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Effects of Antiinflammatory Agents on Acetaldehyde Induced Cytotoxicity

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Acetaldehyde 유도 세포독성에 대한 항염증제의 영향

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ABSTRACT—In order to get informations on the development of alcohol induced cardiovascular disorders, primary cultured vascular smooth muscle cells (PVSMC) were treated with acetaldehyde, one of the most reactive metabolites of ethanol. Acetaldehyde caused the striking release of lactate dehydrogenase (LDH) from PVSMC and it stimulated the prostaglandin synthesis in the same system. But it didn't induce cyclooxygenase activity. Lipoxygenase inhibitors-propyl gallate and nordihydroguaiaretic acid could reverse the effect of acetaldehyde, but dexamethasone, a phospholipase A_2 (PLA_2) inhibitor and cyclooxygenase inhibitors except indomethacin could not protect the cells from acetaldehyde toxicity. These results indicate that enhanced prostaglandin synthesis by acetaldehyde is not a direct cause of cell death, but secondary effect due to the activation of PLA_2 and also, the roles of the lipoxygenase metabolites and/or PLA_2 activity itself might be more important in the cytotoxicity of acetaldehyde.

Keyword □ **Acetaldehyde, prostaglandin synthesis, LDH release**

Chronic alcohol abusers may develop clinical signs of cardiac dysfunctions, and up to 50 percent of the difference between normal death rates and those of alcohol dependent individuals and heavy drinkers may be attributed to cardiovascular disorders.[1] Alcohol abuse affects the cardiovascular system in several ways.[2-5] Alcohol can affect the heart muscle itself, producing cardiomyopathy and cardiac arrhythmias. Chronic alcohol consumption is associated with a significant increase in hypertension and may play an important role in ischemic heart disease and cerebrovascular disorder including stroke. Although many reports on relationship between alcohol ingestion and the development of alcohol-induced cardiovascular disorders are coming out, the action mechanism and pathologic processes still remain to be elucidated.[6] This study was stimulated to determine whether acetaldehyde-highly reactive metabolite of ethanol, could exert the same effects as ethanol in vascular smooth muscle. Quite strikingly, it caused dramatic increase of prostaglandin synthesis in primary cultured vascular smooth muscle cells (PVSMC) and this result was thought to be somehow associated with cell death process. Thus, this experiment was designed to determine whether alterations in prostaglandin synthesis is a direct cause of acetaldehyde induced cytotoxicity or simply result of it.

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Material and Methods

Materials

Indomethacin, aspirin, sod. salicylate, dexamethasone, propyl gallate, nordihydroguaiaretic acid (NDGA) and bacterial lipopolysaccharide were obtai-

ned from Sigma Chemical (St. Louis, USA). Dulbecco's MEM (DMEM), Ham's 12 media and fetal bovine serum were purchased from GIBCO (Grand Island, USA). And all other reagents were of first grade.

Isolation and cultivation of smooth muscle cells

Rats were maintained on a standard chow diet (Samyang Feed Prod., Korea) were sacrificed after an overnight fast with tap water provided. Rats were anesthetized with diethyl ether and the chest and abdomen were wiped down with 70% ethanol. Rats were then sacrificed by exanguation from the abdominal aorta. The chest cavity was opened and the thoracic aorta was excised using asceptic techniques and placed in Ham's F12 media (GIBCO, Grand Island, NY, USA). Isoaltion and cultivation of the cells were done according to the modified method of Thyberg et al.[7] Under asceptical condition, connective tissue and small arteries were removed from aorta. A longitudinal slit was made in the aorta and placed in 0.1% collagenase solution in Ham's F12 media. After one hour treatment, the inner lining of the aorta was scraped with a rubber policeman to remove the endothelial cells and the adventitia was pulled away. Next, the remaining medial layer was placed in fresh 0.1% collagenase solution and cut into approximately 1 mm^2 pieces and allowed to digest overnight in 5% CO_2/95% air at 37℃. After overnight incubation, the digested cells were spun down for 5 minutes and resuspended in Dulbecco's modified essential media (DMEM, GIBCO) containing 10% fetal calf serum and 1% antibiotic mixture (Pen-Strep, GIBCO). The isolated smooth muscle cells were then placed in a sterile 60 mm petri dish (Corning, NY) and allowed to attached while incubating in 5% CO_2/95% air at 37℃. Cells were maintained until confluent with the media being changed twice a week. Second or third passage PVSMC were used throughout the study and cells were starved serum for 48 hours before acetaldehyde treatment. Cells were treated with acetaldehyde and samples for 12 hours unless otherwise mentioned and the spent media were saved for biochemical analysis.

Determination of Prostaglandin Formation

Media from PVSMC which received experimental treatment was obtained and quantitation of the major eicosanoid produced (6-keto-$PGF_{1\alpha}$) was determined by radioimmunoassay (RIA). Both accumulation levels and cyclooxygenase activity were measured. Accumulated levels refer to the concentration of 6-keto-$PGF_{1\alpha}$ produced during specified incubation period. Cyclooxygenase activity was assessed by treating PVSMC with 10 mM arachidonic acid (NuCheck Prep., Minnessota, USA) for ten minutes.

Determination of Lactate Dehydrogenase (LDH) Activity

As a cytotoxicity index, LDH activity released into the media was determined according to the procedure described elsewhere.[8]

Statistical Analysis

All values are the mean± SE. Statistical analysis of the data was performed using Student's t-test by finding the standard error of the difference between two means and testing the size of the difference by this standard error. Differences between values were considered significant if $p<0.01$.

Results and Discussion

Ongoing clinical studies throughout the world indicate a higher than normal incidence of hemorrhagic stroke and other intracranial bleeding among heavy users of alcohol.[1] Such stroke like episodes often appear within 24 hours of drinking binge, and several investigators have suggested that excessive alcohol consumption predisposes to stroke and sudden death.[1] In a recent prospective study among middle aged women, Stampfer et al[5] found that moderate alcohol consumption, defined as three to nine drinks a week, was associated with increased risk of a hemorrhage beneath the arachnoid membrane, which is the middle one of three membranes covering the brain and spinal cord. In animals, moderate amounts of alcohol can cause spasm in cerebral blood vessels.[2] The alcohol-stroke relation may be partially explained by association of both drinking and stroke with hypertension and a bleeding tendency due to alcohol.[3] Although many reports on as-

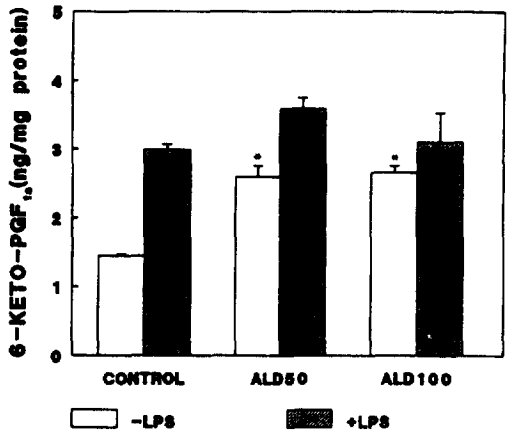

Fig. 1. Effect of acetaldehyde on prostaglandin synthesis of primary cultured vascular smooth muscle cells. ALD50 and ALD100 denote 50 mg% and 100 mg% of acetaldehyde respectively. Bacterial lipopolysaccharide (LPS) was added at the concentration of 10 μg/ml. *P<0.01 vs control.

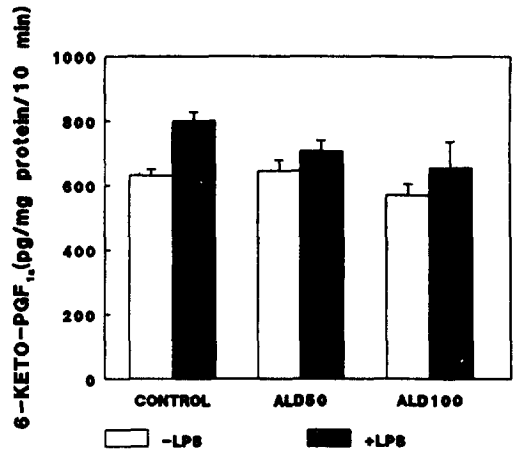

Fig. 2. Effect of acetaldehyde on the activity of prostaglandin H synthase of primary cultured vascular smooth muscle cells. ALD50 and ALD100 denote 50 mg% and 100 mg% of acetaldehyde respectively. Bacterial lipopolysaccharide (LPS) was added at the concentration of 10 μg/ml.

sociations of alcohol ingestion and cardiovascular dysfunction have been published, the overall pathologic processes of alcohol induced disorders are still unclear. Recent studies show that the heart directly metabolizes ethanol and resulting metabolites may induce dysfunction in the mitochondria.[3,4] Thus, in order to get information on the biochemical process of the alcohol induced vascular dysfunction, we tried to determine whether acetaldehyde, one of the most reactive metabolites of ethanol could explain the effects of alcohol on primary cultured vascular muscle cells (PVSMC). Our preliminary study showed that alcohol stimulated the prostaglandin synthesis in PVSMC (Data are not shown). Acetaldehyde also increased the 6-keto-$PGF_{1\alpha}$ synthesis in the same system (Fig. 1), and this effect was comparable to that of bacterial lipopolysaccharide (LPS) which is the most potent stimulator so far tested in PVSMC (our unpublished result). But the combination treatment of acetaldehyde and LPS did not show any further increase compared to individual treatment. In contrast to LPS, acetaldehyde did not induce cyclooxygenase activity under this experimental condition, rather, there was a decreasing tendency (Fig. 2). And also, unlike LPS treatment, acetaldehyde caused the striking alteration of cell morphology which might be the sign of cell injury. Thus, we assumed that the increase of prostaglandin synthesis in this system might be somehow correlated with cell death. In fact, it is well recognized that tissues are known to respond to mechanical or chemical disturbance with the generation of prostaglandins and it is only during injury and inflammation that high levels of prostaglandin can be measured.[9,10] Thus, lactate dehydrogenase (LDH) release was de-

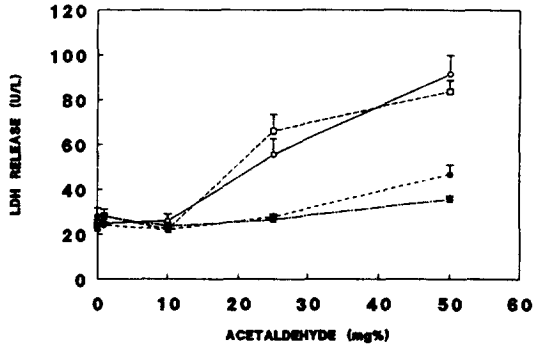

Fig. 3. Effect of indomethacin on the acetaldehyde induced cytotoxicity. Indomethacin (IM) was added at the concentration of 100 μg/ml. bacterial lipopolysaccharide (LPS) was added at the concentration of 10 μg/ml. □ acetaldehyde + LPS, ■ acetaldehyde + LPS + IM, ○ acetaldehyde, ● acetaldehyde + IM

Table 1. Effects of antiinflammatory agents on acetaldehyde induced cytotoxicity

Group		LDH Release (U/L)
Control		25.2± 1.6
25 mg% Acetaldehyde treated		
Control		65.8± 8.9
Indomethacin	100 μM	21.9± 2.3*
Dexamethasone	20 μM	36.9± 3.8*
	2 μM	56.4± 3.4
n-Propyl Gallate	200 μM	25.5± 2.9*
NDGA	100 μM	35.2± 0.2*
Aspirin	1 mM	69.8± 2.9
	500 μM	63.5± 5.2
	100 μM	66.1± 5.9
Salicylic Acid	500 μM	66.7± 3.7
	100 μM	55.1± 3.9
Imidazole	20 mM	49.5± 5.7**
	10 mM	57.6± 8.0
	20 μM	55.2± 8.0

Values are means± SE. Results are obtained from three separate experiments. NDGA means a nordihydroguaiaretic acid. *$P<0.01$, **$P<0.05$

termined whether acetaldehyde injured PVSMC, and if it does, whether that injury can be protected by the treatment of indomethacin which is a potent cyclooxygenase/lipoxygenase inhibitor. As shown in Fig. 3, acetaldehyde increased the release of LDH activity from PVSMC and indomethacin could almost completely reverse this effect. And in the case of 25 mg% acetaldehyde treatment, indomethacin could protect the change of cell morphology. LPS alone did not elicit any release of LDH from PVSMC (data are not shown) and combination treatment of LPS and acetaldehyde failed to increase further release of LDH comared with acetaldehyde alone. In order to determine whether this protective effect is specific to indomethacin only, various inhibitors of eicosanoid metabolism were tested. Interestingly, inhibitors of lipoxygenase pathway (PG and NDGA) were more effective than others (Table 1). Salicylic acid and aspirin which are specific inhibitors of cyclooxygenase did not show any preventive effect at all, and imidazole, which is a specific inhibitor of thromboxane synthetase, showed negligible effect. And also, dexamethasone did exert minimal protective effect against acetaldehyde induced cytotoxicity. Tested inhibitors, by themselves, did not have any noticeable effect on LDH release.

There is increasing evidence that the mechanisms of chemically mediated cell death are common to a wide variety of cell types and to a large number of toxic compounds. Among them, the perturbation of Ca^{2+} homeostasis is recognized to be particularly important.[11] The most likely connecting link in the mechanism of cytotoxicity between an increase in the cytosolic free Ca^{2+} concentration and cell death is the Ca^{2+} activation of phospholipase A_2 (PLA_2) and the conversion of the released arachidonic acid into eicosanoids which in some way as yet unknown cause cell death.[10-13] And also, lysophospholipids, another resulting products of PLA_2, are known to be potent cytotoxic agents by themselves.[13] In fact, aldehydes are known to disrupt the cellular Ca^{2+} homeostasis by releasing Ca^{2+} from endoplasmic reticulum.[13] Indomethancin is an fairly specific inhibitor of cyclooxygenase, but at high concentration, it can inhibit lipoxygenase and even PLA_2. And also, PG and NDGA can inhibit not only lipoxygenase but PLA_2. Thus, our experimental results described above could be, at least in part, explained by these facts. That is, enhanced prostaglandin synthesis by acetaldehyde is not a direct cause of cell death but secondary effect due to PLA_2 activation. Rather, the role(s) of lipoxygenase metabolites and/or PLA_2 activity itself appears to be more important in the cytotoxicity of acetaldehyde. But, in the consideration of antioxidant nature of these effective compounds, it could not be exlcuded that other explanations are possible. The primary products of both 5-lipoxygenase and cyclooxygenase pathways are hydroperoxides, which in the presence of Fe^{2+} possibly initiate or sustain free radical chains.[10] During the reduction of these peroxides, catalysed by prostaglandin hydroperoxidase, release of a highly oxidizing species, possibly heme associated ferryl species is also observed.[14] Thus, in order to delineate the precise cytotoxic mechanism of acetaldehyde, a possible involvement of free radicals should be addressed in the future study.

Acknowledgement

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국문요약

본 연구는 ethanol의 reactive metabolite인 acetaldehyde의 일차 배양 혈관 평활근 세포에 대한 독성 발현 양식을 규명하기 위한 연구의 일환으로, prostaglandin 합성과 세포독성과의 관련성 여부를 확인하기 위하여 수행되었다. Acetaldehyde는, 일차 배양 혈관 평활근 세포에서의 prostaglandin 합성을 현저히 증가 시켰으며, 이때, cyclooxygenase activity는 큰 변화 없거나 오히려 감소 시키는 경향을 보였다. Cyclooxygenase inhibitor인 indomethacin은 acetaldehyde에 의한 LDH release를 현저히 차단 시켰으며, aspirin 및 salicylic acid는 전혀 영향을 주지 못했다. Phospholipase A_2 (PLA_2) inhibitor로 알려져 있는 dexamethasone은 유의적인 세포 독성 경감 작용을 보이지 못하였으며, Lipoxygenase inhibitor들인 NDGA, propyl gallate 등은 현저한 독성 경감 효과를 보였다. 이상의 결과로부터 acetaldehyde에 의한 일차 배양 혈관 평활근 세포에서의 prostaglandin 합성 증가는 Cell death에 대한 직접적인 원인이 아님을 추론할 수 있었으며, PLA_2/lipoxygenase inhibitor들의 강력한 세포 독성 경감 작용으로 미루어 볼 때, acetaldehyde에 의한 세포 독성은 lipoxygenase 대사 산물 혹은 PLA_2의 직접 작용에 기인할 가능성을 확인할 수 있었다.

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