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Endogenous inhibitors of nitric oxide synthesis and lipid peroxidation in hyperlipidemic rabbits[1]

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KEY WORDS thoracic aorta; vasodilation; vitamin E; atherosclerosis; lipid peroxidation; N^G, N^G-dimethylarginine

AIM: To examine whether the elevation of endogenous N^G, N^G-dimethylarginine (DMA) content is related to lipid peroxidation in the high lipid-fed rabbit. **METHODS**: In high lipid diet-fed rabbits, concentrations of serum cholesterol, triglyceride, malondialdehyde (MDA), and DMA were measured, and endothelium-dependent relaxation to acetylcholine (ACh) was tested. **RESULTS**: After 6-wk on a high lipid-diet, the levels of serum total cholesterol, triglyceride, MDA, and DMA were increased *vs* those in control group (MDA was $2.88 \pm s\ 0.20$ *vs* 1.54 ± 0.13 nmol·L^{-1}, $P < 0.01$ and DMA was 1.51 ± 0.07 *vs* 0.75 ± 0.13 μmol·L^{-1}, $P < 0.01$), while the endothelium-dependent vasodilation in the isolated thoracic aorta was impaired (the maximal response to ACh was 45.59 ± 3.10 *vs* 76.93 ± 5.68 %). Supplementation with vitamin E decreased MDA and DMA content and improved the endothelium-dependent vasodilation. **CONCLUSIONS**: An increase in serum concentration of DMA may be secondary to the elevation of lipid peroxides in the hyperlipidemic rabbit.

l-Arginine is the precursor of nitric oxide (NO) synthesis and *l*-arginine analogues such as methylated arginine can inhibit NO synthesis[1]. N^G, N^G-dimethylarginine (DMA), an endogenous inhibitor of NO synthesis, is present in blood of both humans and animals[2,3]. Recently, we have found that chronic hyperlipidemia induced the elevation of DMA content concomitantly with an increase in plasma concentration of lipid peroxides[4]. Others have demonstrated that supplementation with dietary *l*-arginine improves endothelium-dependent vasodilation and alleviates histomorphologic changes of atherosclerosis in the hyperlipidemic rabbit[5]. The objective of this study, therefore, was to explore whether the content of DMA is correlated with lipid peroxidation in the high fat, high cholesterol fed rabbit.

MATERIALS AND METHODS

Drugs and chemicals N^{ω}, N^G-Dimethylarginine, *o*-phthaldialdehyde and phenylephrine (Sigma); thiobarbituric acid (Fluka); acetylcholine is of analytical grade, vitamin E (Xiamen Fish Liver Oil Factory, China) and cholesterol (Biochemical Agent Co Shanghai Institute of Zheng Xiang Chemical Agent Research, lot № 930219).

Lipid feeding protocol Rabbits (♂, $n = 24$, weighing $2.0 \pm s\ 0.2$ kg) fed a laboratory semisynthetic diet for 6-wk were randomly divided into 3 groups: A) Control group; B) Added 1 % cholesterol, 5 % lard, and 15 % egg for 3-wk and omitted cholesterol for the second 3 wk; C) Received high lipid diet plus vitamin E 200 mg·kg^{-1}·d^{-1} for 6-wk.

Morphological examination After 6 wk, the rabbits were anesthetized with sodium pentobarbital 30 mg·kg^{-1}, iv. Blood samples were collected from the carotid artery, and upper thoracic aortae were opened longitudinally and fixed in methyl aldehyde. The color slides of the aortae stained with hematoxylin and eosin were observed with light microscope for intimal lesions and endothelial integrity.

Vascular reactivity The thoracic aorta was placed in Krebs' solution (NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 11.5 mmol·L^{-1}). The aorta was cut into rings (4 mm), taking special care not to touch their luminal surface. The rings were mounted in organ chambers (30 mL) aerated with 95 % O_2 + 5 % CO_2 at 37 ℃. Isometric force was recorded by a force transducer and a polygraph. The rings were equilibrated for 90 min after a loading of 6.5 *g*. Rings were contracted by the EC_{50} concentration of phenylephrine, and then relaxation to ACh was tested. Relaxation was calculated as a % of the contraction elicited by phenylephrine.

In the study of effect of exogenous DMA on vasodilator responses to ACh, the thoracic aorta from control rabbit was exposed for 30 min, and the agents remained in the perfusate

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for the remainder of the study.

Biochemical assays Blood samples were kept in ice-bath for 24 h and then centrifuged at 2500 × g for 20 min. Serum levels of total cholesterol, triglycerides, and creatine were measured by routine methods. The content of thiobarbituric acid reactive substance reflecting level of lipid peroxide was measured by a spectrofluorometer[6] and expressed as the amount of malondialdehyde (MDA).

To 1.0 mL of the serum, 1.0 mL of 15 % trichloroacetic acid was added, and the mixture was left in ice for 10 min. The precipitated protein was removed by centrifugation at 2500 × g for 15 min, and the supernatant was used for measurement of DMA with HPLC[7]. HPLC was carried out using a Shimadazu LC-6A liquid chromatograph. o-Phthaldialdehyde adducts of methylated amino acids and internal standard DMA produced by pre-column mixing were monitored using a model RF 530 fluorescence detector set at λ_{ex} 330 and λ_{em} 420 nm on a resolve C_{18} column. Samples were eluted from the column using a linear gradient containing solvent A composed of NaAc-MeOH (80:20, v/v) 0.05 mol·L^{-1} and solvent B composed of NaAc-MeOH (20:80, v/v) 0.05 mol at a flow-rate of 1.0 mL·min^{-1}.

Statistical analysis Statistical analysis was performed with one way ANOVA, and Tukey's test was used to determine differences between groups.

RESULTS

Morphological studies The atherosclerotic plaques of aortae from rabbits receiving high lipid diet were severer than those receiving vitamin E + high lipid diet. In both groups intimal thickenings were constituted by agglomerates of cells separated by a negligible amount of interstitial material such as collagen fibrils. Large roundish cells with a foamy cytoplasm and round nucleus constituted the main cell type in the intima. No pathological change was found in the aortae from control group.

Serum lipid profile After 6-wk feeding, serum total cholesterol and triglycerides levels were higher in high lipid-fed rabbits compared with control rabbits. The content of MDA in the plasma was also raised in the high lipid-fed rabbits. Supplementation with vitamin E attenuated the elevation of MDA, but did not affect total cholesterol and triglycerides (Tab 1).

Creatine and DMA in serum The serum creatine was not significantly different between 3 groups (Tab 1).

DMA concentrations were increased in the high lipid-fed rabbits compared with those of control group. However, supplementation with vitamin E markedly decreased the elevation of DMA level elicited by chronic hyperlipidemia (Tab 1).

Tab 1. Effect of vitamin E on serum contents of total cholesterol (TC), triglycerides (TG), creatine (Cre), malondialdehyde (MDA) and dimethylarginine (DMA) in normal (Control), high lipid (Lipid) and vitamin E + high lipid (Vit E + lipid) diet-fed rabbits. $n = 7-8$, $\bar{x} \pm s$. **[a] $P > 0.05$, [b] $P < 0.05$, [c] $P < 0.01$ *vs* control; [d] $P > 0.05$, [e] $P < 0.05$, [f] $P < 0.01$ *vs* lipid.**

	Control	Lipid	Vit E + Lipid
TC (mmol·L^{-1})	0.81 ± 0.11	2.13 ± 0.27[c]	1.74 ± 0.36[e]
TG (mmol·L^{-1})	0.39 ± 0.04	1.10 ± 0.14[c]	0.84 ± 0.16[e]
Cre (μmol·L^{-1})	70.72 ± 4.72	76.33 ± 4.94[a]	67.04 ± 5.35[a]
MDA (nmol·L^{-1})	1.54 ± 0.13	2.88 ± 0.20[c]	1.85 ± 0.07[f]
DMA (μmol·L^{-1})	0.75 ± 0.13	1.51 ± 0.07[c]	1.17 ± 0.07[e]

Vasodilator responses In aorta rings from normal diet-fed rabbits, ACh evoked endothelium-dependent relaxation in a concentration-dependent manner, which was impaired in the high lipid-fed rabbits. Supplementation with vitamin E prevented impairment of endothelium-dependent relaxation. The maximal response to ACh was reduced in the high lipid-fed rabbits, while the reduction in the maximal response to ACh of aortae from the rabbits supplemented with vitamin E was less compared with those from rabbits fed high lipid alone (Fig 1).

To confirm the role of DMA in inhibition of endothelium-dependent relaxation, exogenous DMA 0.8 and 1.5 μmol·L^{-1} (similar to endogenous DMA levels in serum of control and high lipid-fed rabbits, respectively) was tested. DMA 1.5 μmol·L^{-1} inhibited ACh-induced vasodilation, while DMA 0.8 μmol·L^{-1} did not influence the vasodilator responses to ACh (Fig 2).

DISCUSSION

Treatment with N^G-nitro-*L*-arginine methyl ester, an exogenous inhibitor of NO synthesis, deteriorates impairment of endothelium-dependent relaxation and atherosclerotic lesions in hyperlipidemic rabbits[8,9]. In the present study, vasodilator

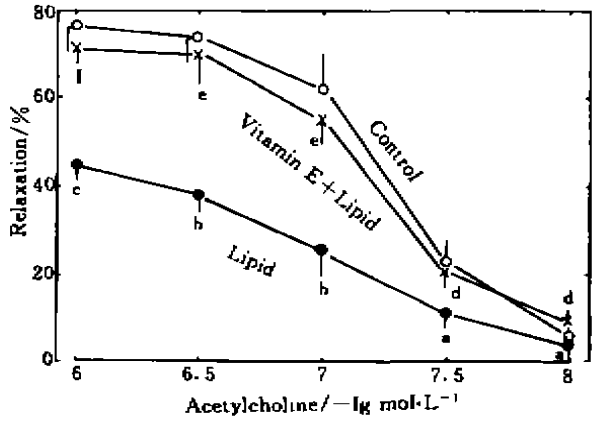

Fig 1. Effects of vitamin E on endothelium-dependent relaxation to ACh in isolated rabbit thoracic aortae from control diet ($n = 6$), high lipid diet ($n = 6$), and vitamin E + high lipid diet-fed rabbits ($n = 6$). $\bar{x} \pm s$.
$^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control;
$^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* lipid.

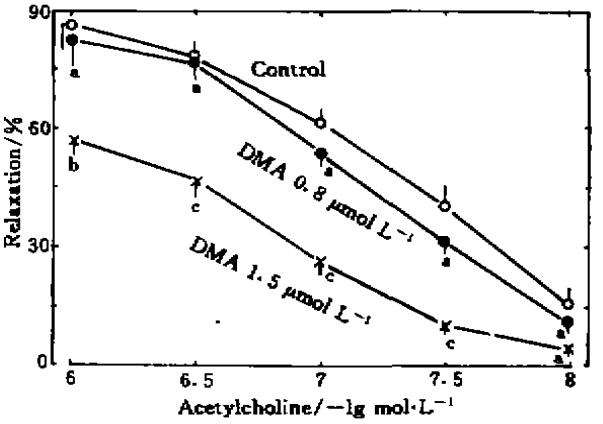

Fig 2. Effects of exogenous dimethylarginine (DMA) on endothelium-dependent relaxation to ACh in isolated thoracic aortae from normal rabbits. $n = 4-6$. $\bar{x} \pm s$.
$^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

responses to ACh in isolated aortae of hyperlipidemic rabbits were impaired with the elevation of DMA level. It is probable that hyperlipidemia induces the increase of endogenous DMA, with a subsequent reduction of synthesis and/or release NO, contributing to the formation of atherosclerosis.

Exposure of intact vessels to oxidized LDL results in impairment of endothelium-mediated relaxation[10]. As others have reported previously[11], supplementation with vitamin E, a potent chain-breaking antioxidant, decreased the serum level of MDA and prevented the severity of atherosclerotic lesions in hyperlipidemic rabbits. It is of interest that vitamin E decreased lipid peroxides (MDA) level and reduced concentrations of DMA in the hyperlipidemic rabbit. The data suggest that impairment of endothelium-dependent relaxation elicited by hyperlipidemia may be due to stimulation of DMA production via elevation of lipid peroxides, resulting in reduction of NO synthesis. But the precise mechanisms for increased DMA content in the high lipid-fed rabbits remain to be determinated.

In conclusion, the present results suggest that: (1) chronic hyperlipidemia in rabbits causes an increase in the content of endogenous DMA which may be a contributing factor in atherogenesis, and (2) the elevation of DMA content may be secondary to the increase of lipid peroxides.

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高胆固醇血症家兔 NO 合成酶抑制物含量与脂质过氧化的关系

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高脂血病 一氧化氮合成酶

关键词 胸主动脉; 血管舒张; 维生素 E; 动脉粥样硬化; 脂质过氧化; 二甲基精氨酸

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目的: 探讨高脂饲养家兔血中 NO 合成酶抑制物二甲基精氨酸(DMA)含量变化与脂质过氧化的关系. **方法**: 检测高脂饲养家兔血清总胆固醇、甘油三脂、丙二醛(MDA)及 DMA 含量, 并观察离体胸主动脉内皮依赖性舒张反应. **结果**: 高脂饲养家兔血脂、血清 MDA 和 DMA 含量比正常组增加(MDA 为 2.88 ± s 0.20 *vs* 1.54 ± 0.13 nmol·L^{-1}, $P < 0.01$, DMA 为 1.51 ± 0.07 *vs* 0.75 ± 0.13 μmol·L^{-1}, $P < 0.01$), 胸主动脉舒张反应降低(最大舒张% 为 45.59 ± 3.1 *vs* 76.93 ± 5.68 %). 维生素 E 抑制 MDA 升高的同时降低 DMA 含量及改善内皮舒张功能. **结论**: 高脂血症家兔血清 DMA 含量的升高可能与脂质过氧化的增加有关.

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Heterogeneity of human platelet density subpopulations in aggregation, secretion of ATP, and cytosolic-free calcium concentration

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KEY WORDS blood platelets; platelet aggregation; calcium; adenosine diphosphate; adenosine triphosphate; thrombin; serotonin

AIM: To investigate thrombin (500 U·L^{-1})-, ADP (0.1 - 30 μmol·L^{-1})-, and 5-hydroxytryptamine (5-HT, 3 μmol·L^{-1})-induced aggregation, secretion of ATP and cytosolic-free calcium mobilization in density subpopulations of human washed platelets. **METHODS**: Using Percoll discontinuous gradient. **RESULTS**: The human platelets were separated into high density (HD), intermediate density (ID), and low density (LD) subpopulations, and their sizes were diminished with decreasing density ($r = 0.978$, $P < 0.01$). The magnitude of aggregations by thrombin, ADP, and 5-HT was more significant in HD platelets than that in LD platelets ($P < 0.01$). The amount of secretion of ATP induced by thrombin and ADP in HD platelets was also much higher than that in LD platelets ($P < 0.01$), except for 5-HT which did not cause the ensuring release reaction in any subpopulation of human platelets. Thrombin (1500 U·L^{-1})-, ADP (μmol·L^{-1})-, and 5-HT (3 μmol·L^{-1})-induced cytosolic-free calcium mobilization was evaluated as well. Results showed that the resting level of cytosolic-free calcium concentration ($[Ca^{2+}]_i$) was the same in all subpopulations, about 80 - 90 nmol·L^{-1}. However, the level of $[Ca^{2+}]_i$ mobilization was entirely different, heightened with increasing density. **CONCLUSION**: The function of HD platelets was much stronger and more active than that of LD platelets in human.

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The previous studies suggested that platelet function was different with platelet density in rabbits[1,2] and the density was depends on the composition of subcellular components[3], especially on the