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Rosiglitazone reduces serum homocysteine levels, smooth muscle proliferation, and intimal hyperplasia in Sprague-Dawley rats fed a high methionine diet

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Abstract

Homocysteine (Hcy) is a metabolite of the essential amino acid methionine. Hyperhomocysteinemia is associated with vascular disease, particularly carotid stenosis. Rosiglitazone, a ligand of the peroxisome proliferator-activated receptor γ, attenuates balloon catheter–induced carotid intimal hyperplasia in type 2 diabetic rats.

We studied 4 groups (n = 7 per group) of adult female Sprague-Dawley rats fed (a) powdered laboratory chow (control), (b) control diet with rosiglitazone (3.0 mg/kg/d), (c) diet containing 1.0% L-methionine, and (d) diet containing methionine and rosiglitazone. After 1 week on high methionine diet, the rats were administered an aqueous preparation of rosiglitazone by oral gavage. One week after initiation of rosiglitazone, balloon catheter injury of the carotid artery was carried out using established methods, and the animals continued on their respective dietary and drug regimens for another 21 days. At the end of the experimental period, blood samples were collected, and carotid arteries and liver were harvested. Serum Hcy increased significantly on methionine diet compared with controls (28.9 $\pm$ 3.2 vs 6.3 $\pm$ 0.04 μmol/L). Development of intimal hyperplasia was 4-fold higher in methionine-fed rats; this augmentation was significantly reduced ($P < .018$) in rosiglitazone-treated animals. Rosiglitazone treatment significantly ($P < .001$) suppressed Hcy levels and increased the activity of the Hcy metabolizing enzyme, cystathionine-β-synthase in the liver samples. Hcy (100 μmol/L) produced a 3-fold increase in proliferation of rat aortic vascular smooth muscle cells; this augmentation was inhibited by incorporating rosiglitazone (10 μmol/L).

After balloon catheter injury to the carotid artery of animals on a high methionine diet, there was an increase in the rate of development of intimal hyperplasia consistent with the known effects of Hcy. It is demonstrated for the first time that the peroxisome proliferator-activated receptor γ agonist rosiglitazone can attenuate the Hcy-stimulated increase in the rate of development of intimal hyperplasia indirectly by increasing the rate of catabolism of Hcy by cystathionine-β-synthase and directly by inhibiting vascular smooth muscle cell proliferation. These findings may have important implications for the prevention of cardiovascular disease and events in patients with hyperhomocysteinemia (HHcy).
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1. Introduction

Homocysteine (Hcy) is an amino acid formed during the metabolism of the methionine to cysteine. Hyperhomocysteinemia (HHcy), or increased serum concentration of Hcy, is generally recognized as an independent risk factor for coronary, cerebral, and peripheral atherosclerosis [1,2]. The circulating levels of plasma Hcy can increase because of defective metabolism. The metabolic alterations could either be (i) acquired, as are the cofactor (vitamin) deficiencies [3], or (ii) because of mutations in genes coding for the enzymes of metabolism of Hcy [4,5]. Environmental influences and medications could contribute to variations in the levels of

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Hcy [6]. Moreover, cellular export mechanism can contribute to Hcy levels, and these are known to increase in various conditions [7]. The content of Hcy is fairly low in food items regarded as having low methionine content, and therefore in general, the dietary contribution of Hcy may not be significant [8].

HHcy is associated with cardiovascular disease (CVD), particularly in subjects with significant carotid stenosis [9,10]. Clinical and epidemiological studies have shown that Hcy measured in serum or plasma is a strong predictor of CVD risk [11,12]. The risk of CVD appears to be greatest in patients who have HHcy after a methionine load [13,14]. HHcy can be moderate (16-30 μmol/L), medium (30-100 μmol/L), or severe (>100 μmol/L) [15]. Mild to severe HH has been reported to cause pathological changes in the vascular wall, neural tube formation, and kidney function. Previous workers have shown that plasma total Hcy (tHcy) level is a strong predictor of mortality in patients with angiographically confirmed coronary artery disease [16]. Elevated serum tHcy is associated with sudden death and is especially associated with diabetes [17]. In addition, elevated serum tHcy is associated with an increase in fibrous plaques and a relative decrease in thin-cap atheromas [18]. It is also reported that in the absence of other known risk factors, HHcy stimulates the expression of monocyte chemoattractant protein-1, vascular cell adhesion molecule-1, and E-selectin in vivo, leading to increased monocyte adhesion to the aortic endothelium, which may significantly contribute to the development of atherosclerosis by facilitating monocyte/macrophage infiltration into the arterial wall [19]. HHcy can be induced experimentally by dietary manipulation with methionine [20]. The progression of events involved in carotid restenosis in experimental HHcy can be studied by injuring the carotid artery. Studies have shown that HHcy increases intimal hyperplasia after a carotid endarterectomy in a rat model [21,22].

Ligands that bind peroxisome proliferator-activated receptors (PPARs) activate increased transcription of target genes by binding to specific sequences in transcription regulatory regions of the gene. Two classes of therapeutic agents selectively bind to subtypes of the PPAR: hypolipidemic drugs (fibrates, agonists of PPAR-α) and the insulin-sensitizing drugs (thiazolidinediones, agonists of PPAR-γ). The beneficial effects of PPAR-γ ligands include improving insulin sensitivity, decreasing hyperinsulinemia, increasing high-density lipoprotein levels, lowering blood pressure, decreasing the generation of reactive oxygen species, and improving vascular reactivity. The PPAR-γ ligand, rosiglitazone, has been shown to have protective effects on the vessel wall [23-25]. We have observed that rosiglitazone but not fenofibrate attenuated intimal hyperplasia independently of insulin, glucose, and triglyceride levels in the Zucker fatty rat [23]; this is a model of insulin resistance and mild hyperglycemia [26]. Because these effects of rosiglitazone appear to be, at least in part, independent of insulin resistance (data on lean Zucker rat [23]), we tested the hypothesis that rosiglitazone would alleviate the circulating levels of tHcy and attenuate the augmented development of intimal hyperplasia in a model of dietary HH.

2. Materials and methods

This study was approved by the Institutional Animal Care and Use committee of the Tulane University Health Sciences Center, New Orleans, La.

2.1. Animals and feeding schedule

Ten-week-old female Sprague-Dawley rats from Harlan Laboratories (Indianapolis, Ind), were divided into 4 groups (n = 7), and fed a diet with or without methionine. The methionine diet was prepared by adding 10 g of L-methionine (Sigma Chemical Company, St Louis, Mo) to 1.0 kg of powdered feed. After 1 week on the diet, an aqueous preparation rosiglitazone (3.0 mg/kg/d) or equal volume of water was administered by oral gavage for 1 week before surgery, and 3 weeks after. All rats had free access to the feed and water and were maintained in a 12-hour day/light cycle.

2.2. Reagents

Rosiglitazone (Avandia) for animal experiments was purchased from GlaxoSmithKline Pharmaceuticals, Philadelphia, Pa. For in vitro experiments, rosiglitazone was obtained from Alexis Corporation Lausen, Switzerland. L-Homocysteine-thiolactone and other chemicals were purchased from Sigma-Aldrich, St Louis, Mo. Homocysteine thiolactone was converted to L-homocysteine using a standard method [27].

2.3. Balloon catheter injury

Balloon catheter injury of 1 of the carotid arteries was induced as previously published [28]. Briefly, a 2.0-mm balloon catheter was introduced through the femoral artery to the left carotid. The balloon was inflated to 4 atmospheres for 20 seconds and then deflated to 2 atmospheres and dragged down to the aorta. Rats were killed 3 weeks after injury.

2.4. Sample collection

A baseline sample of blood for measurement of serum Hcy was collected before the beginning of the experiment (under isoflurane anesthesia). At the end of the experiment, rats were killed using a carbon dioxide chamber for harvesting tissues. Blood, carotid arteries, and liver tissue were obtained from each rat, immediately processed, and specimen was stored under appropriate conditions until analyzed.

2.5. Histology and morphometric measurements

The carotid arteries were separated and flushed with zinc-buffered formalin for processing by conventional

methods for dehydration. They were cut from top into 4 equal segments and placed sequentially for embedding in paraffin. Sections of 6 μm were cut and stained with hemotoxylin and eosin for microscopy and analyzed with a magnification of ×10. Computerized digital microscopic software (Image-Pro plus 4) was used to obtain measurements of the intimal and medial areas [23]. The intima/media (I/M) ratio was calculated for all specimens for comparison between treatment groups.

Rat aortic vascular smooth muscle cells (RAVSMCs) were obtained from 4-month-old male Sprague-Dawley rats weighing approximately 350 to 500 g. The rats were anesthetized with pentobarbital sodium (35 mg/kg IP), and aortae were removed and placed in Medium 199 (Sigma Chemical). The vessel was incubated in a collagenase solution (200 U/mL collagenase type I, 0.4 mg/mL trypsin inhibitor) for 30 minutes at 37°C. The tunica adventitia was removed from the aortae, and then aortae were cut longitudinally. A sterile cotton swab was used to remove the endothelial lining. The vessel was then sectioned into small pieces and placed in a collagenase-elastase solution (200 U/mL collagenase type I, 15 U/mL elastase type III) for 2 hours. The tissue was washed in Medium 199 supplemented with 10% fetal bovine serum (FBS) containing penicillin (100 U/mL) and streptomycin (200 μg/mL). The tissue pieces were plated on a 25-cm^2 cell culture flask. The flask was placed in a humidified incubator (95% air, 5% CO_2) at 37°C. The tissue segments were allowed to attach for 5 to 7 days, after which the medium in the flask was aspirated. Fresh Medium 199, 10% FBS containing penicillin and streptomycin was added. Upon reaching 70% confluency, the cells were passaged (0.25% trypsin, 0.053 mmol/L EDTA; GIBCO, Grand Island, NY). The identity of the cells was confirmed by the morphology in addition to immunohistological studies showing that >95% stained positive for smooth muscle–specific α-actin [29]. The VSMCs were placed into primary culture at a constant density of 2×10^4 cells/cm^2 in multiwell plates in Medium 199 10% FBS in an atm of 95% air, 5% CO_2 at 37°C. Experiments were carried out between passages 2 and 3.

The effects of Hcy and/or rosiglitazone were monitored by direct cell enumeration and measurement of DNA synthesis. The VSMCs were seeded at low density (3×10^4 cells per 30-mm diameter plate) and grown for 24 hours and exposed to either Hcy (100 μmol/L) and/or rosiglitazone (10 μmol/L) for 72 hours. The cells were counted at 24-hour intervals using an inverted phase contrast microscope with cell quantification optics. For DNA synthesis, cells were washed twice with 10% FBS containing Medium 199 and labeled with 1 μCi of [methyl-^{3}H]-thymidine (20 Ci/mmol DuPont/NEN, Boston, Mass) in Medium 199 10% FBS for additional 18 hours. The [^{3}H]-thymidine incorporation was analyzed by standard liquid scintillation techniques.

2.6. Hcy levels and cystathionine-β-synthase activity

The Hcy levels were measured by high-pressure liquid chromatography according to an established method [30]. Cystathionine-β-synthase (CβS) activity was assayed in the liver samples by a previously published procedure [31].

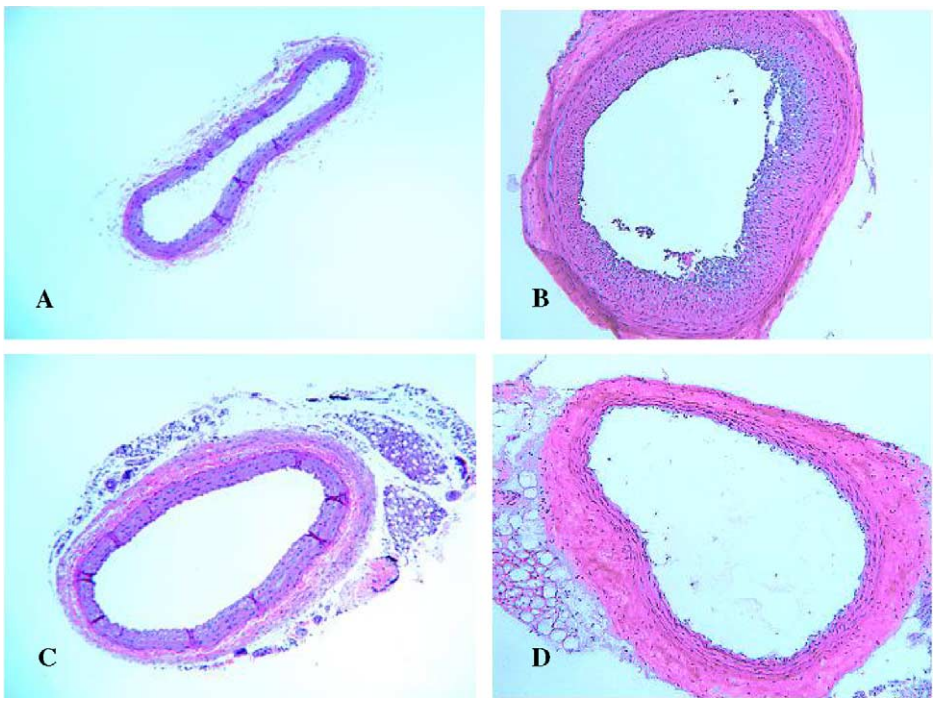

Fig. 1. Hemotoxylin and eosin–stained carotid arteries (balloon catheter–injured) from Sprague-Dawley rats on (A) control diet, (B) methionine diet, (C) control diet with rosiglitazone, and (D) methionine diet with rosiglitazone.

2.7. Statistical analyses

All data were expressed as the mean ± SEM. Computer-assisted statistical analysis (Sigma Stat 2.0 statistical program) was used for 1-way analysis of variance and Tukey test.

3. Results

3.1. Histology

Representative micrographs of sections of the carotid arteries from rats killed 21 days after the balloon catheter–induced injury are presented in Fig. 1. Micrograph A is from a rat fed the control diet; development of intimal hyperplasia occurred over the 3-week period, and the I/M ratio was 0.13. There was no significant change in the medial area after catheter injury. Micrograph B is from an animal on the methionine diet; the I/M ratio was 1.37. Micrograph C is from an animal that was on the control diet given rosiglitazone; the I/M ratio was 0.19. Micrograph D is from an animal on the methionine diet with rosiglitazone; the I/M ratio was 0.17. The animal on the methionine diet exhibited augmented development of intimal hyperplasia, and this augmentation was attenuated by treatment with rosiglitazone.

3.2. Development of intimal hyperplasia

Summary data for the effect of rosiglitazone on intimal hyperplasia are presented in Fig. 2. The group of animals receiving control diet had an I/M ratio of 0.18 ± 0.05; the group on control diet with rosiglitazone exhibited an I/M ratio of 0.21 ± 0.03; this was not statistically significant, as compared with the control diet group without rosiglitazone ($P < .997$). In the group on methionine diet, however, the development of intimal hyperplasia was markedly augmented, an I/M ratio of 0.82 ± 0.21 which was significant, as compared with the animals on control diet ($P < .005$). Administration of rosiglitazone to the group receiving methionine diet inhibited the development of intimal hyperplasia seen in the presence of the methionine diet alone, I/M ratio of 0.28 ± 0.12 ($P < .01$). Moreover, the development of intimal hyperplasia in the group with methionine diet plus rosiglitazone treatment was not statistically different from that seen in the group on the control diet without rosiglitazone (0.28 ± 0.12 vs 0.18 ± 0.05, respectively [$P < .918$]). The I/M ratio was zero in the uninjured carotid (data not shown).

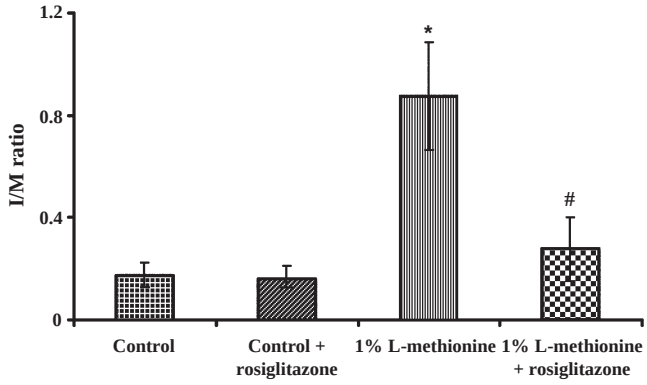

Fig. 2. Summary data for the effect of rosiglitazone on the rate of development of intimal hyperplasia in the carotid artery after balloon catheter injury. *In animals fed a high methionine diet, the development of intimal hyperplasia was significantly augmented ($P < .005$). #Administration of rosiglitazone along with the methionine diet inhibited the increase of intimal hyperplasia seen in the presence of the methionine diet alone ($P < .01$).

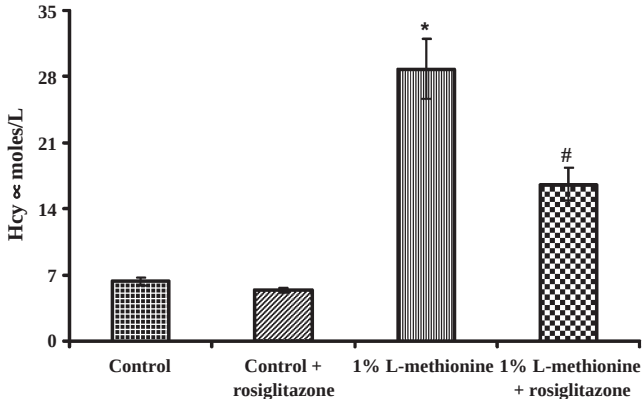

Fig. 3. Total serum Hcy levels in rats on different dietary and treatment regimens. Rosiglitazone had no effect on Hcy levels in animals on a control diet. *Animals on the methionine diet exhibited significantly enhanced levels of Hcy, compared with animals on the control diet ($P < .002$). #Administration of rosiglitazone to the animals on the methionine diet significantly reduced the serum Hcy ($P < .001$ compared with the methionine diet alone); however, the Hcy levels remained statistically elevated as compared with animals on the control diet ($P < .002$).

3.3. Hcy levels

Summary data for serum Hcy are presented in Fig. 3. On the control diet, Hcy levels were 6.3 ± 0.04 μmol/L. Administration of rosiglitazone to animals on the control diet did not affect the Hcy levels, 5.1 ± 0.6 μmol/L. The animals on methionine diet exhibited markedly enhanced

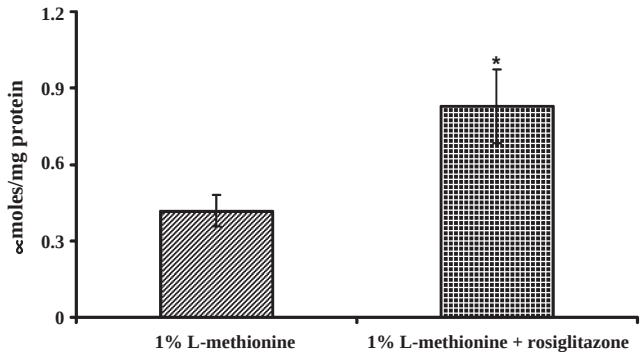

Fig. 4. The activity of CβS in liver homogenates from animals fed a high methionine diet alone or with rosiglitazone. *Rosiglitazone significantly increased the activity of CβS in theses animals ($P < .05$).

levels of Hcy, 28.9 ± 3.2 μmol/L, and was statistically significant compared with animals on control diet (P < .002). Administration of rosiglitazone to the animals on methionine diet significantly reduced the serum Hcy, 16.4 ± 1.7 μmol/L. This was statistically significant compared with the methionine diet alone (P < .001); however, the Hcy levels remained statistically elevated as compared with animals on the control diet (P < .002).

3.4. CβS activity

The activity of this enzyme in the animals receiving the methionine diet was 0.35 ± 0.05 μmol/mg protein (Fig. 4). Rosiglitazone augmented the activity of CβS in the animal on the methionine diet, 0.57 ± 0.1 μmol/mg protein.

3.5. VSMC proliferation and DNA synthesis

The proliferation of VSMC under control conditions was normalized to 100%, and the mitogenic effects at each time point are represented as a percent of control (Fig. 5). Exposure to Hcy (100 μmol/L) for 72 hours significantly augmented cell proliferation to 26% ± 9% of baseline P < .05 compared with control; the addition of rosiglitazone (10 μmol/L) significantly inhibited this Hcy-induced augmentation, reducing it to control levels, 92% ± 5%, P < .05 compared with Hcy alone. The VSMC proliferation was significantly attenuated by rosiglitazone alone—77% ± 6% as compared with control. DNA synthesis in the VSMCs was monitored by ^{3}H-thymidine incorporation, and the counts per minute (cpm) under control conditions were normalized to 100%. Exposure to Hcy (100 μmol/L) significantly augmented DNA synthesis, 24% ± 4.5% (P < .01) (Fig. 6); rosiglitazone (10 μmol/L) inhibited Hcy-induced stimulation of DNA synthesis to levels below that of control, 76% ± 1% (P < .05). Moreover, rosiglitazone alone inhibited DNA synthesis under control conditions, 67% ± 2% (P < .01). Rosiglitazone in a similar manner inhibited the stimulatory effect of Hcy on both DNA synthesis and proliferation of VSMCs.

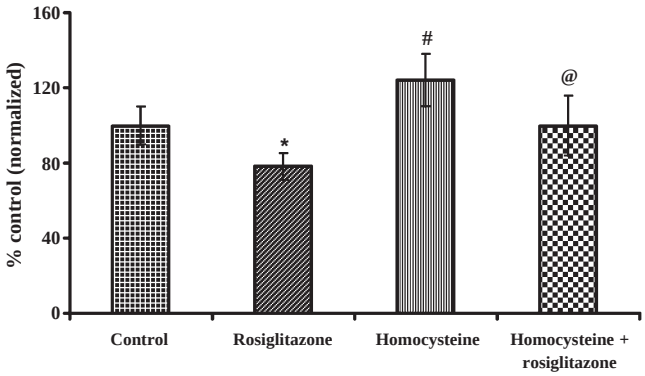

Fig. 5. Effect of Hcy alone, rosiglitazone alone, or in combination on Hcy induced VSMC proliferation over 72 hours. #Hcy alone significantly increased proliferation (P < .01). *Rosiglitazone significantly (P < .01) decreased proliferation compared with control. @The addition of rosiglitazone to Hcy significantly decreased the Hcy-induced proliferation to control levels compared with Hcy alone (P < .01).

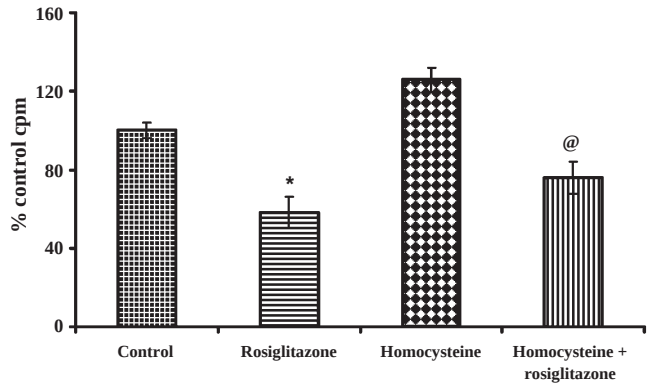

Fig. 6. Effect of rosiglitazone (10 μmol/L) on Hcy (100 μmol/L)-induced RAVSMC DNA synthesis was assessed by incorporation of ^{3}H-thymidine into VSMC measured by liquid scintillation spectrometry at 72 hours. The cpm under control conditions was normalized to 100%. Results are presented as percentage of control of cpm. *Rosiglitazone significantly decreased DNA synthesis. #Hcy significantly augmented DNA synthesis compared with control (P < .01). @Rosiglitazone decreased Hcy-stimulated DNA synthesis as compared with control as well as compared with Hcy alone (P < .05).

4. Discussion

This is the first study to show that a PPAR-γ agonist attenuates the intimal hyperplasia after arterial balloon catheter injury in a model of diet-induced HHcy. In addition, data on PPAR-γ–mediated decrease in serum tHcy levels in methionine-fed animals with rosiglitazone are presented for the first time. When compared with Sprague-Dawley rats fed the control diet (I/M ratio 0.18 ± 0.05), a significant increase in the hyperplastic response was seen in rats on methionine diet (I/M ratio 0.82 ± 0.21). This effect was significantly reduced by rosiglitazone (I/M ratio 0.28 ± 0.12). The present model of angioplasty-induced injury uses the catheter approach routinely used in human subjects. This approach avoids the additional and more global injury to the carotid associated with both endartectomy and/or a direct approach where the catheter is inserted through an incision in the carotid.

The methionine diet resulted in an increase in plasma Hcy compared with the controls (28.9 ± 3.2 vs 6.3 ± 0.04 μmol/L). The elevation (an independent risk factor for CVD) is similar to, and in the range seen in clinical cases. The intimal hyperplasia after catheter injury in the group fed methionine diet was 4-fold higher than controls (I/M ratio 0.82 ± 0.21 vs 0.2 ± 0.04). Treatment with rosiglitazone reduced the Hcy levels to 16.4 ± 1.7 μmol/L and reduced the I/M ratio to 0.27 ± 0.12. The reduction in intimal hyperplasia in response to rosiglitazone was not significantly different from the group on the control diet. However, the Hcy levels, although significantly reduced, remained elevated as compared with the control diet group (P <

.001). In some respects, this observation on serum tHcy levels is not surprising in that these animals were continuously on a high methionine diet, and the levels after treatment are analogous to those in human beings who have a normal methionine load test. We know of no treatment currently available that will reduce Hcy levels after a methionine load to that seen in either animals or human beings who have not been similarly loaded, and we therefore consider this posttreatment level to be the maximum possible.

This is the first study to demonstrate that rosiglitazone stimulated the activity of CβS in high methionine diet-fed animals and is in line with our earlier report on troglitazone [32], as well as rosiglitazone in animals not fed methionine [33]. As this enzyme catalyzes the irreversible conversion of Hcy to cystathionine, these data suggest that the reduction in serum Hcy levels in the present study was caused, at least in part, by increased metabolism of Hcy in the rosiglitazone-treated group. Recent data highlight the importance of CβS with an increase in atherosclerosis in mice genetically modified to have a deficiency of this enzyme [34]. Furthermore, this enzyme is critical in lowering serum tHcy after a methionine load. High methionine intake has also been shown to accelerate atherosclerosis [35]. As has been reported, there is little activity of CβS in vascular tissue [36], and therefore, we have measured the activity of this enzyme only in the liver where it is most abundantly expressed and active [37,38].

It is well established that after balloon catheter injury, there is proliferation of VSMCs [39]; therefore, we chose to study the effects of Hcy and rosiglitazone on these cells, in vitro. The inhibition of Hcy-induced proliferation of VSMCs by rosiglitazone is reported for the first time in the present study. The proliferation of VSMCs is a documented response to arterial injury and is thought to be important in the development of restenosis and atherosclerosis. People with diabetes have an increased risk of CVD, which could result from accelerated coronary atherosclerosis. Our data are in corroboration with previous studies on exposure of VSMCs to Hcy (100 μmol/L) that resulted in increased cell proliferation [40]. It is known that Hcy could exert either promotional or a suppression effect on mitogenesis in a cell type–specific manner. Where it elicits proliferation of smooth muscle cells, it is inhibitory to the growth of endothelial cells and NIH/3T3 cells as reported by earlier workers [41]. The inhibition of proliferation of VSMCs by PPAR-γ ligands has been well documented [42-44]. In the present study, the increase was from the normalized control proliferation of 100% to 140% $\pm$ 11%. We extended the earlier studies in that 10 μmol/L rosiglitazone completely inhibited the proliferative response caused by Hcy, 92% $\pm$ 5%. As one of the mechanisms by which intimal hyperplasia can be reduced is inhibition of VSMC proliferation, these data show an additional effect by which rosiglitazone may be attenuating the development of intimal hyperplasia. These data also suggest that rosiglitazone is an inhibitor of Hcy-induced cell growth but may not be just a competitive inhibitor of the effect of Hcy itself as Hcy is present in a 10-fold excess than rosiglitazone. Interestingly, rosiglitazone inhibited proliferation under control conditions suggesting the hypothesis that, in addition to other mechanisms, it may be inhibiting VSMC directly through a step in the cell cycle.

The VSMC proliferation data are corroborative with inhibition of DNA synthesis. Hcy stimulated ^{3}H-thymidine incorporation in VSMCs, and rosiglitazone completely inhibited this. Rosiglitazone also inhibited ^{3}H-thymidine incorporation in control cell cultures. These data confirm the hypothesis that, in addition to increasing Hcy metabolism via CβS, rosiglitazone may inhibit the intimal hyperplasia directly by inhibiting VSMC proliferation and DNA synthesis at the level of the cell cycle.

The PPAR-γ ligands have been previously reported to decrease intimal hyperplasia in animal models of diabetes and insulin resistance, as well as decreasing stent reocclusion in human beings after angioplasty and stent implantation [23,42-44]. We have recently demonstrated that rosiglitazone reduces intimal hyperplasia to a much greater extent than PPARα ligands, and this effect was independent of insulin, triglyceride, and glucose levels suggesting a possible direct effect on VSMC proliferation and DNA synthesis [23]. A similar effect was seen in lean Zucker rats, demonstrating that the effect of rosiglitazone is independent of its effects on ameliorating insulin resistance, again supporting the hypothesis that rosiglitazone is inhibiting VSMC proliferation and DNA synthesis [23]. The present data support this hypothesis and extend to a different model.

Our data are important in light of recent clinical trials demonstrating that, although Hcy remains a risk factor for CVD and stroke, vitamin therapy has been disappointing [45,46]. Alternative strategies such as the use of a PPAR-γ agonist offer a previously unrecognized therapeutic option.

In summary, this is the first report to demonstrate that a PPAR-γ agonist can reduce the Hcy augmentation of catheter-induced vascular injury and also reduce the elevation of serum Hcy levels induced by dietary methionine. This reduction is caused in part by the stimulation of the activity of CβS activity by the PPAR-γ agonist. Moreover, the reduction in intimal hyperplasia by the PPAR-γ agonist is caused, at least in part, by inhibition of proliferation and DNA synthesis in VSMCs. These findings may have important implications for preventing CVD and cardiovascular events in patients with HHcy.

Acknowledgments

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