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Induction of hepatic inducible nitric oxide synthase by cholesterol *in vivo* and *in vitro*

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Abbreviations: NOS, nitric oxide synthase; LPS, lipopolysaccharide; EE, 17α-ethinyl-estradiol; TBARS, thiobarbituric acid reactive substances; LDL, low density lipoprotein; LDLR, LDL receptor; LysoPC, lysophosphatidylcholine

Abstract

Cholesterol-rich diet impairs endothelial NO synthase (eNOS) and enhances inducible NOS (iNOS) expression. In this study, we investigated effects of cholesterol on iNOS expression in high-fat-fed rat models, HepG2 and RAW264.7 cells. The high-fat diet increased the plasma total cholesterol level 6-7 fold and low-density lipoprotein cholesterol level (LDL-C) approximately 70 fold and slightly increased the level of lipid peroxidation as determined by thiobarbituric acid-reactive substance assay. The high-fat diet also increased plasma nitric oxide (NO) concentrations up to 5 fold, and induced iNOS mRNA expression in liver. The contractile responses of the endothelium-denuded thoracic aortic rings to phenylephrine were significantly damaged in high-fat-fed rats when assessed by organ chamber study. Treatment with estrogen for 4 days failed to reduce iNOS expressions as well as aortic contractility, although it improved lipid profiles. In cultured HepG2 or murine macrophage RAW264.7 cells, 3 days treatment with either 25-hydroxycholesterol or 7-ketocholesterol induced iNOS mRNA expression, as determined by RT-PCR. Our data suggested that the chronic exposure of hepatocytes and macrophage cells to high concentration of cholesterol or oxysterols may induce iNOS expression and subsequent synthesis of NO, which may be important in the pathogenesis of atherosclerosis.

Keywords: iNOS, expression, HepG2, rat, cholesterol

Introduction

Nitric oxide (NO), which is a short-lived free radical, important signaling molecule and potent vasodilator (Ignarro *et al*., 1987), influences physiological processes in every organ and tissue (Li and Billiar, 1999). It exhibits a remarkably wide spectrum of functions, including roles in neurotransmission and memory formation, prevention of blood clotting, regulation of blood pressure, and mediation of the bactericidal and tumoricidal activity of macrophages (Nathan and Hibbs, 1991). NO is enzymatically synthesized by the oxidation of the terminal guanido-nitrogen atom of L-arginine, which is catalyzed by three different isoforms of nitric oxide synthase (NOS) (Forstermann *et al*., 1995). The constitutive isoforms of NOS are found predominantly in endothelial cells (eNOS or NOS-III) (Schmidt and Murad, 1991) and in the neuronal cells (nNOS or NOS-I) (Lowenstein *et al*., 1992). The inducible isoform, iNOS (or NOS-) is expressed in several cell types including macrophages (Lyons *et al*., 1992) hepatocytes (Geller *et al*., 1993) and vascular smooth muscle cells (Nakayama *et al*., 1992) in response to cytokines. Production of NO by constitutive eNOS is primarily controlled by change of intracellular calcium levels. But iNOS is calcium independent and is mainly controlled at the transcriptional level. Ghafourifar and Richter (1997) reported for the first time the presence of NOS in mitochondria (mtNOS), which was localized in the inner mitochondrial membrane and was dependent on Ca^{2+}. The function of mtNOS was suggested to control mitochondrial respiration and mitochondrial trans-membrane potential.

The biological functions of individual NOS have been clarified by the use of various NOS-knockout mice. nNOS KO mice were resistant to brain damage caused by vascular strokes, showing nNOS is crucial in mediating stroke damage (Nelson *et al*., 1995). eNOS KO mice demonstrated the involvement of eNOS in normal blood pressure regulation since their aortic rings displayed no relaxation in response to acetylcholine and their mean arterial blood pressures

were 35% higher than the control (Huan *et al*., 1995). iNOS KO mice revealed the participation of iNOS in inflammatory responses since they had markedly reduced defenses against microorganism and against tumor cells (Laubach *et al*., 1995; MacMicking *et al*., 1995). On the other hand, iNOS KO mice were resistant to carrageenan inflammation and hypotension evoked by bacterial endotoxin (LPS), suggesting that iNOS plays an important role in vascular smooth muscle dilation as well as inflammatory response (Wei *et al*., 1995).

The vascular activity of NO has been reported to show conflicting effects in the process of atherosclerosis. Modified LDL was reported to impair the endothelium-dependent NO-mediated relaxation in atherosclerotic vessel (Kugiyama *et al*., 1990). However, NO was also reported to show anti-atherogenic activities, such as attenuation of platelet- and monocyte-endothelial cell interactions, and inhibition of smooth muscle cell proliferation. Therefore, NO must be a double-edged knife in physiology, since both the abundance and the paucity of NO cause diseases. It is believed that NO produced by ecNOS at low level maintains normal function of tissues like endothelium, while NO produced by iNOS at high level in defenses against bacteria attack also destroys the host itself. That is, overproduction of NO by iNOS has been linked to a variety of clinical inflammatory diseases, including septic shock and arthritis. The conflicting effects of NO in atherosclerosis could be due to the NO's dual function.

Several recent studies provided evidence for an effect of estrogen on the expression of iNOS (Kauser *et al*., 1998; Cho *et al*., 1999). The expressions of iNOS in T-lymphocytes and macrophages of cholesterol-fed rabbit (Esaki *et al*., 1997) and atheroscleotic vessels from cholesterol-fed rabbits (Behr *et al*., 1999) have recently been observed, however, the induction of iNOS by cholesterol in liver tissue from high-fat diet model has yet been reported.

In the present study, we investigated the effects of high-fat diet and estrogen on the expression of iNOS, production of NO, and its relation to aortic constriction of rat. Our results demonstrated that high-fat diet induced the level of iNOS mRNA and subsequent NO production *in vivo* and the treatment of 17α-ethinyl-estradiol did not reverse iNOS level, although it normalized the plasma cholesterol level. In both HepG2 and RAW264.7 cells as *in vitro* model, 25-hydroxycholesterol was found to induce iNOS mRNA. Since the isolated aortae from high fat-fed rats showed impairment of the adrenergic receptor-α-mediated contractility, disturbance of NO production system by high fat diet might have contributed to the development of atherosclerosis.

Materials and Methods

Materials

17α-ethinyl-estradiol (EE), 25-hydroxycholesterol, 7-keto-cholesterol, acetylcholine chloride, phenylephrine, and other reagent were purchased from Sigma Chemical Co (St. lous, MO, USA). Cell culture materials were purchased from GIBCO Laboratories (Rockville, MD, USA). Reverse transcriptase was purchased from Promega (Madison, WI, USA).

Animals

Male Sprague-Dawley rats (body weight, 250 g) were divided into four groups (n=4/group); N, fed normal rat chow and treated with vehicle; NE, fed normal rat chow and treated with EE; HF, fed high-fat diet and with vehicle; HE, fed high-fat diet and treated with EE. The animals were allowed free access to water and diet. After 40 days, rats were subcutaneously injected with EE (10 mg/kg) dissolved in propyleneglycol for 4 consecutive days, while control rats were treated with propyleneglycol only. At the end of experiment, animals were sacrificed and liver was removed immediately and stored in liquid nitrogen until isolating total RNA. Serum was collected individually for determination of total cholesterol, HDL cholesterol, and triglyceride.

Analysis of plasma cholesterol

Levels of plasma total cholesterol and LDL cholesterol (LDL-C) were determined enzymatically using a kit purchased from Sigma Co. LDL-C was calculated using the Friedwalds formula (Friedewald *et al*., 1972).

Thiobarbituric acid-reactive substances (TBARS) assay

Liver tissues were homogenized in 2 volumes of cold 10% (w/v) trichloroacetic acid (TCA) to precipitate protein. The precipitate was centrifuged and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) thiobarbituric acid in a boiling water bath for 60 min. Samples were centrifuged for 5 min and absorbance of the supernatant was determined at 532 nm (Han *et al*., 1999). TBARS concentration was calculated as malon-dialdehyde per mg liver protein using extinction coefficient of ε=1.53 x 10^5 $M^{-1}cm^{-1}$.

Measurement of serum nitrite and nitrate (NOx)

The blood samples were collected from retroorbital sinuses of the animals. Four microliter of serum samples were injected into 20 ml of hot (90-95°C) 0.1M vanadium (III) solution dissolved in 2N HCl in NO analyzer. NO, which is released by reduction of both nitrite and nitrate

under these conditions, was transferred from solution into the gas phase by a steady flow of helium. NO was determined by chemiluminescence method with Antek Model 7020 (Houston, TX, USA) (Kang *et al*., 1999)

Cell culture and RNA extraction

HepG2 or mouse murine RAW264.7 cells (ATCC TIB-71) were seeded at 1×10^6 cells/60 mm plate in DMEM supplemented with 10% FBS. After 12-16 h, cells were washed with DPBS, transferred to phenol red-free MEM with 10% FBS, and then were treated with 25-hydroxycholesterol (10 μg/ml) for 5 days. The cells were harvested at indicated times (1 to 5 days) and total RNAs were isolated by the acid guanidium-phenol-chloroform method (Jeong *et al*., 2002). As a positive control for iNOS, the LPS (100 ng/ml)/ IFN-γ (100 units/ml)-treated RAW264.7 cells were used.

RT-PCR

Two microgram of total RNA was reverse transcribed in a 25 μl reaction containing 5x reverse transcriptase buffer, 25 mM dNTPs, random hexamers (0.5 μg/μl), 20 units RNase inhibitor and 200 units M-MLV reverse transcriptase at 37°C for 1 h followed by incubation for 5 min at 70°C. PCR was performed in a 50 μl volume using 2 μl of the reverse transcription reaction plus 1 unit of Taq polymerase, 10x buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$ and 0.2 μM of each primer under following conditions: 94°C for 4 min; followed by 35 cycles of 94 for 30 s, 54°C for 30 s, and 72°C for 1 min; followed by 7 min at 72°C. The primers to amplify 400bp fragment of the human iNOS and rat iNOS were 5'-atc tac aaa gtc cga cat cc-3' (human-sense), 5'-ttg gtg aac ttc cac ttg ct-3' (human-anti-sense), 5'-tca tga tga aag aac tcg gg-3' (rat-sense), 5'-gaa ggt gag ttg aac aag ga-3' (rat-anti-sense). The β-actin was used as a control. After agarose gel electrophoresis, the EtBr-stained band intensities were quantified using an image analyzer.

Immunohistochemical staining

LDL receptor knock out mice were fed with high fat diet for 4 weeks and liver tissues were immediately placed in a solution of 1% paraformaldehyde in PBS. After 6h, tissues were transferred and washed at 4°C in several changes of PBS containing 0.45 M sucrose and 0.01% sodium azide. The tissues were frozen and cryostat sections were taken for immunohistochemical staining with anti-murine iNOS antibody (Transduction Laboratory, Lexington, KY, USA)

Preparation of rat aortas and vasoconstriction in rat aortic rings

Thoracic aortae were rapidly removed from rats and placed in ice-cold Krebs solution composed of 118.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.016 mM Ca-EDTA and 11.1 mM glucose as previously described (Kim *et al*., 2000). The aortae were cut into eight rings (2-3 mm) after whose loose connective tissues were cleaned. The endothelium from the intimal surface of the vessels was mechanically removed by gentle rubbing. The aortic rings were suspended in organ chamber containing Krebs solution bubbled with O_2 containing 5% CO_2. The cumulative concentration-dependent response curves to phenylephrine (10^{-8} to 10^{-5} M) were obtained using a physiograph (Narco-Bio system). All experiments were carried out in the presence of indomethacin (10^{-5} M) to prevent the production of vasoactive prostanoids. In some cases, 10 mM KCl was added to the bath in order to block voltage-dependent calcium channel.

Statistical analysis

All results are expressed as mean±SEM. Statistical analysis of the variation among the groups was performed with Duncan's multiple range tests. A *p* value lower than 0.05 was considered to be statistically significant.

Results

Quantification of plasma cholesterol and TBARS levels

Plasma total cholesterol and low-density lipoprotein LDL-C levels of HF rats increased about 6 fold and 70-fold, respectively, compared with N rats (Table 1). The estrogen treatment reduced total cholesterol level of NE and HE groups to 30% of N group and restored the LDL-C levels to normal in the both groups. TBARS, hepatic lipid peroxidation products, were increased 2 fold and 2.7 fold in the liver of HF rats and HE rats, respectively, indicating that high fat diet increased the lipid peroxidation while estrogen administration did not affect it.

Table 1. The effects of high fat diet and estrogen on plasma lipid concentrations and hepatic TBARS in rats

Groups	Total C	LDL-C	TBARS
N	101.5 ± 9.4^{a}	$7.8 \pm .7^{a}$	1.16 ± 0.11^{a}
NE	30.0 ± 3.2^{a}	8.7 ± 0.5^{a}	1.36 ± 0.26^{a}
HF	628.9 ± 64.7^{b}	556.3 ± 69.0^{b}	2.37 ± 1.21^{ab}
HE	35.1 ± 4.2^{a}	15.4 ± 3.3^{a}	3.27 ± 1.62^{b}

Data represent mean±SEM (n=5). Total C: total cholesterol, LDL-C: low density lipoprotein cholesterol, TBARS: thiobarbituric acid reactive substances, N, normal diet group; NE, normal diet group treated with 17α-ethinyl estradiol (EE, 10 mg/kg per day, s.c) for 4 consecutive days; HF, high-fat diet group; HE, high fat diet group injected with EE similarly as NE. Means with the same alphabets within a column are not significantly different at $p<0.05$ as determined by Duncan's test

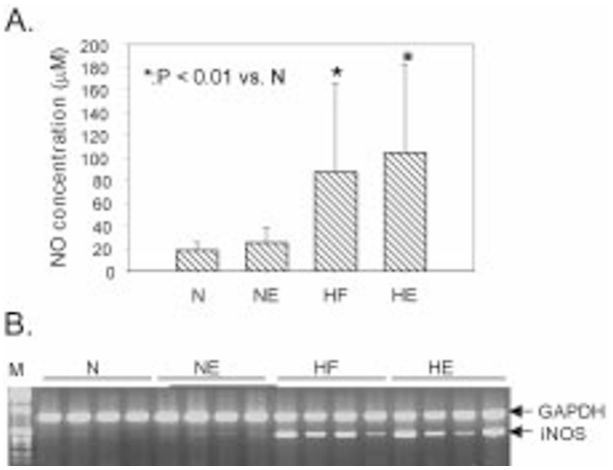

Figure 1. Effects of high fat diet and estrogen on the serum NOx concentrations and hepatic iNOS mRNA in rats. The animal groups are same as described in Table 1. A. Serum NOx was determined by Antek NO analyzer. Values are mean±SD (n=7). * represents the statistical significance by student t-test ($p<0.05$). B. Total RNA from the rat livers was subjected to RT-PCR analysis using specific primers for rat iNOS and GAPDH and analyzed on 1% agarose gel. Abbreviation for each group is the same as in the legend for Table 1.

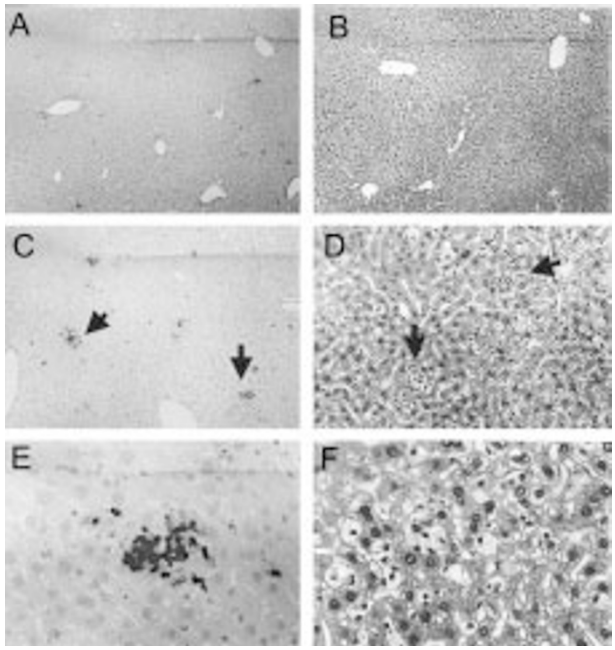

Figure 2. Immunohistochemical staining of iNOS on the liver isolated from LDLR-KO mouse. Liver sections either from normal diet-fed LDLR-KO (A, B) or high fat-fed LDLR-KO mouse (C to F) were immunostained with anti-mouse iNOS antibody (A,C,E) or stained with hematoxylin and eosin (B,D,F). The arrows indicate the iNOS positive cells. Original magnification A to C, x40; D, x100; E and F, x200

Induction of NO production and iNOS by high-fat diet in rat models

Plasma nitric oxide (NO) concentrations from rats were increased up to 5 fold in high-fat diet-fed rats (HF and HE) showing no effect of estrogen treatment (Figure 1A). To elucidate the cause of increased amount of NO in high-fat diet group, the expression of iNOS was investigated by RT-PCR of total RNA from liver, since the 5 fold increase of plasma NO might not be attainable simply by endothelial NOS alone. Indeed, hepatic iNOS expression was dramatically induced in high-fat diet

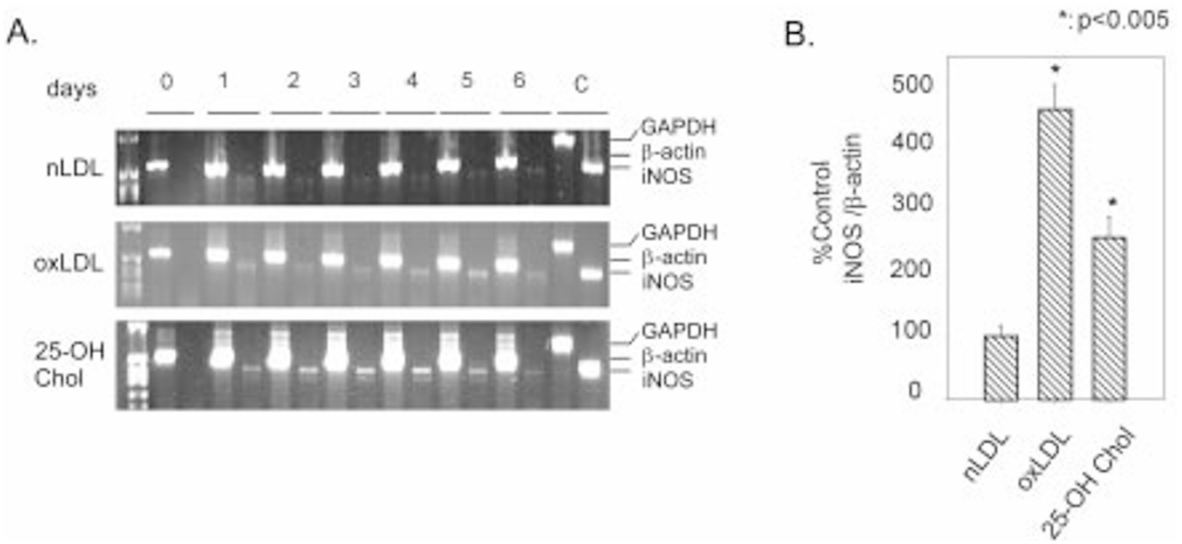

Figure 3. Induction of iNOS mRNA by 25-hydroxycholesterol in HepG2 cells. A. HepG2 cells in phenol-red free MEM plus 10% FBS were treated for 0 to 6 days with nLDL (10 μg/ml), oxLDL (100 μg/ml), or 25-hydroxycholesterol (25-OH Chol, 10 μg/ml). As a positive control (C), macrophage cells incubated with LPS (100 ng/ml) and IFN-α (100 units/ml) for 1day were utilized. The iNOS, β-actin or GAPDH mRNA levels were determined by quantitative RT-PCR. B. The relative levels of iNOS and β-actin at 1 day on panel A were quantified by scanning densitometry. Each point represents the mean±SD of three separate experiments.

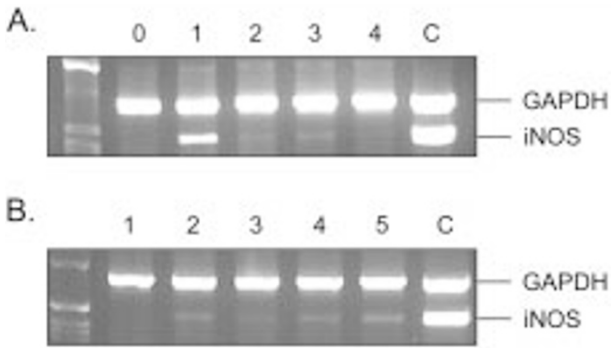

Figure 4. Induction of iNOS mRNA by 25-hydroxycholesterol in RAW264.7 cells. A. Raw 264.7 cells were cultured in DMEM, and at 50% confluency the medium was changed to phenol-red free MEM plus 10% FBS. RT-PCR analysis was performed using the cells, which were untreated (lane 0), incubated with 25-hydroxycholesterol (10μg/ml) for 2 days (lane 1), with 7-ketocholesterol (30μM) for 3 days (lane 2), 5 days (lane 3) and 6 days (lane 4). Lane C is a positive control that was treated with LPS (100 ng/ml) and IFN-γ (100 units/ml) for 1 day. B. Time-dependent induction of iNOS mRNA by 25-hydroxycholesterol (10μg/ml). lane 1, 0 h; lane 2, 24 h; lane 3, 36 h; lane 4, 48 h; lane 5, 60 h.

groups, whereas it was hardly observed in normal diet groups (Figure 1B). The estrogen administration did not restore the plasma NO or iNOS mRNA levels to the normal, although it normalized the plasma cholesterol concentrations in HE rats. It is of interest to note here that in hypercholesterolemia the production of NO is repressed by promoting the formation of inhibitory complex of caveolin and endothelial NOS (eNOS) (Ferson *et al*., 1999) and that the estrogen treatment alleviates atherosclerosis by induction of eNOS translocation (Goez et al., 1999). However, the extent of alteration of plasma NO by eNOS in *in vivo* system might be negligible and NO produced by eNOS works only on local vessel cells. Therefore, it was most likely that in the present study high-fat diet increased the plasma NO concentration by induction of iNOS.

The hepatic iNOS induction is independent of LDLR

The uptake of plasma LDL into hepatocytes is mediated by LDL receptor (LDLR), therefore, the impairment of LDL receptor in high fat diet-fed rats may block hepatic iNOS expression. Thus, we examined the iNOS expression in liver tissues, which were isolated from the high-fat diet-fed LDLR-knock out mice, by immunohistochemical staining. As shown in Figure 2, strong staining of iNOS proteins was observed at localized cells, possibly macrophages, implying that high-fat diet might have induced hepatic macrophage iNOS in the absence of LDLR function.

25-hydroxycholesterol induced iNOS mRNA in both HepG2 and RAW264.7 cells

In order to identify which component of lipids induced the expression of hepatic iNOS in rats, the iNOS mRNAs were determined by RT-PCR after treating HepG2 hepatoma cells or RAW264.7 murine macrophage cells with several different cholesterol compounds. Since lysophosphatidylcholine (LysoPC), a component of oxidized LDL, activates nuclear factor-κB(NF-κB) in resting macrophages (Han *et al*., 2001), which in turn induces iNOS transcription, it was expected that LysoPC might increase the expression of hepatic iNOS. Unexpectedly, chronic exposure of both cell types to LysoPC (10 μM) did not induce the iNOS mRNA, while 25-hydroxycholesterol (10 μg/ml) and 7-ketocholesterol

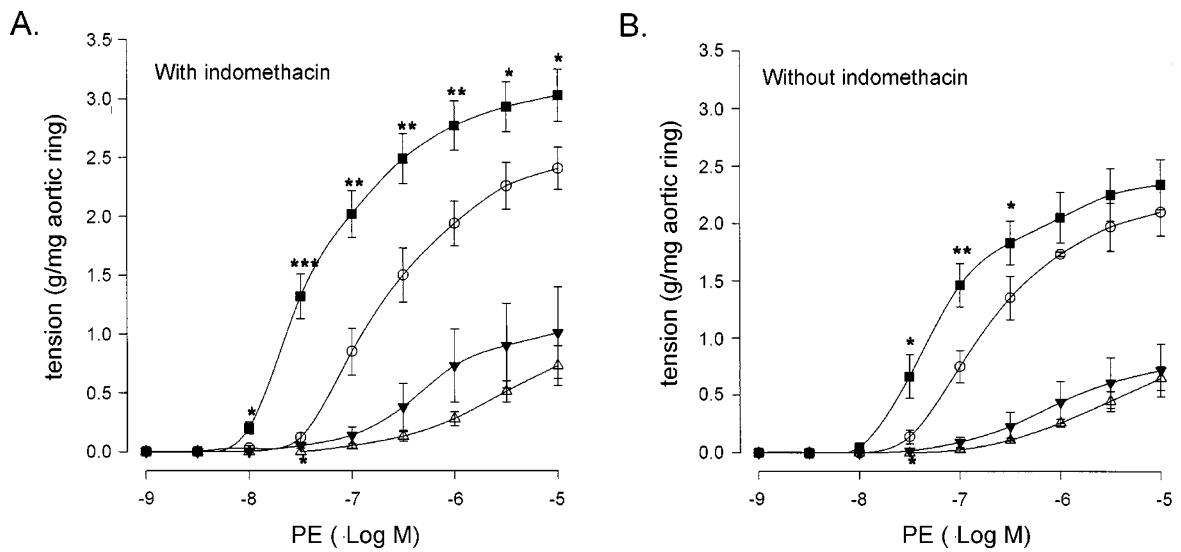

Figure 5. Phenylephrine-induced contractile response of endothelium-denuded aortae from rat. The aortic rings isolated from N (○), NE(4) (■), HF (△) and HE(4) (▼) were suspended in an organ chamber. The cumulative concentration dependent response curve to phenylephrin was obtained using physiograph in the presence (A) or absence (B) of indomethacin. Data represent mean±S.D. (n=6)

(30 μM) with less extent increased the iNOS transcription in HepG2 (Figure 3) and RAW264.7 cells (Figure 4). The longer the cells were incubated with the oxysterols, the more iNOS mRNA was produced. These results suggest that hepatic iNOS transcription is activated by chronic exposure to oxysterols via a mechanism distinguished from one previously reported.

The effect of increased NO in blood on aorta contraction

We investigated the effects of high fat diet-induced serum NO production on α_2-adrenergic receptor mediated contraction of aorta using organ bath experiment. As shown in Figure 5, endothelium-denuded aortic rings from high-fat diet group failed to show contractile response to phenylephrine (PE) in both the absence and presence of indomethacin. This was possibly due to the fact that the exposure of aortae to high concentrations of NO for extended periods impaired the function of α_2-adrenergic receptor. In order to investigate whether the high-fat diet inhibited the action of voltage dependent calcium channel, we induced the contraction by treatment with 60 mM KCl. The result showed that normal and high-fat diet group had same reaction pattern (data not shown) leading us to conclude that the high fat diet and its subsequent production of systemic NO did not damage the function of voltage dependent calcium channel.

Discussion

Although hypercholesterolemia is known to be associated with an impaired endothelial NO production and consequent alteration in eNOS abundance and activity has been proposed to constitute early events in the development of atherosclerosis (Feron *et al*, 1999), there are few reports on the iNOS expression. For example, iNOS staining was observed in the intima of atherosclerotic vessels obtained from long-term cholesterol-fed rabbits (Esaki *et al*., 1997; Behr *et al*., 1999). When lesions were more advanced, iNOS expression was more intense and diffused in the media, suggesting a link between the severity of the lesion and the iNOS expression (Behr *et al*., 1999).

In this study, we provided biochemical and functional evidence that the high fat diet, the high level of LDL-cholesterol, increased the production of NO in liver cells by upregulating the iNOS expression. Our results identified 25-hydroxycholesterol as one of possible culprits to induce the iNOS expression in both HepG2 and RAW264.7 macrophage cells. Based on several independent experiments on induction of iNOS transcription, NF-κB is a well-known major transcription factor in its regulation (Xie *et al*., 1992). We reported previously that an oxidized form of phosphatidylcholine, LysoPC, activated the nuclear translocation of NF-κB and NF-κB-mediated tran-sactivation in resting macrophages, however, LysoPC did not affect the iNOS expression in HepG2 as well as macrophage cells. These results suggested that pathway(s) other than NF-κB mediated the induction of iNOS expression by the high fat diet in liver tissues. There are several reports showing that estrogen may be a modulator of iNOS expression and activity, although the mechanism has yet been understood (Kauser *et al*., 1998; Zancan *et al*., 1999). It was obvious that the estrogen effects were not mediated by NF-κB, however, other possibilities are still open to discussion. Presently, it is of interest to note that administration of estrogen to high fat-fed rats reduced the plasma LDL-C, while it did not change the levels of plasma NO and hepatic iNOS mRNA. If cholesterol itself turned on the transcription of iNOS in liver, the estrogen treatment is expected to decrease the NO production by shutting-off the iNOS transcription. However, the result was opposite. Mechanism of high cholesterol diet and estrogen effects on the iNOS induction needs to be studied further.

It is known that the activated iNOS expression and its subsequent production of NO exert various influences on the pathogenesis of atherosclerosis. Certainly, the chronic inhibition of NO production in the early stages of the disease has been shown to promote atherosclerosis (Naruse *et al*., 1994). On the other hand, in the presence of superoxide radicals, NO may affect a number of pathological events such as lipid peroxidation or cellular damage, and has an effect on the athero-sclerotic process in later stages of the disease (Beckmann *et al*., 1990).

In comparison with normal diet group, our results showed that contractile response to phenylephrine was significantly reduced in endothelium-denuded aortic rings of the high fatdiet rat. The hyporesponsiveness of endothelium-denuded aortae to vasoconstrictor was limited to phenylephrine-mediated constriction, but not to voltage-dependent calcium channel. Similarly, the exposure of arteries to LPS and/or cytokines such as interleukin-1β and tumor necrosis factor α for several hours or direct injection of LPS leads to an attenuation of the contractile response to vasocontractive agents. It is, therefore, possible that the high fat-diet or plasma NO directly impairs the contractile units of aortic smooth muscle and dysfunction in vasoconstriction may contri-bute to develop atherosclerosis.

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