Original article

γ-Tocopherol, but not α-tocopherol, potently inhibits neointimal formation induced by vascular injury in insulin resistant rats

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Abstract

Insulin resistance may enhance the neointima formation via increased oxidative stress. However, clinical trials investigating the benefit of antioxidant therapy with α-tocopherol showed negative results. Recent studies showed that chemical characteristics of γ-tocopherol are distinct from those of α-tocopherol. We hypothesized that γ-tocopherol is superior to α-tocopherol in preventing the neointima growth after arterial injury in insulin resistance. Male rats were fed with standard chow or a high fructose diet for induction of insulin resistance. Thereafter, the left carotid artery was injured with a balloon catheter. After 2 weeks, the carotid arteries were harvested and histomorphometrically analyzed. The neointima–media ratio of the injured artery was significantly greater in insulin resistance group ($n=8$, 1.33 ± 0.12) than in normal group ($n=10$, 0.76 ± 0.11, $p<0.01$). γ-Tocopherol (100 mg/kg/day) reduced the ratio ($n=5$, 0.55 ± 0.21, $p<0.01$ vs. insulin resistance group), while α-tocopherol was without effect ($n=7$, 1.08 ± 0.14). The quantification of plasma phosphatidylcholine hydroperoxide, an indicator of systemic oxidative stress, and dihydroethidium fluorescence staining of the carotid artery, an indicator of the local superoxide production, showed that oxidative stress in the systemic circulation and local arterial tissue was increased in insulin resistance. Both tocopherols decreased plasma phosphatidylcholine hydroperoxide, but failed to suppress the superoxide production in the carotid arteries. Increased 3-nitrotyrosine in neointima by insulin resistance was greatly reduced only by γ-tocopherol. In conclusion, γ-tocopherol, but not α-tocopherol, reduces the neointima proliferation in insulin resistance, independently of its effects on superoxide production. The beneficial effect may be related with its inhibitory effects on nitrosative stress.

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1. Introduction

Insulin resistance (IR) which latently progresses in subjects with multiple risk factors, enhances vascular oxidative stress [1] and causes neointimal proliferation which are relevant to atherosclerosis and restenosis after angioplasty [2]. As increasing evidence has shown that oxidative stress plays an important role in the neointima proliferation [3], antioxidant therapy may be a promising strategy for the prevention of atherosclerotic diseases. However, randomized clinical studies investigating the benefit of antioxidant therapy with vitamin E for the prevention of cardiovascular diseases showed negative results [4].

Vitamin E consists of four tocopherol homologues, and among them α-tocopherol has always been used for supplementation in clinical studies [4,5]. On the other hand, γ-tocopherol has received less attention. Recent evidence

demonstrated that the pharmacokinetics of γ-tocopherol are quite different from those of α-tocopherol [6,7]. In addition, γ-tocopherol has several specific pharmacological properties such as anti-inflammatory and anti-proliferative effects in addition to its antioxidative effects [8,9]. Accordingly, it is worthwhile to compare α- and γ-tocopherol in terms of their preventive effects on neointima formation.

On the basis of these findings, we tested the hypothesis that γ-tocopherol supplementation is superior to α-tocopherol in inhibiting the enhanced neointima formation following vascular injury in an insulin resistant state. We found that γ-tocopherol, but not α-tocopherol, greatly reduces the neointima proliferation following the vascular injury in insulin resistance, independently of its effects on superoxide production, and that γ-tocopherol potently reduces nitrosative stress.

2. Materials and methods

The present studies were approved by the institutional ethics committee for animal experiments. The investigation confirms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.1. Animal model and oral tocopherol supplementation

Eight-week-old male Sprague–Dawley rats (SLC Inc, Hamamatsu, Japan) were divided into 2 groups that were fed with standard chow (Normal group) or high fructose chow containing 62.5% fructose (IR group). The latter group was divided into the following 3 subgroups: (1) IR-vehicle group in which the vehicle for tocopherols (corn oil) was given, (2) IR-α group in which α-tocopherol (100 mg/kg/day) was orally supplemented, and (3) IR-γ group in which γ-tocopherol (100 mg/kg/day) was orally supplemented from 3 days before the vascular injury and continued until the end of experiment. γ-Tocopherol was generously donated by Eisai (Tokyo) and α-tocopherol (DL-all-*rac*-α-tocopherol) was purchased from Sigma (St. Louis, MO). After 4 weeks from the randomization, we induced left carotid arterial injury in all rats as previously described [10]. Briefly, after the induction of anesthesia with pentobarbital (50 mg/kg, i.p.), a midline cervical incision was made. A 2F Fogarty balloon catheter (Edwards Lifescience LLC, Irvine, CA) was introduced into the left external carotid artery and passed three times through the common carotid artery with the balloon distended sufficiently with saline to generate resistance. The balloon inflation pressure ranged from 1.0 to 1.3 atm. We confirmed that the extent of the balloon distension was similar in each procedure by observing with a dissecting microscope.

2.2. Blood pressure measurement and blood chemistry assay

At 14 days after the carotid arterial injury, the rats were fasted for 12 h. Systolic blood pressure was measured by the tail-cuff method (MK-2000, Muromachi kikai, Tokyo, Japan). Then, the rats were anesthetized with pentobarbital (50 mg/kg,

i.p.) and blood samples were collected via the lower abdominal aorta for measuring insulin (RIA), glucose (EIA), and free fatty acid (EIA). After this procedure, the rats were euthanized. The obtained blood samples were immediately centrifuged and the plasma fraction was stored at −80 °C until analysis. The insulin resistance index assessed by the homeostasis model assessment (HOMA-IR) was calculated as follows [11]:

$$\text{HOMA-IR} = \text{FIRI} \times \text{FPG}/22.5$$

where FIRI is the fasting plasma insulin level (μU/mL) and FPG is the fasting plasma glucose level (mmol/L).

2.3. Histomorphometric study

At 14 days after inducing the carotid arterial injury, perfusion fixation with 4% paraformaldehyde was performed. The injured common carotid arteries were embedded in paraffin for sectioning (thickness: 4 μm), and hematoxylin–eosin staining was performed. Two sections from each vessel were histomorphometrically analyzed as previously described [12]. The carotid arterial cross-sectional images were observed with a light microscope (BX51, Olympus, Tokyo) and digitally recorded with a digital camera (DP70, Olympus). Obtained images were analyzed with an image analyzing system (AxioVision 4.4, Carl Zeiss, Jena, Germany). The cross-sectional areas of the neointima and media were measured and the neointima–media ratio was calculated. Two persons who were blinded to the experimental protocols measured the morphometric parameters independently each other.

2.4. Plasma PCOOH measurement

Phosphatidylcholine hydroperoxide (PCOOH) is the primary peroxidized product of phosphatidylcholine and is the most reliable parameter of lipid peroxidization [13]. The plasma PCOOH was measured to evaluate the systemic oxidative stress. Blood samples were collected from 4 groups of rats (Normal: $n=3$, IR-vehicle: $n=5$, IR-γ: $n=5$, IR-α: $n=7$) at 6 h after the carotid arterial injury. The obtained blood samples were immediately centrifuged and the plasma fraction was collected. The total lipid extracts were obtained from 0.4 mL of plasma [14]. Obtained lipid extracts were redissolved with chloroform/methanol (2:1, v/v) and 40 μL of the solution was used for analysis. The plasma PCOOH concentrations were measured by chemiluminescence high-performance liquid chromatography (CL-HPLC), as previously reported [15]. The CL-HPLC system consisted of a Jasco HPLC system (Japan Spectroscopic Co., Tokyo, Japan) combined with a CLD-100 chemiluminescence detector (Tohoku Electronic Industrial Co. Ltd., Sendai, Japan) and a Jasco UV detector (UV-875) equipped with a Jasco Finepak SIL NH2-5 column (*n*-propylamine-bound silica column, 5 μm particle size, 250×4.6 mm, Japan Spectroscopic Co.). The HPLC mobile phase consisted of 2-propanol/methanol/water (135:45:20, v/v/v), and the flow rate was 1.0 mL/min. The chemiluminescence reagent was prepared by dissolving 2.5 μg/mL cytochrome *c*

(from horse heart, type 4; Sigma) and 0.25 μg/mL luminol (3-aminophytaloyl hydrazine; Wako Pure Chemical, Osaka, Japan) in 50 mM borate buffer (pH 10) and was added at a flow rate of 1.4 mL/min. A calibration curve was made for the PCOOH prepared by photo-oxidation of synthetic phosphatidylcholine (2-lionoleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine, Sigma).

2.5. Determination of plasma and carotid artery tocopherol concentrations

Plasma samples were collected for measurement of tocopherols from the 3 groups of insulin resistant rats ($n=5$ in each group) at 6 h after carotid arterial injury. Tocopherols in the plasma samples were extracted as described previously [16]. Plasma (0.2 mL) was mixed with 0.9% NaCl solution (0.8 mL) and 2,2,5,7,8-pentamethyl-6-chromanol (PMC: 1 nmoL, Wako Pure Chemical, Tokyo, Japan) as an internal standard. After vortexing, hexane (3 mL) was added. The sample solution was centrifuged at 2500 rpm for 5 min and the upper hexane layer was collected. The collected hexane layer was evaporated under N_2, and the dried residue was redissolved in 50 μL of hexane. In each case, 20 μL of sample solution was injected into the HPLC system.

Carotid arteries were collected for the quantitation of tocopherols from the 3 groups of rats (IR-vehicle: $n=6$, IR-γ: $n=8$, IR-α: $n=8$) at 6 h after vascular injury. Two vessels each were pooled for the analysis. Tocopherols in the carotid arterial samples were extracted as described previously [16]. Carotid arteries (10 mg) were mixed with 0.9% NaCl solution (0.1 mL), 6% ethanolic pyrogallol (1 mL), and 60% KOH (0.2 mL). PMC (1 nmoL) was added as an internal standard. All contents were saponified at 70 °C for 30 min. Then, 0.9% NaCl solution (4.5 mL) and 10% ethylacetate/hexane solution (3 mL) were added and centrifuged at 2500 rpm for 5 min. The collected hexane layer was evaporated under N_2, and the dried residue was redissolved in 50 μL of hexane. In each case, 20 μL of sample solution was injected into the same HPLC system as for measurement of plasma tocopherol concentration.

The concentrations of tocopherol in the plasma and carotid arterial tissue were similarly measured using an HPLC system as previously reported [16]. The HPLC column was a SILICA SG120A (5 μm particle size; 250 × 4.6 mm, Shiseido Co. Ltd., Tokyo, Japan), the column mobile phase was hexane/2-propanol (1000:5, v/v), and the flow rate was 1.0 mL/min. Postcolumn fluorescence detection was carried out using a RF-10AXL fluorescence detector (Shimazu Co., Kyoto, Japan). The excitation wavelength was 298 nm and emission fluorescence was detected at the wavelength of 325 nm. The tocopherol concentrations of the samples were determined using the known concentration of PMC as an internal standard by HPLC with fluorometry.

2.6. In situ superoxide detection (dihydroethidium staining)

We evaluated the in situ production of superoxide using the oxidative fluorescent dye dihydroethidium (DHE, Sigma) in 4 groups (Normal: $n=7$, IR-vehicle: $n=11$, IR-γ: $n=7$, IR-α: $n=6$) as previously reported [17]. Briefly, at 14 days after the arterial injury, the left common carotid arteries were harvested and frozen in optimized cutting temperature compound (Sakura Finetechnical Co. Ltd., Tokyo) with liquid nitrogen. Carotid arterial ring segments were cut into 20 μm thick sections. DHE (2 μmol/L) was topically applied to each tissue section. Slides were incubated in a light-protected humidified chamber at 37 °C for 30 min. Images were obtained with a laser scanning confocal microscope equipped with a krypton/argon laser (MRC-1024, BioRad, Hercules, CA). The excitation wavelength was 488 nm, and emission fluorescence was detected with a 585-nm long-pass filter. The ethidium fluorescence images were analyzed with LaserSharp software (version 2.1, BioRad). Quantitative analysis was performed by using the mean fluorescence intensity from each arterial segment which was standardized with that of the uninjured carotid arteries of Normal group.

2.7. Lucigenin chemiluminescense

Superoxide production in the carotid artery was measured using the lucigenin-enhanced chemiluminescence method as described previously [18]. Three days after the arterial injury, the carotid arteries were rapidly isolated and placed in ice-cold modified Krebs–HEPES buffer (pH 7.4) containing (in mmol/L): NaCl 119, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1, $CaCl_2$ 1.9, $NaHCO_3$ 25, glucose 11.1, and HEPES 20. Then, excessive adventitia and connective tissue were removed and cut into 3–5 mm ring segments. Carotid arterial segments were equilibrated in Krebs–HEPES buffer gassed with 95% O_2 and 5% CO_2 at 37 °C for 30 min. The vessel segments were then gently transferred to a polypropylene tube. Lucigenin-enhanced chemiluminescence from arterial segments was measured for 10 min in buffer (2 mL) containing lucigenin (5 μM, Sigma) by luminometer (AT-2200, ATTO Co., Tokyo). After measurement of the baseline superoxide production, NADPH(100 μM, Sigma)-induced superoxide production was measured for 10 min. The chemiluminescence count was expressed as counts per minute per milligram of dry weight of vessel.

2.8. Nitrotyrosine immunostaining

We performed immunohistochemistry for 3-nitrotyrosine to investigate the involvement of reactive nitrogen species. After removal of paraffin and rehydration, the carotid arterial sections were treated with 10 mmol/L citrate and microwave-heated for antigen retrieval. The sections were incubated with anti-3-nitrotyrosine rabbit IgG (5 mg/mL, Upstate Biotechnology, Lake Placid, NY) in PBS with 0.5% bovine serum albumin overnight at 4 °C, after nonspecific binding was blocked with 10% goat serum. Endogenous oxidation was blocked with 2% H_2O_2 PBS, and the tissue sections were incubated for 30 min at room temperature with anti-rabbit IgG secondary antibody with use of Histofine (Nichirei, Tokyo). 3,3′-Diaminobenzidine (Dojin, Kumamoto), a peroxidase substrate, was used for visualization of the positive immunoreactivity as brown color.

2.9. Measurement of plasma IL-1β and TNF-α

To evaluate the effect of the tocopherol supplements on systemic inflammation, plasma IL-1β and TNF-α were measured at 3 days after vascular injury in the 3 insulin resistant groups ($n=5$ in each group). The plasma IL-1β and TNF-α levels were measured with an ELISA kit (Biosource Inc. Camarillo).

2.10. Real-time reverse transcription polymerase chain reaction (real-time RT-PCR)

We analyzed the expression of cell cycle-related genes in the carotid artery. We measured the mRNA expressions of cyclin D_1, a cyclin-dependent kinase inhibitor p21^{Cip1} and a tumor suppressor gene p53 with quantitative real-time RT-PCR methods. The common carotid artery samples were isolated from the 3 groups of insulin resistant rats ($n=4$ in each group) at 3 days after the arterial injury. Total RNA was extracted from a pool of 3 carotid arteries from separate rats using an RNase-Free DNase Set (Qiagen) combined with an RNeasy Mini Kit (Qiagen). Reverse transcription was performed with 1 μg of total RNA. Real-time quantitative PCR was performed using SYBR green with a LightCycler (Roche, Basel, Switzerland). The primer sets used for RT-PCR were as follows; cyclin D_1 (GenBank accession no. NM171992): 5′-TACCGCACAACG-CACTTTC-3′ (sense), 5′-GCCTTGGGATCGATGTTCTG-3′ (antisense); p21^{Cip1} (GenBank accession no. NM080782): 5′-GAAAACGGAGGCAGACCAGC-3′ (sense), 5′-GCTAA-GGCAGAAGATGGGGA-3′ (antisense); p53 (GenBank accession no. NM030989): 5′-TCCGAGAGCTGAATGAGGC-3′ (sense), 5′-TATGGCGGGACGTAGACTGG-3′ (antisense); GAPDH (GenBank accession no. AF106860): 5′-TGAACGG-GAAGCTCACTGG-3′ (sense), 5′-TCCACCACCCTGTTGC-TGTA-3′ (antisense). After an initial denaturation step at 95 °C, amplification was performed using 40 cycles of denaturation (95 °C), annealing (60 °C), and extension (72 °C). The calculated relative amount of target gene product was standardized with the amount of the GAPDH-PCR product.

2.11. Statistical analysis

All data were expressed as mean±SEM. Statistical analyses were performed with GraphPad Prism ver. 3.02 (GraphPad Software, San Diego, CA). Comparisons between groups were made by ANOVA and Student's *t* test with a Bonferroni correction for multiple comparisons. For the comparison of morphometrical analysis by two observers, we performed linear regression analysis. A value of $p<0.05$ was considered significant.

3. Results

3.1. Animal characteristics

The animal characteristics are shown in Table 1. IR-vehicle group had significantly higher concentration of insulin and

Table 1
Animal characteristics

	Normal	IR-vehicle	IR-γ	IR-α
Body weight (g)	390±11	384±10	387±10	407±8
Insulin (μU/mL)	26.4±5.1	92.8±18.7*	98.4±45.1	106.3±24.4
HOMA-IR index	12.4±2.6	51.3±13.0*	51.5±25.3	57.6±14.2
Glucose (mmol/L)	10.3±0.5	12.0±0.6	11.3±0.9	11.8±0.9
Free fatty acid (mEq/L)	0.37±0.04	0.72±0.19	0.44±0.06	0.47±0.05
Systolic blood pressure (mm Hg)	123±3	143±3*	124±4†	125±3†

Values are means±SEM. IR: insulin resistance, HOMA-IR: homeostasis model assessment-insulin resistance. *$p<0.05$ vs. Normal group, †$p<0.05$ vs. IR-vehicle group.

higher HOMA-IR value without increase in plasma glucose. It is characteristic to the prediabetic insulin resistant state. Neither the α- nor γ-tocopherol supplementations had any effect on the plasma insulin concentration or HOMA-IR. Although free fatty acid also tended to be higher in IR-vehicle group, it did not attain statistical significance.

Blood pressure was significantly higher in IR-vehicle group than in Normal group and the oral tocopherol supplementations, both α- and γ-tocopherol, significantly decreased the systolic blood pressure compared with the IR-vehicle group, but there was no difference between the two tocopherols in terms of blood pressure-lowering effects.

3.2. Analysis for neointima formation

Representative images of the common carotid arterial cross-sections at 14 days after arterial injury are shown in Fig. 1. The results of the histomorphometrical analyses are shown in Fig. 2. The morphometrical data by two observers were almost identical (Fig. 2A, $r^2=0.978$, $p<0.0001$, $Y=0.9938X+0.0038$).

Insulin resistance did not enhance neointima formation in noninjured carotid artery (Figs. 1A, C). Vascular injury produced neointima formation (Fig. 1B) and insulin resistance markedly enhanced the proliferative responses (Figs. 1D, 2B, D). The oral supplementation of γ-tocopherol markedly reduced the neointima area and neointima–media ratio (Figs. 1F, 2B, 2D), while α-tocopherol did not have such effects (Figs. 1H, 2B, D). There were no differences in media area among all groups (Fig. 2C).

3.3. Tocopherol concentrations in plasma and carotid artery

Plasma and carotid arterial tocopherol concentrations in each group are shown in Table 2. The oral supplementations of α- and γ-tocopherols produce plasma α- and γ-tocopherol concentrations, respectively. α-Tocopherol supplementation tended to decrease plasma γ-tocopherol concentration, although it did not attain statistical significance.

In the carotid artery, γ-tocopherol supplementation produced marked increase in γ-tocopherol level in the vessels, especially in the injured vessels. In contrast, no enhanced accumulation of α-tocopherol in the carotid arteries was observed by α-
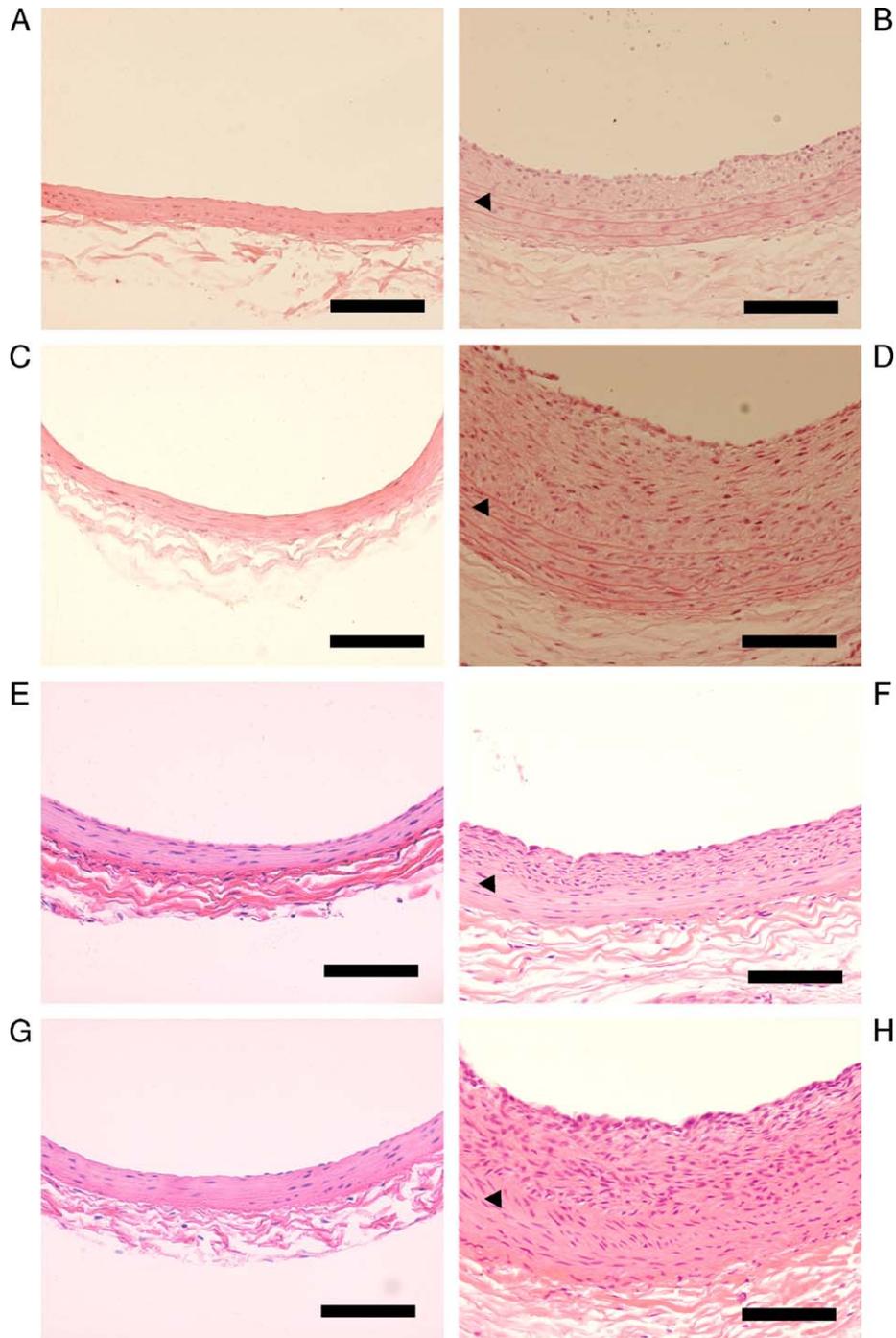
Fig. 1. Representative images of uninjured (A, C, E, G) and njured (B, D, F, H) carotid arterial cross-section at 14 days after arterial injury from Normal group (A, B), IR-vehicle group (C, D), IR-γ group (E, F), and IR-α group (G, H). Arrowheads show internal elastic lamina of carotid artery. Scale bar: 100 μm.

tocopherol treatment despite the fact that the plasma level of α-tocopherol was increased twofold. α-Tocopherol tended to produce the decrease in tissue γ-tocopherol level, although it did not attain statistical significance.

3.4. Evaluation of systemic and tissue oxidative stress

The plasma PCOOH level was significantly higher in IR-vehicle group than in Normal group (Fig. 3A), indicating elevated systemic oxidative stress in the insulin resistant rats.

Both α- and γ-tocopherol supplementations significantly decreased the plasma PCOOH level (Fig. 3A). There was no difference in the PCOOH levels between the IR-α and IR-γ groups.

Insulin resistance also enhanced the superoxide production in both uninjured and injured carotid arteries (Fig. 3B). However, the superoxide production in the injured carotid arteries evaluated by dihydroethidium stain or lucigenin chemiluminescence was not decreased in either α- or γ-tocopherol treatment (Figs. 3C, D).
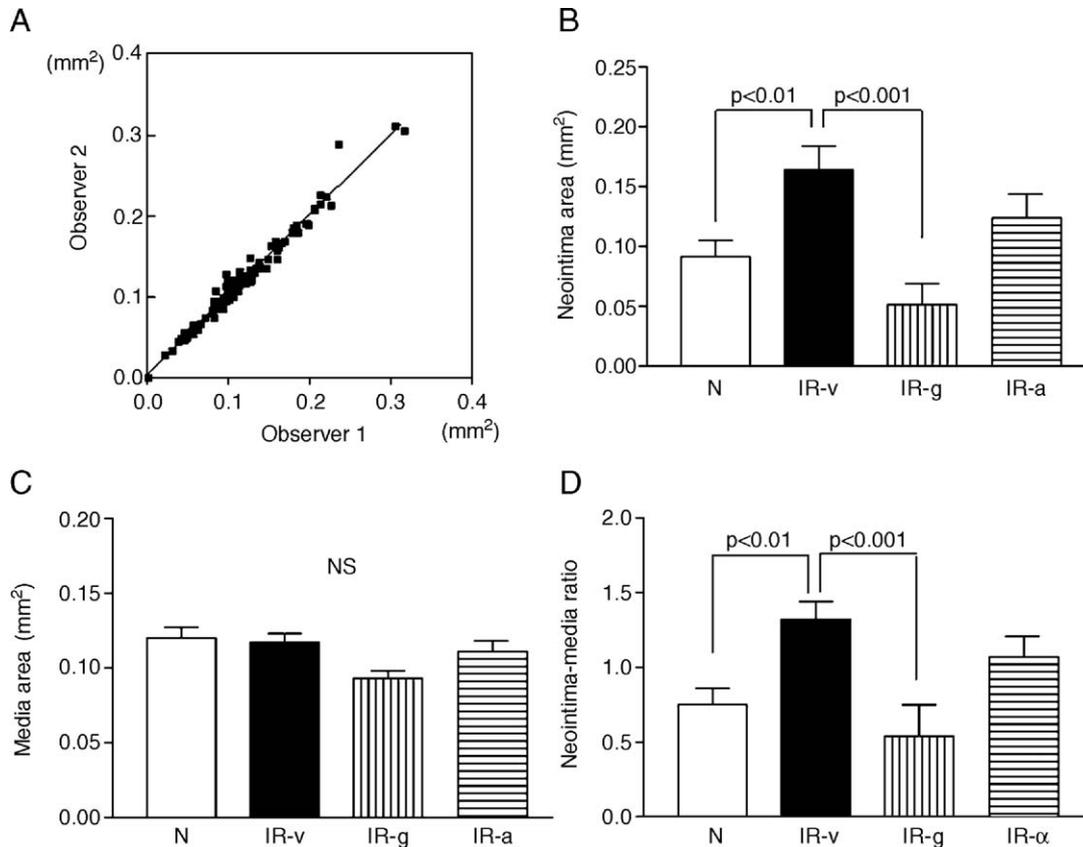
Fig. 2. The morphometrical data (the neointima area and the media area) measured by two observers are shown (A) and the regression line was very close to the line of identity. Neointimal area (B), medial area (C), and neointima/media ratio (D) are shown. N: Normal group, $n=10$; IR-v: IR-vehicle group, $n=8$; IR-γ: γ-tocopherol group, $n=5$; IR-α: α-tocopherol group, $n=7$.

3.5. Effect of tocopherol supplementation on inflammatory state

Neither α- nor γ-tocopherol supplementation decreased IL-1β (28.8 ± 3.7 pg/mL in the IR-vehicle group, 29.2 ± 5.2 pg/mL in the IR-γ group, 17.5 ± 4.6 pg/mL in the IR-α group, $n=5$, NS) or TNF-α (20.3 ± 4.8 pg/mL in the IR-vehicle group, 16.1 ± 4.1 pg/mL in the IR-γ group, 13.1 ± 4.0 pg/mL in the IR-α group, $n=5$, NS), suggesting no difference in the systemic inflammatory state among these 3 groups.

3.6. Effect of tocopherol supplementation on cell cycle-related gene expression

Cyclin D_1 was upregulated by carotid arterial injury in insulin resistant rats (Fig. 4A). The cyclin D_1 mRNA expression level was significantly lower in the IR-γ group than in the IR-α group, although there was no statistical difference between IR-vehicle group and IR-γ group.

Messenger RNA expression of p21^{Cip1} and p53 gene, which are known to suppress cell growth, was significantly decreased in the injured arteries compared with the uninjured arteries of

Table 2
Plasma and carotid arterial tocopherol concentrations

(A) Plasma

	IR-vehicle ($n=5$)	IR-γ group ($n=5$)	IR-α group ($n=5$)
α-tocopherol (nmol/mL)	32.00 ± 2.17	31.97 ± 4.59	65.81 ± 15.17*
γ-tocopherol (nmol/mL)	1.44 ± 1.03	11.06 ± 4.13*	0.12 ± 0.03

(B) Carotid artery

	IR-vehicle ($n=6$)		IR-γ group ($n=8$)		IR-α group ($n=8$)	
	Uninjured	Injured	Uninjured	Injured	Uninjured	Injured
α-tocopherol (pmol/mg tissue)	47.3 ± 1.1	126.7 ± 50.1	60.8 ± 20.3	107.7 ± 21.9	118.9 ± 27.1	97.0 ± 13.5
γ-tocopherol (pmol/mg tissue)	3.7 ± 1.1	3.8 ± 1.8	12.5 ± 3.0*	29.0 ± 5.5†	1.8 ± 0.5	1.2 ± 0.1

*$p<0.05$ vs. IR-vehicle uninjured artery, †$p<0.05$ vs. IR-vehicle injured artery.
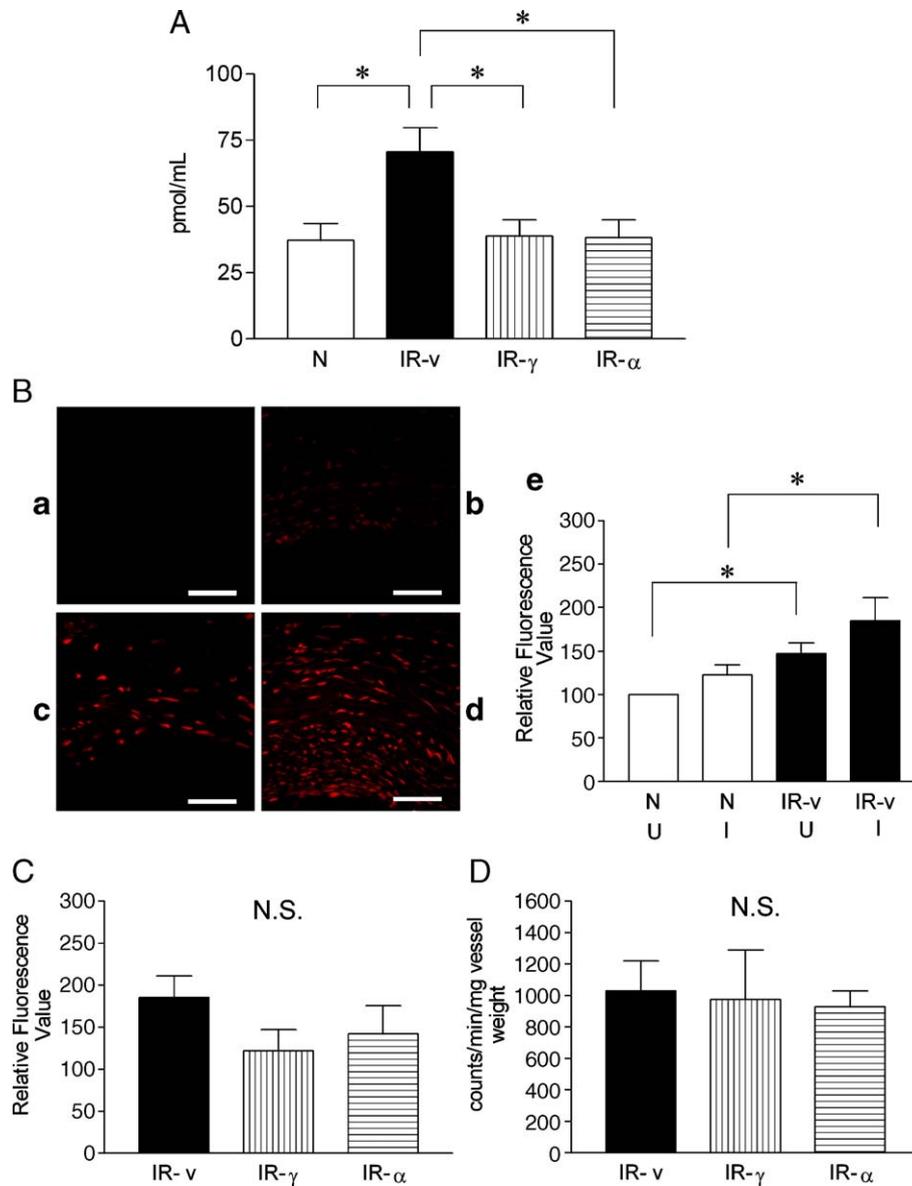∗$p<0.05$ vs. IR-vehicle group.

Fig. 3. The plasma PCOOH concentration of each group is shown in panel A. Representative dihydroethidium fluorescent confocal micrographs of uninjured (a, c) and injured (b,d) arteries from Normal (a,b) and IR-vehicle (c,d) groups and the quantitative data (e) are shown in panel B. The effect of α- or γ-tocopherol supplementation on injured carotid arterial oxidative stress which was evaluated by dihydroethidium fluorescence is shown in panel C. Superoxide production of the injured arteries in response to NADPH (100 μmol/L) evaluated by lucigenin chemiluminescence is shown in panel D. Scale bar: 50 μm. N: Normal group; IR-v: IR-vehicle group; IR-γ: γ-tocopherol group; IR-α: α-tocopherol group. U: uninjured carotid artery; I: injured carotid artery. *$p < 0.05$.

the IR-vehicle groups (Figs. 4B, C). Neither α- nor γ-tocopherol upregulated those mRNA expressions.

3.7. Effect of tocopherol supplementations on nitrotyrosine staining

Representative images for 3-nitrotyrosine immunostaining are shown in Fig. 5. The stain intensity was increased in insulin resistant rats compared with normal rats, especially in neointima area (Figs. 5A, B). α-tocopherol did not reduce the intensity (Fig. 5C). In striking contrast, γ-tocopherol markedly reduced the 3-nitrotyrosine stain intensity (Fig. 5D). We investigated three vessels for each group, and the results were consistent.

4. Discussion

The major findings of the present studies are as follows. First, the neointima formation in response to carotid arterial injury was significantly increased and both the systemic and the carotid arterial oxidative stress were enhanced in the insulin resistant rats. Second, the oral γ-tocopherol supplementation was effective for the inhibition of neointima formation after carotid arterial injury in the insulin resistant rats, but α-tocopherol was without effect. Third, neither α-tocopherol nor γ-tocopherol treatment reduced the superoxide production at the arterial tissue level, while both tocopherols reduced systemic oxidative stress. Fourth, only γ-tocopherol markedly reduces the 3-nitrotyrosine immunoreactivity in the neointima,
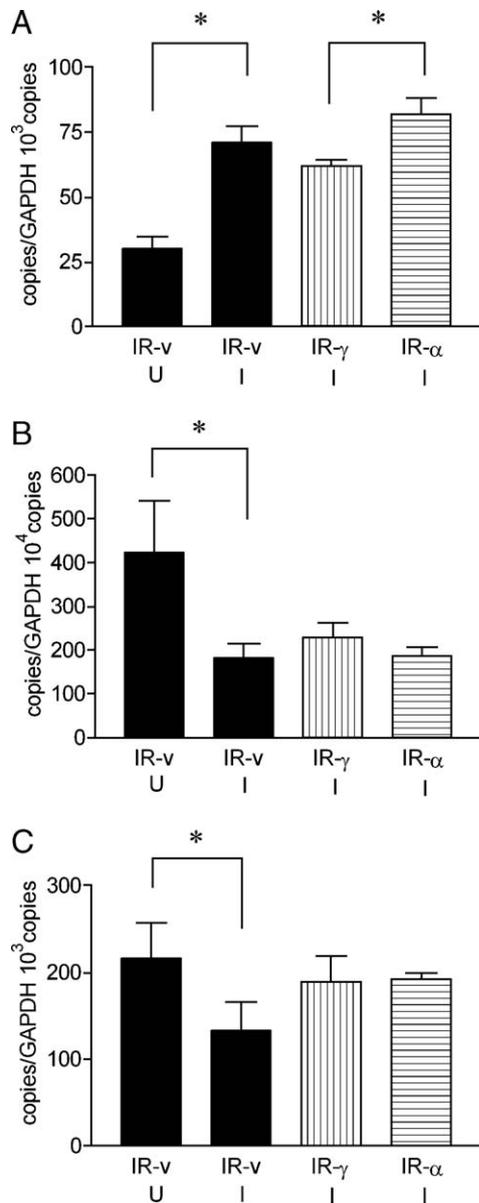
Fig. 4. Cell cycle-related genes cyclin D_1 (A), $p21^{Cip1}$ (B), and p53 (C) mRNA ($n=4$ in each group) levels are shown. Cyclin D_1 mRNA level was lower in the IR-γ group than in the IR-α group. IR-v: IR-vehicle group; IR-γ: γ-tocopherol group; IR-α: α-tocopherol group; U: uninjured carotid artery; I: injured carotid artery. $*p<0.05$.

suggesting its potent inhibitory effect on reactive nitrogen species. Fifth, the expression of cyclin D_1 mRNA was lower in the γ-tocopherol group than in the α-tocopherol group.

4.1. Considerations for insulin resistant rat model

The fructose-fed rat has been widely used as an insulin resistance model [19], and the present rat model also exhibited hyperinsulinemia, normoglycemia, high HOMA-IR index, and hypertension, which are characteristic to prediabetic insulin-resistant state. We used the plasma PCOOH level as a systemic oxidative stress marker and we evaluated the injured arterial oxidative stress with the ethidium staining and the lucigenin

chemiluminescence method. In the present study, we confirmed that oxidative stress both in the systemic circulation and in the vascular tissue was enhanced in the insulin resistant state, which is consistent with earlier studies [1,20].

4.2. Effect of orally supplemented tocopherols in insulin resistance

In the present study, since neither α- nor γ-tocopherol significantly changed the plasma levels of insulin, glucose, or free fatty acid, the tocopherols themselves did not affect the insulin resistance per se. In contrast, α- and γ-tocopherol supplementations decreased the blood pressure to an equal extent (Table 1). We speculate that both α- and γ-tocopherol supplementations normalized the elevated vascular tone in the insulin resistant state. The decreased systemic oxidative stress evidenced with the decreased plasma PCOOH level may have inhibited the nitric oxide quenching by superoxide anions. In spite of the decrease in the systolic blood pressure, neointima formation in response to the carotid injury was not ameliorated in the α-tocopherol group. Thus, the anti-proliferative effect of γ-tocopherol is likely to be independent of its blood pressure lowering effect.

As shown in Fig. 3A, the amount of α- or γ-tocopherol used in this experiment was enough to improve the systemic oxidative stress in an insulin resistance state. However, neither α- nor γ-tocopherol reduced the local oxidative stress in the vascular tissue despite their lipophilic property. It is possible that the tissue oxidative stress is much more resistant to antioxidant agents than the systemic oxidative stress. Similar observations with α-tocopherol were reported by Micheletta et al. [21] in human vessel walls. Since there was no difference in the inhibitory effect on tissue superoxide production between α- and γ-tocopherol groups, the remarkable difference in the inhibitory effect on neointima formation cannot be attributed to its inhibitory effects on the superoxide production.

The pro-inflammatory cytokines IL-1β and TNF-α are reported to be correlated with the extent of neointima in injured vessel [22,23]. However, in the present study, there were no differences in the plasma TNF-α or IL-1β level between the α- and γ-tocopherol groups. And so, the anti-proliferative effects of γ-tocopherol are unlikely to be associated with its anti-inflammatory effect in our model.

4.3. Possible mechanisms of the superiority of γ-tocopherol compared to α-tocopherol

To our knowledge, the superiority of γ-tocopherol to α-tocopherol in in vivo vascular responses has not been reported previously. Recently, it was reported that γ-tocopherol possesses several specific properties and pharmacokinetics distinct from those of α-tocopherol. There are several possible mechanisms that may explain the superiority of γ-tocopherol in the inhibition of neointima formation.

First, γ-tocopherol may have a specific antioxidant effect for free radical scavenging. γ-Tocopherol has an unsubstituted position in its chemical structure and is able to trap lipophilic
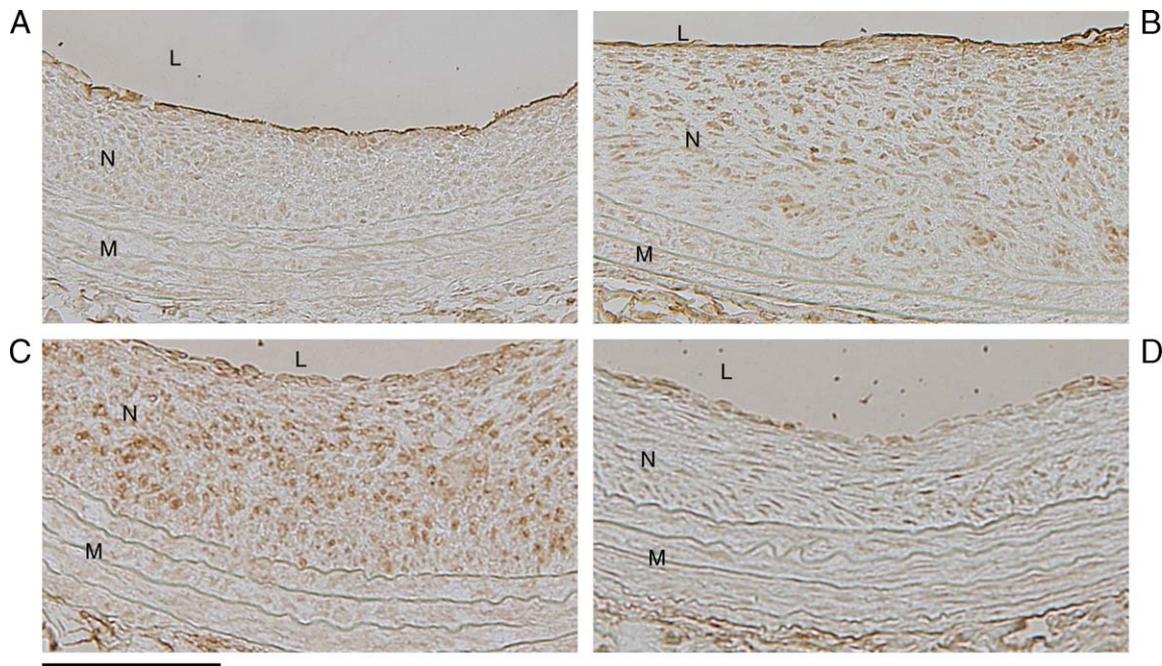
Fig. 5. Representative images of the immunochemical staining for 3-nitrotyrosine in injured arteries are shown. (A) Normal group, (B) Inslin resistant group, (C) α-tocopherol group, (D) γ-tocopherol group. Only γ-tocopherol reduced the 3-nitrotyrosine stain (brown) intensity which was enhanced by insulin resistance. L: carotid arterial lumen, N: neointima, M: media.

electrophiles such as reactive nitrogen species, including peroxynitrite and nitrogen dioxide [24,25]. Peroxynitrite has been known as a potent agent of LDL oxidation in atherosclerotic vascular lesions [26]. γ-Tocopherol may reduces nitrosative stress by preferentially detoxifying reactive nitrogen species. The present immunohistochemical finding showed that γ-tocopherol strikingly reduces the stain intensity of 3-nitrotyrosine in neointima which was increased by insulin resistance. These findings are in line with the recent evidence that γ-tocopherol inhibits protein nitration produced by inflammation [27].

Second, γ-tocopherol is known to exert direct anti-proliferative effects in several human cancer cells. In human prostate cancer cells, γ-tocopherol potently inhibits cell proliferation and retards the cell cycle at the G_1 phase via reductions of cyclin D_1 and cyclin E levels compared with α-tocopherol [9]. At the G_1 phase, the cell cycle is regulated by multiple factors, such as cyclin D/E, and cyclin-dependent kinases $p21^{Cip1}$, $p27^{Kip1}$, and p53 [28]. Since these regulators are also involved in the process of vascular neointima formation [29,30], we analyzed the gene expression of cyclin D_1, $p21^{Cip1}$, and p53 in the injured carotid arteries in vivo. We found a tendency that γ-tocopherol decreased and α-tocopherol increased the injured arterial cyclin D_1 mRNA expression and the difference between the two tocopherols was statistically significant. This different effect between α- and γ-tocopherol on cell cycle retardation may at least partly account for the different effect on the neointima formation.

Third, γ-tocopherol shows different biological kinetics from those of α-tocopherol in vivo. Although γ-tocopherol absorbed from the gut can be transported by sharing a part of α-tocopherol transfer protein (α-TTP) with α-tocopherol, the binding activity to α-TTP of γ-tocopherol is only 9% of the α-tocopherol binding activity [31]. As shown in Table 2, the γ-tocopherol concentration tended to decrease when α-tocopherol was supplemented. Furthermore, γ-tocopherol preferentially accumulated in the injured artery, while α-tocopherol supplementation did not result in such preferential accumulation in the injured vascular wall. Although the mechanism of the preferential accumulation of γ-tocopherol in injured vessels remains to be determined, it may relate to the unique transportation of γ-tocopherol, which is unrelated to α-TTP, and it may explain the vasculoprotective effect of γ-tocopherol.

Fourth, γ-tocopherol is reported to inhibit platelet aggregation more efficiently than α-tocopherol [32]. It is likely that circulating platelets attach to the injured vessel luminal surface leading to their aggregation after the deendothelialization. Activated platelets release several potent mitogenic cytokines such as PDGF and TGFβ [33]. These molecules can induce vascular smooth muscle proliferation. Therefore, γ-tocopherol may indirectly inhibit the neointima formation after vascular injury via the suppression of platelet activation.

Effect of α-tocopherol on smooth muscle proliferation is controversial. Tasinato et al. [34] previously reported that α-tocopherol inhibits the smooth muscle cell (SMC) proliferation, and Lafont et al. [35] demonstrated the inhibitory effect of α-tocopherol on neointima formation in the hypercholesterolemic rabbit. In contrast, it is reported that α-tocopherol does not inhibit rabbit SMC proliferation that is stimulated by serum [35]. Furthermore, Upston et al. [36] reported that intimal proliferation following vascular injury is even increased with α-tocopherol supplementation in cholesterol-fed rabbit. The

mechanisms of the smooth muscle proliferation may be diverse according to experimental models.

4.4. Clinical implications

The present data direct us to reconsider the present antioxidant therapy. Antioxidant therapy has been believed to be a promising approach for several cardiovascular diseases including atherosclerosis and postangioplasty restenosis. There are several basic [32] and clinical studies [5] in which the effects of vitamin E on atherosclerotic diseases were evaluated. However, the clinical benefit of vitamin E therapy has been controversial [4]. Most previous studies analyzed only the effect of α-tocopherol and, therefore, it remains unknown whether other tocopherol homologues exert vasculoprotective effects or not. The present study provides evidence that γ-tocopherol is much superior to α-tocopherol in inhibiting the neointimal proliferation, and that only γ-tocopherol possesses potent inhibitory effect on protein nitration. There is evidence that the intake of vitamin E from food, which contains mostly γ-tocopherol, is more effective than the intake of α-tocopherol supplementation for the prevention of coronary heart diseases [37]. Intensive γ-tocopherol supplementation sufficient to induce tissue γ-tocopherol accumulation may be useful as a therapeutic strategy for reducing morbidity and mortality of cardiovascular disease.

Special characters

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Maths:
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Acknowledgments

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