 production after 40-min exposure to the atmosphere (Fig. 2C) without affecting PLA2 activity (Fig. 2B).

Stimulating effect of 9-ONA on arachidonate cascade in blood. In the above results, the increases of PLA2 activity and TxB2 production followed by lipid peroxidation, suggested that one of the peroxidation products was a factor stimulating the activity of PLA2, the key enzyme of the arachidonate cascade, and induced TxB2 production. One of the major products of lipid peroxidation, 9-ONA, was added to fresh blood, and PLA2 activity...
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and the TxB2 level determined in blood (Fig. 4). 9-ONA significantly increased PLA2 activity (Fig. 4A) and the TxB2 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 PLA2 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 TxB2 by around 50% (from 588 ± 27 ng/L of control to 904 ± 36 ng/L). These results clearly showed that 9-ONA activated PLA2 and induced the production of TxB2.

Fresh blood was placed under nitrogen and compared in PLA2 activity and TxB2 production to those in blood under the atmosphere (Fig. 5). PLA2 activity in serum under nitrogen was similar to the activity at 0 time (Fig. 5A). TxB2 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 PLA2 activity to 12.23 ± 0.28 or 13.14 ± 0.42 M/min/ml from 11.43 ± 0.22 M/min/ml, respectively, and TxB2 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 PLA2 activity and then increase TxB2 production.

TxA2 is known to be an inducer of blood clotting, and production of TxA2 in platelets showed a linear relationship with platelet aggregation in vitro. TxB2 is a stable metabolite of TxA2 after playing a signaling role in platelet aggregation. Fig. 6 shows that the addition of 3 μM 9-ONA produced similar aggregation activity in blood with 1 μM/L arachidonic acid which is a potent platelet agonist. This result together with the results in Fig. 5 indicates that 9-ONA induces platelet aggregation by producing TxA2 in the blood.

Discussion

The association of lipid peroxidation has been well recognized in numerous disorders, such as inflammation, atherosclerosis and other degenerative diseases. 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. The primary targets of ROS attack are PUFAs, which usually occupy the sn-2 position in membranous phospholipids. 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 carboxyls such as 4-hydroxynonenal, malonaldehyde and decadienal are highly unstable and easily form protein adducts. Many previous studies have reported that the α, β-unsaturated carboxyls attack the biological components and produce toxicity. 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 \(\alpha\), \(\beta\)-unsaturated carbonyls (Fig. 1).\(^{12-20}\) 9-ONA is relatively stable while \(\alpha\), \(\beta\)-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 validated 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\(_2\) and production induced by 9-ONA, a human trial would be necessary when ROS is generated in the blood circulating system.\(^ {1,21}\) Peroxidation produces various aldehydic products. Among them, \(\alpha\), \(\beta\)-unsaturated carbonyls easily form adducts with biological components,\(^ {6,7,20,44-46}\) 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.

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Abbreviations

9-ONA 9-oxononanoic acid
PLA\(_2\): phospholipase A\(_2\):
PUEA 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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