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Effects of Nonanal, *trans*-2-Nonenal and 4-Hydroxy-2,3-*trans*-nonenal on Cyclooxygenase and 12-Lipoxygenase Metabolism of Arachidonic Acid in Rabbit Platelets

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Abstract

The effects of nonanal, *trans*-2-nonenal and 4-hydroxy-2,3-*trans*-nonenal on the formation of thromboxane B_2 (TXB_2), 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) from exogenous arachidonic acid in washed rabbit platelets were examined.

Nonanal and *trans*-2-nonenal at concentrations ranging from 0·25 to 2 μM inhibited TXB_2, HHT and 12-HETE formation, reducing the amounts of these three arachidonic acid metabolites by 50% at nonanal and *trans*-2-nonenal concentrations of approximately 0·25 μM. The inhibition of TXB_2, HHT and 12-HETE formation induced by 4-hydroxy-2,3-*trans*-nonenal (50% inhibition by 4-hydroxy-2,3-*trans*-nonenal at a concentration of approximately 100 μM) was 400 times weaker than that induced by nonanal and *trans*-2-nonenal.

These results suggest that nonanal and *trans*-2-nonenal can be modulators of platelet arachidonic acid metabolism by affecting the activity of cyclooxygenase and 12-lipoxygenase.

Platelets metabolize free arachidonic acid via the cyclooxygenase and 12-lipoxygenase pathways. Arachidonic acid is converted into thromboxane A_2 (TXA_2) and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) by the cyclooxygenase pathway and into 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) by the 12-lipoxygenase pathway (Hamberg & Samuelsson 1974). TXA_2 is a potent vasoconstrictor and inducer of platelet aggregation and rapidly breaks down to form the stable end-product TXB_2 (Hamberg et al 1975; Ellis et al 1976). 12-HETE has been reported to cause platelet aggregation (Dutilh et al 1978; Sekiya et al 1991) and neutrophil (Turner et al 1975) and aortic smooth muscle cell (Nakao et al 1983) migration.

It is well established that polyunsaturated fatty acids are readily auto-oxidized to hydroperoxides, which are further broken down into a variety of oxidation products, the so-called secondary products of lipid peroxidation, including aldehydes such as alkanals, alk-2-enals and 4-hydroxyalkenals. In previous studies we have shown that lipid peroxidation can modulate arachidonic acid metabolism in rabbit kidney medulla slices (Fujimoto & Fujita 1982; Fujimoto et al 1983) and in washed rabbit platelets (Fujimoto et al 1992). We have, furthermore, also reported that hydroperoxides play an important role in the control of platelet cyclooxygenase and 12-lipoxygenase activity (Fujimoto et al 1992). Little information is yet available about the involvement of aldehydes in arachidonic acid metabolism in platelets and other tissues.

This study was, therefore, undertaken to investigate the effects of nonanal, *trans*-2-nonenal and 4-hydroxy-2,3-*trans*-nonenal (Fig. 1), examples, respectively, of the alkanals, alk-2-enals and 4-hydroxyalkenals generated during lipid peroxidation (Esterbauer 1982; Esterbauer et al 1982, 1991; Koster et al 1986), on the formation of TXB_2, HHT and 12-HETE from exogenous arachidonic acid in washed rabbit platelets.

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Materials and Methods

Materials

TXB_2 and the sodium salt of arachidonic acid were obtained from Sigma (St Louis, MO, USA). HHT was obtained from Cayman (Ann Arbor, MI, USA), and 12-HETE was from Cascade Biochem (Berkshire, UK). 4-Hydroxy-2,3-*trans*-nonenal and 9-anthryldiazomethane were purchased from Funakoshi Pharmaceutical (Tokyo, Japan). Nonanal and *trans*-2-nonenal were obtained from Tokyo Kasei Organic Chemi-

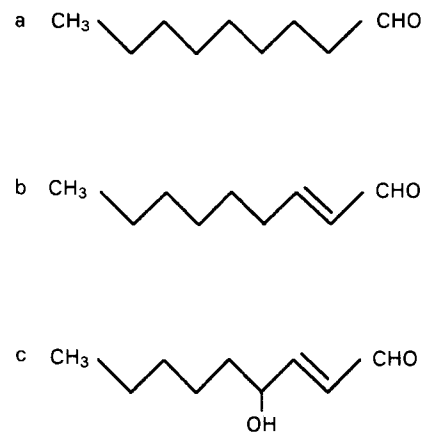

FIG. 1. Structures of (a) nonanal, (b) *trans*-2-nonenal and (c) 4-hydroxy-2,3-*trans*-nonenal.

cals (Tokyo, Japan). The purities of 4-hydroxy-2,3-*trans*-nonenal, nonanal and *trans*-2-nonenal were >98%, >97% and >98%, respectively. All other reagents were analytical grade.

Preparation of platelets

Blood was withdrawn into a 3·8% solution of trisodium citrate (9 : 1, v/v) from the abdominal aorta of male rabbits, 2–2·5 kg, under sodium pentobarbital anaesthesia. Platelets were then collected by differential centrifugation. Whole blood was centrifuged for 10 min at 200 *g* at room temperature and the platelet-rich plasma was withdrawn from above the pelleted erythrocytes. After addition of EDTA (to a final concentration of 1 mM), the platelet-rich plasma was cooled to 0°C and centrifuged at 2000 *g* for 10 min. The platelet pellet was washed twice with 134 mM NaCl, 5 mM glucose, 15 mM Tris-HCl buffer, pH 7·4 (buffer A) containing 1 mM EDTA, and then resuspended in buffer A.

Incubation conditions and measurement of metabolites from arachidonic acid

The washed platelet suspension (3×10^8 platelets) was pre-incubated for 5 min at 37°C in buffer A (1 mL) with or without the indicated concentrations of nonanal, *trans*-2-nonenal or 4-hydroxy-2,3-*trans*-nonenal. Nonanal, *trans*-2-nonenal and 4-hydroxy-2,3-*trans*-nonenal were dissolved in ethanol. The final concentration of ethanol in the platelet suspension was held constant at 1% (v/v) in all experiments. Ethanol at 1% had no effect on arachidonic acid metabolism in platelets. Arachidonic acid (40 μM) was subsequently added to the platelet suspension, and the mixture was incubated at 37°C for 5 min. The reaction was terminated by quickly adding an appropriate amount of 0·5 M HCl to adjust the pH of the reaction mixture to 3·0. The reaction mixture was then extracted with ethyl acetate (3 mL). TXB_2, HHT, 12-HETE and arachidonic acid in the extracted lipid were simultaneously determined by an HPLC method described previously (Fujimoto et al 1990, 1992). Briefly, 12-HETE and HHT were separated by normal-phase chromatography and simultaneously quantitated by means of a UV spectrophotometric detector. TXB_2 and arachidonic acid were esterified with 9-anthryldiazomethane and the esters were separated by reversed-phase chromatography and simultaneously quantitated by spectrofluorometry. Our previous study utilizing indomethacin, an inhibitor of cyclooxygenase (Flower 1974), and quercetin, an inhibitor of lipoxygenase (Hope et al 1983; Nakadate et al 1985), has demonstrated the suitability of this in-vitro system for simultaneous detection of changes in the activities of platelet cyclooxygenase and 12-lipoxygenase (Fujimoto et al 1992).

Statistics

Results are means ± s.e.m. Statistical significance was determined by use of Student's *t*-test.

Results

Fig. 2 shows the effects of nonanal, *trans*-2-nonenal and 4-hydroxy-2,3-*trans*-nonenal on the formation of TXB_2, HHT and 12-HETE from exogenous arachidonic acid (40 μM) in washed rabbit platelets. All the tested substances showed dose-dependent inhibition of the formation of both cyclooxygenase (TXB_2 and HHT) and 12-lipoxygenase (12-HETE) metabolites at concentrations ranging from 0·25 to 2 μM (nonanal and *trans*-2-nonenal) or from 10 to 100 μM (4-hydroxy-2,3-*trans*-nonenal). The concentrations required for 50% inhibition were: nonanal and *trans*-2-nonenal, approximately 0·25 μM, 4-hydroxy-2,3-*trans*-nonenal, approximately 100 μM. Nonanal and *trans*-2-nonenal inhibited the generation of arachidonic acid metabolites at concentrations 400 times lower than 4-hydroxy-2,3-*trans*-nonenal.

The amount of arachidonic acid remaining after incubation was quantified by HPLC (Fig. 2). When nonanal, *trans*-2-nonenal and 4-hydroxy-2,3-*trans*-nonenal were added to the incubation mixture, there was a relative increase in the amount of arachidonic acid concomitant with a decrease in the amount of TXB_2, HHT and 12-HETE. It seems, therefore, that cyclooxygenase and 12-lipoxygenase activities in platelets can be inhibited by these three substances and that inhibition by nonanal or *trans*-2-nonenal is much stronger than that by 4-hydroxy-2,3-*trans*-nonenal.

Discussion

Arachidonic acid is metabolized by two major pathways in platelets, i.e. the cyclooxygenase and 12-lipoxygenase pathways. TXA_2, a metabolite of the cyclooxygenase pathway, is well known to be an endogenous vasoconstrictor and inducer of platelet aggregation (Hamberg et al 1975; Ellis et al 1976). A 12-lipoxygenase product, 12-HETE, has been reported to amplify thrombin-induced aggregation and to counteract the inhibitory effect of cyclic-AMP elevator, PGE_1 on the aggregation (Dutilh et al 1978; Sekiya et al 1991). These metabolites of the cyclooxygenase and 12-lipoxygenase pathways therefore play an important role in the shortening of bleeding time when a vascular bed is injured. It is, on the other hand, well recognized that platelet activation is connected closely with thrombosis and atherosclerosis (Ross 1981; Zucker & Nachmias 1985). Irregular generation of TXA_2 causing platelet aggregation and vasoconstriction is, of course, involved in the initiation and propagation of thrombotic and atherosclerotic disorders. Not only has 12-HETE been reported to give rise to platelet aggregation, it has also been shown to induce migration of smooth muscle cells from media to intima (Nakao et al 1983), an event which is a significant process in the genesis of atherosclerosis. The 12-lipoxygenase pathway has, furthermore, been shown to play an important role in oxidative modification of plasma low-density lipoprotein, which is a contributing factor in the pathogenesis of atherosclerosis (Lenz et al 1990; Thomas & Jackson 1991). These findings imply that dual inhibition of the cyclooxygenase and 12-lipoxygenase pathways in platelets might be beneficial in the treatment of vascular diseases such as thrombosis and atherosclerosis.

Lipid peroxidation often occurs in response to oxidative stress, and a great diversity of aldehydes is formed when lipid hydroperoxides break down in biological systems. It has, on the other hand, been reported that the total level of aldehydes in plasma is about 1 μM under physiological conditions (Esterbauer et al 1991; Siems et al 1996). Some of these aldehydes are highly reactive. The aldehydes most intensively studied are 4-hydroxyalkenals including 4-hydroxy-2,3-*trans*-nonenal; 4-hydroxy-2,3-*trans*-nonenal and other 4-hydroxy-

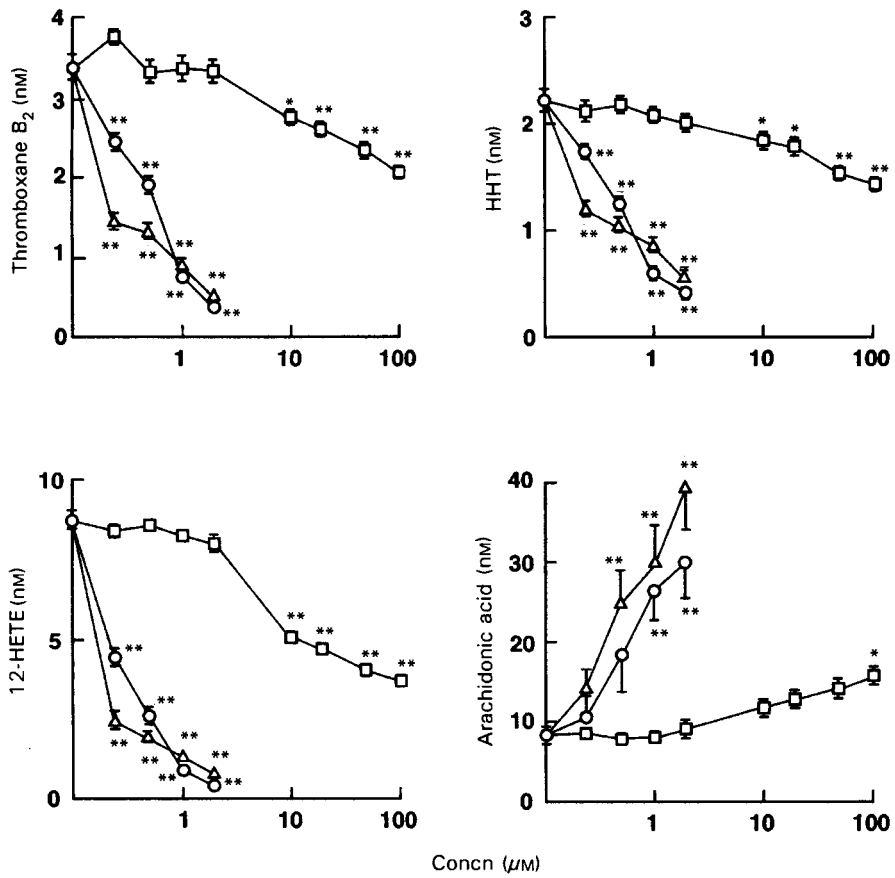

FIG. 2. Effects of nonanal, *trans*-2-nonenal and 4-hydroxy-2,3-*trans*-nonenal on the formation of thromboxane B_2, 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) in washed rabbit platelets. Platelets (3×10^8 mL^{-1}) were preincubated with or without different concentrations of nonanal, *trans*-2-nonenal and 4-hydroxy-2,3-*trans*-nonenal for 5 min at 37°C before incubation with arachidonic acid (40 μM) for 5 min at 37°C. Values represent mean ± s.e.m. of 3–6 experiments. $*P < 0.05$, $**P < 0.01$, significantly different from the corresponding value in the absence of nonanal, *trans*-2-nonenal or 4-hydroxy-2,3-*trans*-nonenal. ○, Nonanal; △, *trans*-2-nonenal; □, 4-hydroxy-2,3-*trans*-nonenal.

alkenals are capable of inducing a variety of powerful biochemical effects such as alterations in the activities of phospholipase C and D, glucose-6-phosphatase, cytochrome P450 and Na^+, K^+-ATPase (Dianzani 1982; Natarajan et al 1993; Ohyashiki et al 1995; Bestervelt et al 1995; Siems et al 1996). In contrast, in these experiments (Dianzani 1982; Natarajan et al 1993; Ohyashiki et al 1995; Bestervelt et al 1995; Siems et al 1996), weaker or no significant effects were observed with alkanals and alk-2-enals including nonanal and *trans*-2-nonanal.

In the present study we have obtained the first evidence that nonanal and *trans*-2-nonenal rather than 4-hydroxy-2,3-*trans*-nonenal, have a potent biochemical function; nonanal and *trans*-2-nonenal inhibited cyclooxygenase and 12-lipoxygenase activity in platelets and the potency of nonanal or *trans*-2-nonenal was much greater than that of 4-hydroxy-2,3-*trans*-nonenal. The finding that cyclooxygenase and 12-lipoxygenase are inhibited by quite low doses of nonanal or *trans*-2-nonenal (50% inhibition at a dose of approximately 0·25 μM) opens the interesting possibility that platelet arachidonic acid metabolism might be modulated by nonanal and *trans*-2-nonenal arising from the decomposition of membrane lipids in conditions of enhanced lipid peroxidation or even during a physiological renewal of lipid-containing membrane particles. Nonanal and *trans*-2-nonenal might, furthermore, also be of potential therapeutic value in the treatment of thrombotic and atherosclerotic disorders.

Further studies are needed to clarify the modulation mechanism; we have, however, provided the first direct evidence that nonanal and *trans*-2-nonenal have the potential to modulate platelet cyclooxygenase and 12-lipoxygenase activity. These observations provide new insight into factors controlling the production of the metabolites derived from arachidonic acid in platelets.

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