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Endothelial Cell Injury caused by 15-HPETE, a Lipoxygenase Product of Arachidonic acid

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Summary. **Among various arachidonic acid metabolites examined, only 15 (S)-hydroperoxy-5, 8, 11, 13-eicosatetraenoic acid (15-HPETE) caused severe injury to bovine endothelial cells in culture. This cellular injury was time- and dose-dependent. When compared with tertiary-butylhydroperoxide (t-BuOOH) and hydrogen peroxide (H_2O_2), it revealed that the injury caused by 15-HPETE was essentially distinct from those by t-BuOOH and H_2O_2 in such respects as temperature dependency and albumin effect. When the endothelial monolayers were exposed to 15-HPETE, prostacyclin (PGI_2) production was markedly suppressed. However, there were discrepancies in time course and dose dependence between 15-HPETE-induced cellular injury and 15-HPETE-induced inhibition of PGI_2 synthesis. In addition, the injurious activity of 15-HPETE was not enhanced even when endothelial monolayers were pretreated with inhibitors of PGI_2 production, suggesting that endogenous PGI_2 does not play any cytoprocective role in endothelial cells at least against such a lipid peroxide as 15-HPETE.**

INTRODUCTION

Endothelial cell injury has been recognized as an initial pathogenic event in various vascular diseases such as thrombosis and atherosclerosis. For treating and/or preventing these diseases, it is very important to elucidate the mechanism of the endothelial injury and to find out some effective strategies to protect endothelia from the injury.

Many chemical and mechanical factors have been reported to be responsible for endothelial cell injury to date. For example, linoleic acid hydroperoxide, which is one of the peroxidation products of fatty acid, was demonstrated to cause endothelial cell injury *in vitro*[1] and *in vivo*.[2-4] However, the detailed mechanism of the cellular injury has not yet been well elucidated, although linoleate hydroperoxide was revealed to inhibit both prostacyclin (PGI_2) production[5] and respiration[6] in endothelial cells.

15(S)-Hydroperoxy-5, 8, 11, 13-eicosatetraenoic acid (15-HPETE), which is a kind of lipid peroxide, is produced from arachidonic acid by 15-lipoxygenase in many tissues and cells including those of the lung and trachea, polymorphonuclear leukocytes, reticulocytes, etc. Endothelial cells,[7,8] as well as vascular smooth muscle cells,[9] have also been found to produce 15-HPETE.

15-HPETE has many biological actions. For example, it potently suppresses PGI_2 production in endothelial cells through the inhibition of the PGI_2 synthetase.[10,11] PGI_2 is a potent vasodilator and the most powerful endogenous inhibitor of platelet aggregation,[12] and it is therefore suggested that the reduced production of PGI_2 due to 15-HPETE results in the pathogenesis of vascular tissues; 15-HPETE has been demonstrated to exist in an atherosclerotic vessel wall.[13,14]

Recently, we found that 15-HPETE caused injury to bovine endothelial cells in culture.[15,16] In the present paper, we will characterize the endothelial cell injury caused by 15-HPETE. We will also describe the effect of endogenous PGI_2 on the 15-HPETE-induced endothelial cell injury.

MATERIALS AND METHODS

Reagents

[^{51}Cr] Sodium chromate (250-500 mCi/mg) and [1-^{14}C] arachidonic acid (58 mCi/mmol) were purchased from Amersham International (Amersham, U.K.). Arachidonic acid and soybean lipoxygenase (type IV) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.) and Dil-acetylated low-density lipoprotein from Paesel GmbH & Co. (Frankfurt, F.R.G.). Various prostaglandins and thromboxanes were generously provided by Ono Pharmaceutical Co. (Osaka, Japan). All other reagents used were of the highest purity available.

Culture of endothelial cells

Endothelial cells were isolated from bovine carotid arteries as previously described.[17] In brief, fresh arteries were rinsed with an Eagle's minimum essential medium (MEM) (Gibco, Grand Island, U.S.A.) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco) and were cut open. Endothelial cells were scraped off from the intimal surface by a surgical blade and they were suspended in MEM supplemented with 20% fetal bovine serum (FBS) (Whittaker M. A. Bioproducts, Walkersville, U.S.A.) and antibiotics. After mild pipetting, the cell suspension was inoculated in tissue culture dishes (Falcone Labware, Division of Becton-Dickinson and Co., Lincoln Park, U.S.A.) and incubated at 37°C in an atmosphere of 95% air and 5% CO_2.

Cell in monolayer were maintained and subcultured in a growth medium, i.e., MEM supplemented with 10% FBS. The cultured cells were identified with endothelial cells by three characteristics: 1) the typical contact-inhibited cobblestone appearance in monolayer, 2) the incorporation of Dil-acetylated low-density lipoprotein and 3) the presence of Weibel-Palade bodies (Fig. 1). Experiments were performed with the cells in the 10th to the 25th passage.

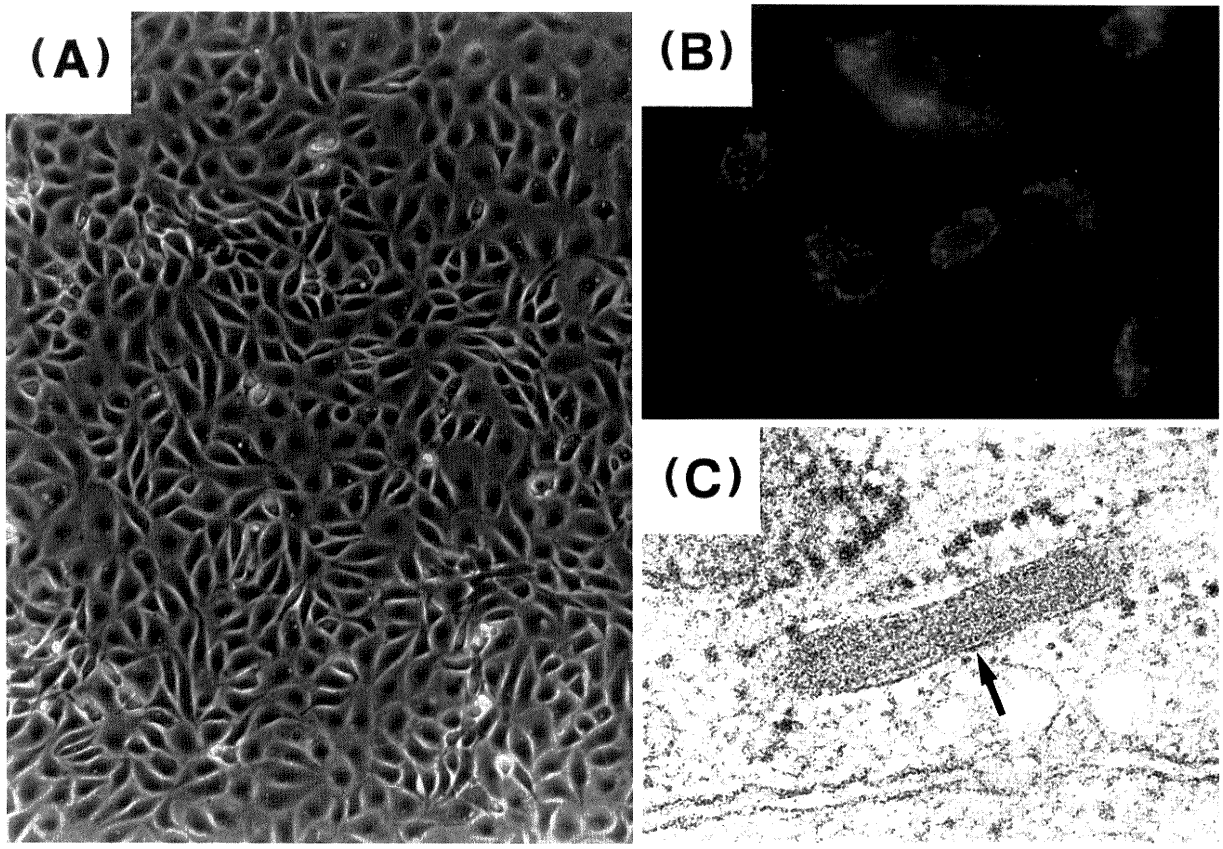

Fig. 1. Cultured endothelial cells derived from bovine carotid artery. The endothelial cells were identified by the typical cobblestone appearance in monolayer (A), the ability to incorporate Dil-acetylated low-density lipoprotein (B) and the presence of Weibel-Palade body indicated by an arrow (C).

Preparation of 15-HPETE

15-HPETE was enzymatically prepared as previously described (16). In brief, arachidonic acid (99% pure) was incubated with soybean lipoxygenase in a 0.1 M Tris-HCl buffer (pH 9.5). The reaction mixture was then acidified and extracted with ethyl ether. The ethereal extract was evaporated and then the residue was subjected to thin-layer chromatography on Silica gel G ($60F_{254}$, E. Merk, Darmstadt, F.R.G.) with the solvent mixture of hexane/ethyl ether/acetic acid (70 : 30 : 1). Silica gel corresponding to a standard of 15-HPETE under UV light was scraped off and then the prepared 15-HPETE in the silica gel was extracted with a mixture of ethyl ether/petroleum ether (1 : 3). After evaporation of the solvent, the purified 15-HPETE was dissolved in absolute ethanol and stored at −20°C until use.

The purity of 15-HPETE thus prepared was examined by reverse-phase high performance liquid chromatography (RP-HPLC) which was performed on a Develosil ODS-5K column (4.6×250 mm, Nomura Chemicals, Seto, Japan) using a mobile phase of methanol/acetonitrile/water/acetic acid (15 : 45 : 35 : 0.01) at a flow rate of 1.5 ml/min (Fig. 2A), and the purity was estimated at more than 95%. The concentration of 15-HPETE was also determined spectrophotometrically by using a molar extinction coefficient at 237 nm of 30000 $M^{-1}cm^{-1}$ [18] (Fig. 2B).

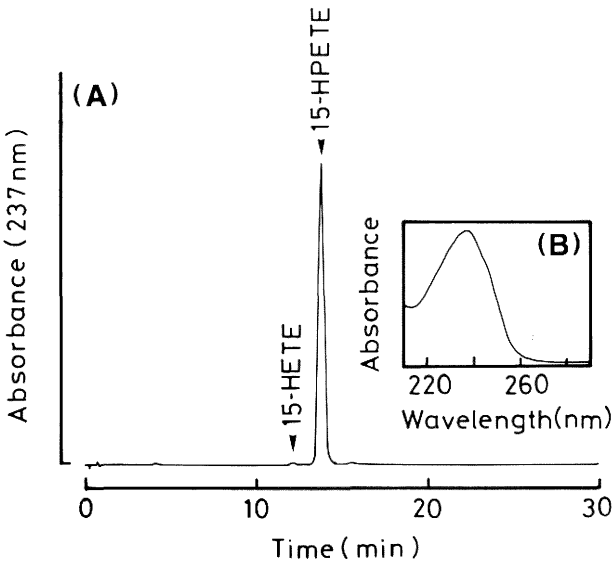

Fig. 2. 15-HPETE preparation. 15-HPETE was prepared enzymatically by incubation of soybean lipoxygenase with arachidonic acid. (A) RP-HPLC profile; (B) UV absorption spectrum.

Estimation of endothelial cell injury

Endothelial cell injury was estimated by the release of ^{51}Chromium (^{51}Cr) as previously described.[16] In brief, confluent monolayers in 24 multi-well dishes (Falcone Labware) were prelabeled with 2 μCi of [^{51}Cr] sodium chromate for 18 hours in the growth medium. After labeling, the cells were washed twice with MEM and then exposed to 15-HPETE or other test compounds for 6 hours in MEM.

After the 6-hour cultivation, aliquots of the culture medium were removed and the ^{51}Cr radioactivities released out of the injured cells were measured by gamma-scintilation spectrophotometer (Packard Auto-Gamma, Packard Instruments Co., Downers Grove, U.S.A.). Results were expressed as the percentage of specific ^{51}Cr-release, calculated as follows: (A-B)/(C-B)×100%, where A represents ^{51}Cr-release due to each test compound, B represents the spontaneous ^{51}Cr-release and C represents the maximum release of ^{51}Cr. Spontaneous and maximum releases were determined in cell monolayers incubated with vehicle solvent and 0.1% Triton X-100, respectively.

Estimation of endothelial PGI_2 production

PGI_2 production by endothelial cells was estimated by the production of 6-keto-$PGF_{1\alpha}$, a hydrolyzed product of PGI_2, as previously described.[19] In brief, confluent monolayers in 90 mm-Petri dish (Falcone Labware) were incubated with various doses of 15-HPETE. After the incubation, the cells were washed with a 0.05 M phosphate buffer (pH 8.2), harvested using a piece of Teflon sheet, and then sonicated. The resulting homogenate was incubated with [^{14}C] arachidonic acid. The reaction products were extracted with ethyl acetate at pH 3 and separated by thin-layer chromatography with the organic phase of ethyl acetate/2, 2, 4-trimethylpentane/water/acetic acid (11 : 5 : 2 : 10). The radioactive products were identified and quantified by a radiochromatogram scanner (Berthold Trace Master 20, Dr. Berthold Institute, Wildbad, F.R.G.).

RESULTS

Effect of 15-HPETE on endothelial cell injury

The effect of various arachidonic acid metabolites on endothelial cell injury was measured by using cultured bovine endothelial cells prelabeled with ^{51}Cr. As shown in Table 1, any prostaglandins and thrombox-

anes examined failed to induce ^{51}Cr-release from endothelial cells. In dramatic contrast, 15-HPETE caused severe ^{51}Cr-release from endothelial monolayers. 15-HETE (15(S)-hydroxy-5, 8, 11, 13-eicosatetraenoic acid), which is a reduced metabolite of 15-HPETE, had no effect at all. Arachidonic acid, a precursor of 15-HPETE, was also ineffective.

Fig. 3 shows the time- and dose-dependency of the endothelial cell injury induced by 15-HPETE. When the cells were exposed to 15-HPETE, there was a time- and dose-dependent increase in ^{51}Cr-release. The injurious activity of 15-HPETE to endothelial cells was also confirmed by the examination of the change in cell morphology by a phase contrast microscopy (Fig. 4). Untreated cells showed typical cobble-

Table 1. Injurious Activities of Various Arachidonic Acid Metabolites to Endothelial Cells

	Specific ^{51}Cr-Release (%)		
PGA_2	−2.5	STA_2	−0.4
PGD_2	−0.5	TXB_2	−0.4
PGE_2	−1.0	15-HPETE	93.8
$PGF_{2\alpha}$	0.6	15-HETE	0.3
PGI_2	0.9	Arachidonic acid	−0.5
6-Keto-$PGF_{1\alpha}$	0.2		

^{51}Cr-Labeled bovine endothelial monolayers were incubated with each compound at 50 μM for 6 hours at 37°C. Each value represents the mean of 6 determinations.

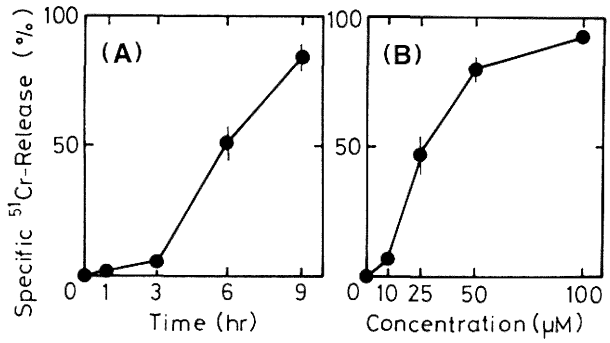

Fig. 3. Time- and dose-dependent injury to endothelial cells induced by 15-HPETE. ^{51}Cr-labeled bovine endothelial monolayers were incubated with 25 μM 15-HPETE for various times (A) or with various concentrations of 15-HPETE for 6 hours (B) at 37°C. Each point represents the mean ±SD, n=6.

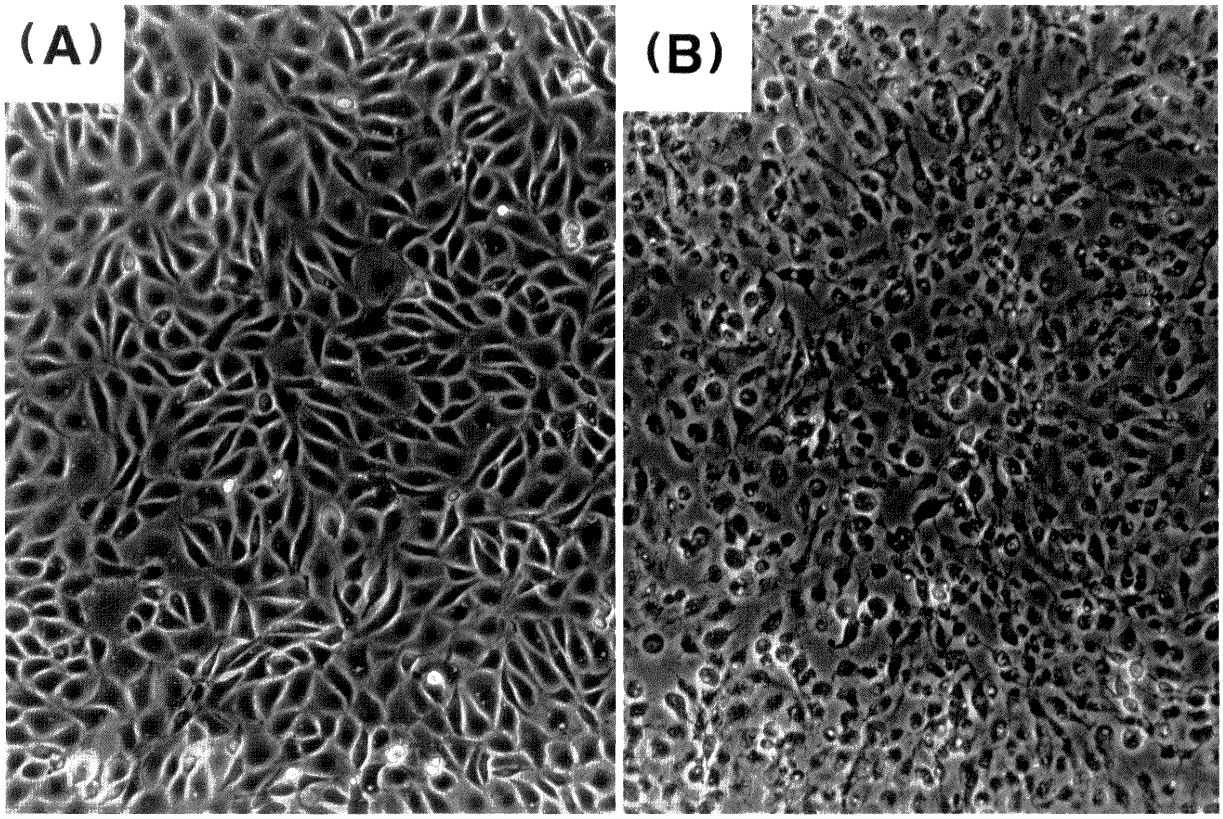

Fig. 4. Change in morphology of endothelial cell monolayers due to 15-HPETE observed by phase contrast microscopy. (A) Untreated cells; (B) Cells exposed to 50 μM 15-HPETE for 6 hours at 37°C.

stone appearance in the monolayer. In contrast, the exposure of endothelial cells to 15-HPETE caused complete detachment of cells from each other and also from the culture dish, followed by cell lysis.

These results suggest that among various arachidonic acid metabolites examined, only 15-HPETE can cause such a severe cell injury as one leading to cell death.

Characterization of 15-HPETE-induced endothelial cell injury

The injurious activity of 15-HPETE to endothelial cells was compared with other peroxides such as hydrogen peroxide (H_2O_2) and tertiary-butylhydroperoxide (t-BuOOH), the latter has been used as a model compound of lipid peroxide. H_2O_2 and t-BuOOH also caused cellular injury in a dose-dependent manner, but their injurious activities were much less potent than that of 15-HPETE (Fig. 5).

In addition, the cellular injury due to 15-HPETE was temperature-dependent. When the temperature was reduced from 37°C to 27°C, endothelial cell injury induced by 15-HPETE significantly decreased (Fig. 6). The similar temperature dependent results were obtained in the cases of H_2O_2 and t-BuOOH. In these cases, the injurious activity was completely abolished at 27°C, even at higher doses as shown in Fig. 6, clearly distinct from the case of 15-HPETE.

Next in order to find out whether the 15-HPETE-induced endothelial cell iujury was energy-dependent, the effect of inhibitors of ATP generation was studied. As shown in Fig. 7, endothelial cell injury due to

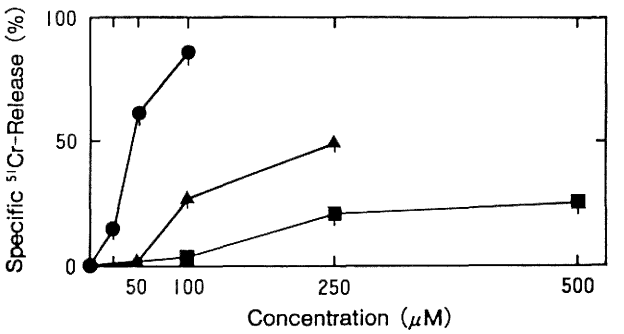

Fig. 5. Comparison of 15-HPETE with t-BuOOH and H_2O_2 in the injurious activity to endothelial cells. ^{51}Cr-labeled bovine endothelial monolayers were incubated with various concentrations of 15-HPETE (circles), t-BuOOH (triangles) and H_2O_2 (squares) for 6 hours at 37°C. Each point represents the mean ±SD, n=3-6.

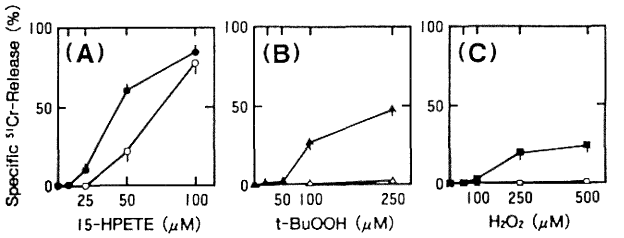

Fig. 6. Effect of temperature on endothelial cell injury induced by 15-HPETE, t-BuOOH and H_2O_2. ^{51}Cr-labeled bovine endothelial monolayers were incubated with 15-HPETE (A), t-BuOOH (B) and H_2O_2 (C) for 6 hours at 37°C (closed symbols) or 27°C (open symbols). Each point represents the mean ±SD, n=3.

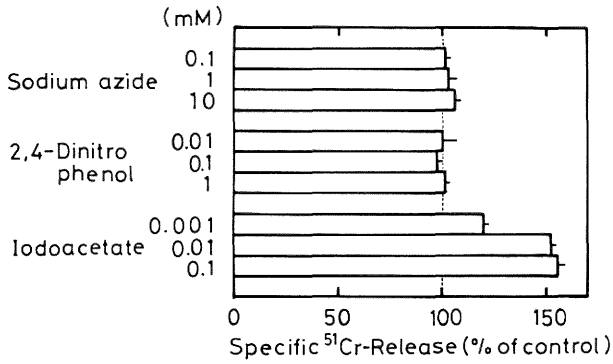

Fig. 7. Effect of inhibitors of ATP generation on endothelial cell injury induced by 15-HPETE. ^{51}Cr-labeled bovine endothelial monolayers were incubated with 25 μM 15-HPETE for 6 hours at 37°C in the presence of each compound. Each value represents the mean ±SD, n=3-6.

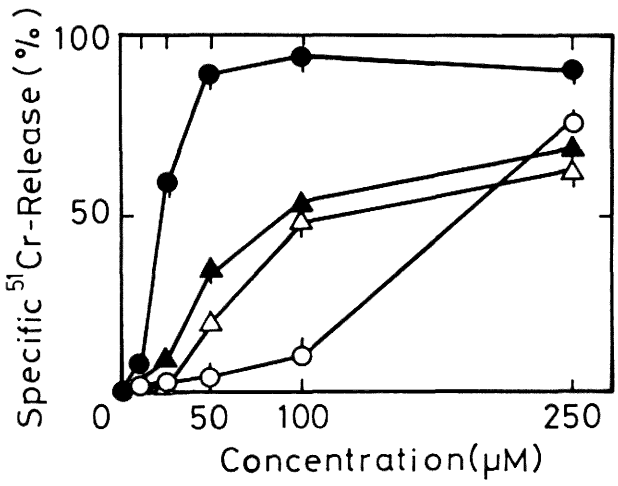

Fig. 8. Effect of FBS on endothelial cell injury induced by 15-HPETE and t-BuOOH. ^{51}Cr-labeled bovine endothelial monolayers were incubated with 15-HPETE (circles) or t-BuOOH (triangles) for 6 hours at 37°C in the absence (closed symbols) or presence of 10% FBS (open symbols). Each point represents the mean ±SD, n=3.

15-HPETE was not inhibited even when the endothelial monolayers were treated with sodium azide or 2, 4-dinitrophenol, which are respiratory inhibitors. In the case of iodoacetate, an inhibitor of glycolysis, a significant increase in the cellular injury was observed.

FBS also affected endothelial cell injury due to 15-HPETE. As seen in Fig. 8, the injurious activity of 15-HPETE was markedly decreased when assayed under a 10% FBS containing medium, while in the case of t-BuOOH serum showed little activity.

These results suggest that endothelial cell injury caused by 15-HPETE may be quantitatively and qualitatively distinct from that by t-BuOOH and H_2O_2.

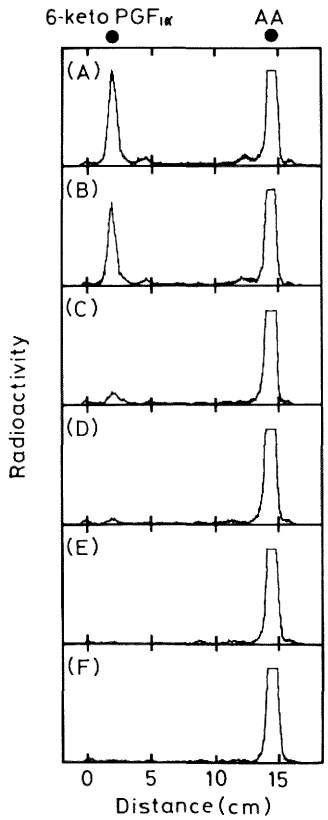

Fig. 9. Effect of 15-HPETE on PGI_2 production by endothelial cells. Confluent monolayers were exposed to 15-HPETE at 0 (A), 1 (B), 5 (C), 10 (D), 25 (E) μM for 1 hour or 25 μM for 6 hours (F) at 37°C and harvested for sonication. The resulting homogenates were incubated with [^{14}C] arachidonic acid, and then the reaction products were extracted and analyzed by radio thin-layer chromatography. The data are representative of the three separate experiments.

Effect of PGI_2 on 15-HPETE-induced endothelial cell injury

As shown in Fig. 9, 15-HPETE treatment of endothelial cell monolayers caused a dose-dependent inhibition of PGI_2 synthesis from arachidonic acid by the cells. When exposed to 25 μM 15-HPETE for 6 hours, there was no PGI_2 production by endothelial cells, with significant increase in ^{51}Cr-release being observed. Treatment of the cells for 1 hour with 25 μM

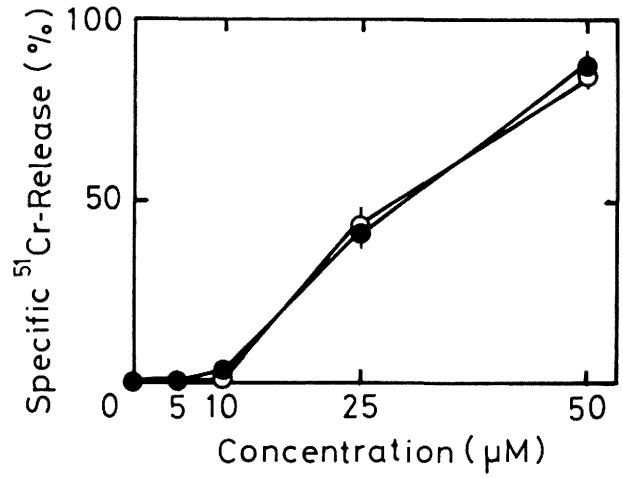

Fig. 10. Effect of aspirin treatment on endothelial cell injury induced by 15-HPETE. ^{51}Cr-labeled bovine endothelial monolayers were incubated with various concentrations of 15-HPETE for 6 hours at 37°C in the absence (closed symbols) or presence (open symbols) of 0.5 mM aspirin. Each point represents the mean ±SD, n=6.

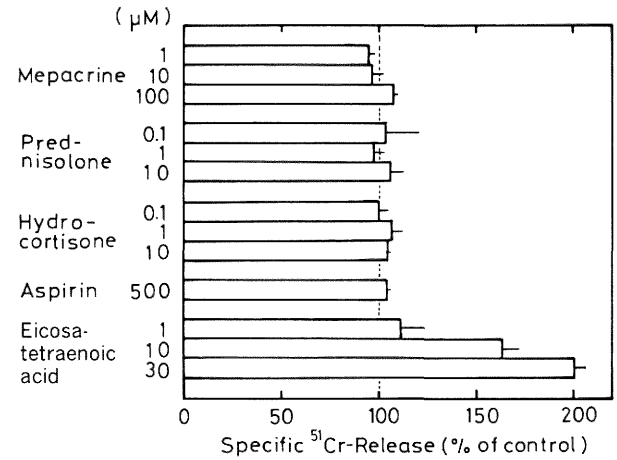

Fig. 11. Effect of inhibitors of arachidonic acid metabolism on endothelial cell injury induced by 15-HPETE. ^{51}Cr-labeled bovine endothelial monolayers were incubated with 25 μM 15-HPETE for 6 hours at 37°C in the presence of each compound. Each value represents the mean ± SD, n=3.

15-HPETE also caused complete inhibition of PGI_2 synthesis, but in this case there was no ^{51}Cr-release from prelabeled cells. Similar results were obtained when endothelial cells were exposed to 5 μM or 10 μM 15-HPETE for 1 hour.

Fig. 10 shows the effect of aspirin, a cyclooxygenase inhibitor, on endothelial cell injury induced by 15-HPETE. Pretreatment of endothelial monolayers with 0.5 mM aspirin blocked PGI_2 production completely (data not shown), but the injurious activity of 15-HPETE was not enhanced even in the presence of aspirin. Effects of some other inhibitors of PGI_2 production were also examined (Fig. 11). Mepacrine, prednisolone and hydrocortisone, which are known as phospholipase A_2 inhibitors, had no effect on 15-HPETE-induced endothelial injury. Eicosatetraenoic acid, a dual inhibitor of cyclooxygenase and lipoxygenase, caused a dose-dependent increase in ^{51}Cr-release induced by 15-HPETE, which was different from the effects of aspirin and other inhibitors described above.

These results suggest that there are some discrepancies in time course and dose dependence between 15-HPETE-induced cellular injury and 15-HPETE-induced inhibition of PGI_2 synthesis by endothelial cells.

DISCUSSION

In the present study, we have demonstrated that 15-HPETE, a lipoxygenase metabolite of arachidonic acid, caused severe injury to cultured endothelial cells in the ^{51}Cr-release assay and phase contrast microscopic observation (Table 1, Fig. 4). This endothelial injury caused by 15-HPETE was time- and dose-dependent (Fig. 3), and its injurious activity was exclusively potent among various arachidonic acid metabolites examined. In contrast to 15-HPETE, 15-HETE had no injurious activity, indicating that this injury may be partly due to radical-related reaction, since it has been believed that hydroxy radicals are produced in the process of conversion of HPETE to HETE. STA_2, a stable analogue of TXA_2, was recently reported to cause injury to human endothelial cells.[20] However, in our ^{51}Cr-release assay system, STA_2 essentially had no effect. The reason for the difference is not clear, but it may be because of the species differences in susceptibility to injury.

To elucidate the mechanism of endothelial cell injury induced by 15-HPETE, we studied H_2O_2- and t-BuOOH-induced endothelial injury. H_2O_2 and t-BuOOH have been extensively utilized as injurious agents to endothelial monolayers[21,22]. H_2O_2 and t-BuOOH also caused endothelial injury in a dose-dependent manner. However, their injurious activities were found to be much less potent than that of 15-HPETE (Fig. 5). In addition, it was revealed that 15-HPETE-induced cellular injury was qualitatively distinct from the injury induced by H_2O_2 and t-BuOOH. As shown in Fig. 8, the injurious activity due to 15-HPETE markedly decreased in the presence of FBS, while that of t-BuOOH did not. Even under an FBS-supplemented condition, t-BuOOH could show the same extent of injury as under the serum-free condition. The reason for this phenomenon is not obvious, but when the incorporation of 15-HPETE into endothelial monolayers was examined, it was found that the incorporation was dependent on FBS concentration (data not shown). This result suggests that the variation seen in the two different types of injurious substances may be at least partly due to the differences in the ability of each substance to be taken up into cellular lipids. It also suggests that the incorporation of 15-HPETE into endothelial cell membrane may be the key point to elucidate the mechanism of 15-HPETE-induced endothelial cell injury. Our preliminary experiments showed that the cellular injury caused by 15-HPETE was enhanced when endothelial monolayers were pretreated with various kinds of polyunsaturated fatty acids which might be able to modify the fluidity of cell membrane. Fig. 6 showed the effect of temperature on the endothelial injury. Cellular damages due to the above-mentioned three peroxides were diminished when the temperature was lowered from 37℃ to 27℃. Especially, in the cases of t-BuOOH and H_2O_2, the injurious activities were completely abolished at 27℃, even at high doses. These results suggest that endothelial cell injury due to 15-HPETE is clearly distinct from that of the other peroxides.

15-HPETE is well known to be an inhibitor of PGI_2 synthetase. Therefore, we next examined the role of endothelial PGI_2 in 15-HPETE-induced endothelial cell injury. Pretreatment of endothelial monolayers with varying concentrations of 15-HPETE resulted in a dose-dependent inhibition of the PGI_2 biosynthesis (Fig. 9). In addition, 15-HPETE was able to induce a time- and dose-dependent injury to endothelial cells, which then led to cell lysis (Fig. 3, 4). However, in contrast to the inhibitory effect of 15-HPETE on PGI_2 synthesis, the injurious effect of 15-HPETE required much more time and much more dose. Moreover, there was no aggravation in the 15-HPETE-induced cellular injury even when endothelial cells were pretreated with various kinds of in-

hibitors of PGI_2 production to suppress endogenous PGI_2 synthesis (Fig. 10, 11). Exogenously added PGI_2 also had no protective effect on the injury due to 15-HPETE (data not shown). The reason for the enhancement of 15-HPETE-induced cellular injury by eicosatetraenoic acid (Fig. 11) is not clear, but it may be caused by its non-specific effect.[3] These results suggest that inhibition of endothelial PGI_2 production by 15-HPETE is not directly associated with endothelial cell injury induced by 15-HPETE.

As we mentioned in our "INTRODUCTION", endothelial cell injury is recognized to be the first step toward the development of atherosclerosis. Because of their location, endothelial linings are always exposed to various aggressive factors, including toxic oxygen metabolites. For example, when neutrophils are activated, reactive oxygen species such as H_2O_2 and lipid peroxides are released out of the cells, and because of the peroxides, highly potent cytotoxicity, the endothelial monolayer may be damaged.[23,24] 15-HPETE, which is also produced in endothelial cells as well as neutrophils when they are stimulated, is a lipid peroxide. As described above, 15-HPETE caused severe injury to endothelial cells. This may occur simply because 15-HPETE is a lipid peroxide. In addition, 15-HPETE inhibited PGI_2 production by endothelial cells. PGI_2 is well known to be a potent inhibitor of platelet aggregation and a vasodilator. In this sense, 15-HPETE can act as an injurious factor to endothelial cell function. Moreover, 15-HETE is reported to be mitogenic for endothelial cells[25] and chemotactic for smooth muscle cells.[26] Additionally, 15-HPETE production[13,14] and reduced PGI_2 production[27,28] have been found to occur in atherosclerotic vessel walls. Therefore, it is suggested that 15-HPETE and its related compounds, which are released from inflammatory cells or even produced in endothelial monolayers, may play a critical role in the pathogenesis of vascular diseases, including atherosclerosis. Further experiments are in progress to clarify the mechanism of 15-HPETE-induced endothelial cell injury and its potential role in atherogenesis.

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