http://www.kidney-international.org

Inhibition of ceramide–redox signaling pathway blocks glomerular injury in hyperhomocysteinemic rats

F Yi[1], AY Zhang[1], N Li[2], RW Muh[2], M Fillet[3], A-F Renert[3] and P-L Li[1,2]

[1]*Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin, USA;* [2]*Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, USA and* [3]*Laboratory of Drug Analysis, University of Liège, Liège, Belgium*

Ceramide-activated NAD(P)H oxidase has been reported to participate in homocysteine (Hcys)-induced abnormal metabolism of the extracellular matrix (ECM) in rat glomerular mesangial cells. However, it remains unknown whether this ceramide–redox signaling pathway contributes to glomerular injury induced by hyperhomocysteinemia (hHcys) *in vivo*. The present study was designed to address this question, defining the role of ceramide and activated NAD(P)H oxidase in the development of hHcys-induced glomerular injury. Uninephrectomized Sprague–Dawley rats were fed a folate-free diet for 8 weeks to produce hHcys and the *de novo* ceramide synthesis inhibitor myriocin or the NAD(P)H oxidase inhibitor apocynin was administrated. Rats with folate-free diet significantly increased plasma Hcys levels, renal ceramide levels, and NAD(P)H oxidase activity accompanied by marked glomerular injury. Treatment of rats with myriocin significantly reduced ceramide levels and improved glomerular injury, as shown by decreased urinary albumin excretion and reduced glomerular damage index. ECM components changed towards to normal levels with decreased tissue inhibitor of metalloproteinase-1 and increased matrix metalloproteinase-1 activity. NAD(P)H oxidase activity and Rac GTPase activity were reduced by 69 and 66%, respectively. In rats treated with apocynin, similar beneficial effects in protecting glomeruli from hHcys-induced injury were observed. These results support the view that *de novo* ceramide production is involved in Hcys-induced NAD(P)H oxidase activity in the kidney of hHcys rats and indicate the important role of ceramide-mediated redox signaling in hHcys-induced glomerular injury in rats.

Kidney International (2006) **70,** 88–96. doi:10.1038/sj.ki.5001517; published online 10 May 2006

KEYWORDS: homocysteine; sphingolipid; oxidative stress; kidney; end-stage renal disease

Correspondence: *P-L Li, Department of Pharmacology and Toxicology, Medical College of Virginia Medical College of Virginia Campus, Virginia Commonwealth University, 410 N. 12th Street, Richmond, Virginia 23298, USA. E-mail: pli@mail1.vcu.edu*

Received 2 September 2005; revised 6 February 2006; accepted 3 March 2006; published online 10 May 2006

Hyperhomocysteinemia (hHcys) is emerging as a critical pathogenic factor in the progression of end-stage renal disease and in the development of cardiovascular complications related to end-stage renal disease.[1–6] We have demonstrated that chronic elevations of plasma Hcys levels even for just 2 weeks induced proteinuria, mesangial (MG) expansion and glomerulosclerosis, without elevation of arterial blood pressure in uninephrotemized rats, indicating that increased plasma Hcys levels importantly contribute to the development of glomerular disease independent of hypertension.[7] Despite substantial evidence indicating the association of hHcys and end-stage renal disease, the mechanisms by which Hcys promotes the development of glomerulosclerosis remains unknown.

Given the similarity of pathological changes between Hcys-induced glomerular injury and Hcys-induced arterial damages such as endothelial injury, cell proliferation, increased matrix formation and aggregated protoglycan,[8–10] the increase in plasma Hcys may also play a crucial role in initiating and facilitating glomerular injury in hypertensive and end-stage renal disease individuals. Moreover, impaired renal function will lead to a further increase in plasma Hcys, which, in turn, exaggerates the progression of glomerular injury, resulting in a vicious cycle and, consequently, in glomerulosclerosis. These Hcys involved a variety of cellular, biochemical, and molecular events are importantly related to local oxidative stress.[11–13] Therefore, the oxidative stress-mediated pathogenic mechanism may contribute to Hcys-induced glomerular injury.

Recently, we have demonstrated that Hcys acutely increases superoxide ($O_2^{\bullet-}$) production via NAD(P)H oxidase and thereby stimulates the formation of tissue inhibitor of metalloproteinase-1 (TIMP-1) in rat MG cells, ultimately resulting in the deposition of collagen.[14] We further demonstrated that Hcys increased *de novo* ceramide synthesis in rat MG cells. This increased ceramide activated Rac GTPase and enhanced NAD(P)H oxidase activity.[15] In previous studies, sphingolipids, in particular, ceramide, play a very important role in cell membrane formation, signal transduction, and plasma lipoprotein metabolisms, all of

which have an impact on the development of atherosclerosis.[16–18] In addition, ceramide is considered as a critical signaling molecule mediating the activation of NAD(P)H oxidase in different cells.[15,19] Therefore, we hypothesized that ceramide-induced NAD(P)H oxidase activity may importantly participate in the development of hHcys-induced glomerular injury. In the present study, we utilized myriocin to inhibit serine palmitoyl-coenzyme A transferase, the key enzyme for *de novo* ceramide synthesis, and examined the role of endogenous-produced ceramide in the development of Hcys-induced glomerulosclerosis in an experimental hHcys animal model and explored related mechanisms. We also treated hHcys rats with an NAD(P)H oxidase inhibitor, apocynin, to observe its beneficial actions on hHcys-induced glomerular injury. Our results provided direct evidence, indicating that increased plasma Hcys stimulates ceramide production and NAD(P)H oxidase activity in the kidney of hHcys rats. We further demonstrated that ceramide-activated NAD(P)H oxidase promotes the formation of TIMP-1 and decrease matrix metalloproteinase-1 (MMP-1) activity, ultimately leading to glomerulosclerosis in hHcys rats.

RESULTS

Plasma total Hcys levels significantly increased in rats with folate-free diet

High-pressure liquid chromatography (HPLC) chromatograms of ABD-F-derivatized standards of several thiol compounds are presented in Figure 1a. Standard thiol solution contains cysteine, Hcys, glutathione, cysteinylglycine, and the internal standard TGA. Hcys had a retention time of 7.4 min, clearly separated from other thiol compounds. In uninephrectomized Sprague–Dawley rats, an 8 week-folate-free diet significantly increased plasma total Hcys levels. Neither apocynin nor myriocin had effect on the increase of Hcys levels induced by a folate-free diet. The results were summarized in Figure 1b. Additionally, two groups of rats were continuously administrated myriocin or apocynin with a normal diet had no significant change in Hcys levels, suggesting that either apocynin or myriocin only does not alter Hcys metabolism.

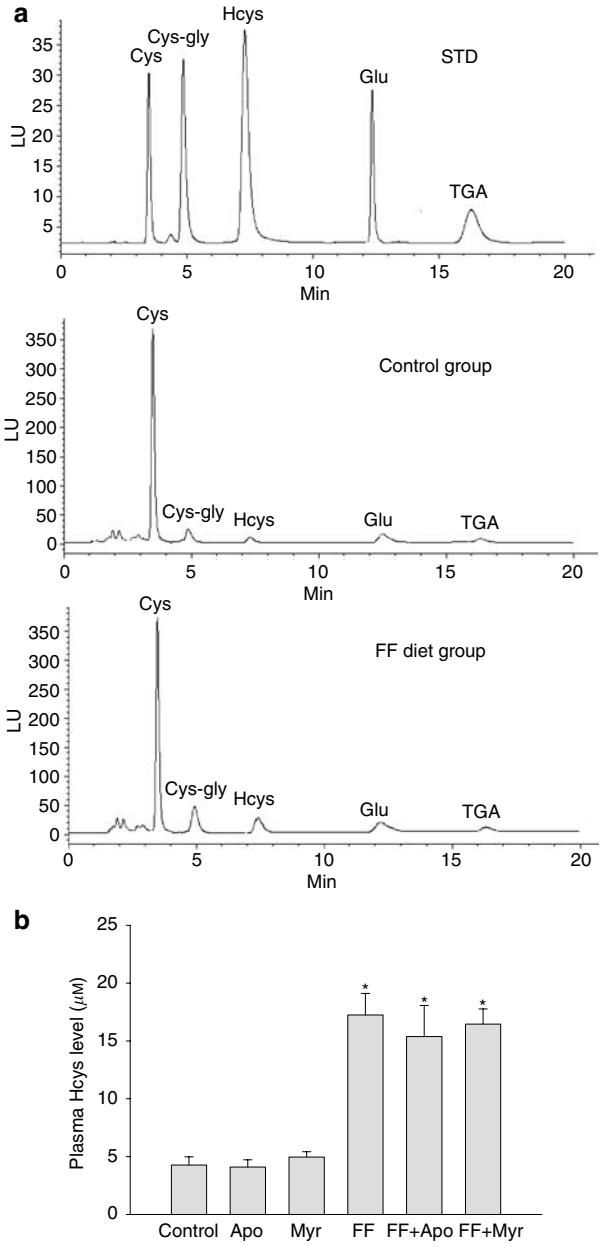

Figure 1 | Fluorescent HPLC chromatograms of Hcys and other thiols from standard solutions and plasma. (**a**) HPLC chromatograms showing standards (STD) containing 40 μM cysteine (Cys), 20 μM Hcys and glutathione (GSH), 5 μM cysteinylglycin (Cysgly), and internal standard, 50 μM TGA and Hcys, and other thiols in the plasma from Sprague–Dawley (SD) rat with normal diet or folate-free diet (FF). (**b**) Average plasma total homocysteine concentrations in different groups of rats on a normal diet with or without myriocin (Myr) and apocynin (Apo) treatments or a folate-free diet with or without Myr and Apo treatments ($n = 7$). *$P < 0.05$ compared with control.

Effect of myriocin and apocynin on glomerular damage induced by hHcys

To determine whether the ceramide–redox pathway contributes to Hcys-induced glomerular injury, we examined urinary protein, albumin excretion, and glomerular morphology in rats fed a folate-free diet. In parallel to increase in plasma total Hcys, both urinary protein and urinary albumin were significantly increased in rats fed a folate-free diet with more dramatic increase in urinary protein (Figure 2).

Morphological analysis showed that glomerular extracellular matrix (ECM) markedly increased and the glomerular mesangium was expanded with hypercellularity, capillary collapse, and fibrous deposition in the glomerulus in hHcys rats (Figure 3a). The semiquantitative injury score of glomeruli with the average glomerular damage index was substantially higher in rats fed a folate-free diet than in rats fed a normal diet (Figure 3b).

However, treatment of hHcys rats with myriocin or apocynin markedly reduced urinary protein excretion, albumin excretion, and glomerular damage index increase induced by hHcys. Myriocin or apocynin alone had no effects on the urinary protein and albumin excretion in rats with a

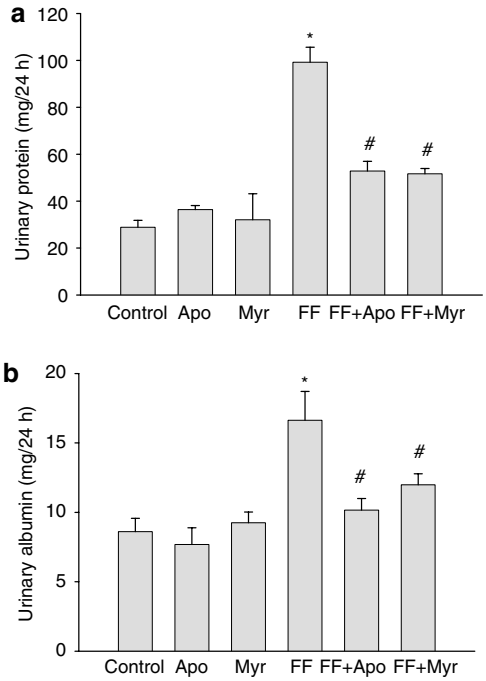

Figure 2 | Measurement of urinary protein and urinary albumin excretion. (**a**) Urinary protein and (**b**) urinary albumin excretion in six different groups of rats fed a normal diet or folate-free diet with or without myriocin and apocynin treatments ($n = 7$). $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with the values obtained from vehicle-treated hHcys rats.

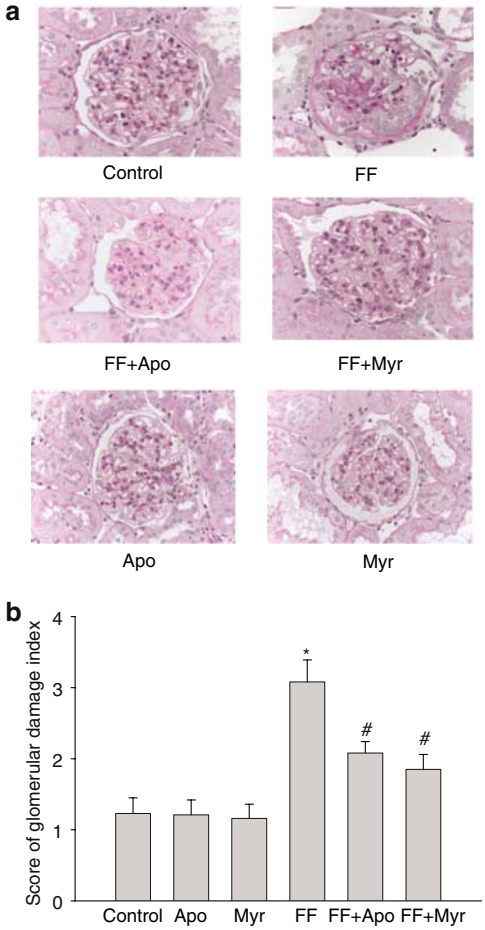

Figure 3 | Morphological features of the glomeruli from different groups of rats including a normal diet or folate-free diet with or without myriocin and apocynin treatments ($n = 7$). (**a**) Photomicrographs (original magnification, $\times 250$) showing typical glomerular structure in control and different treatment groups of rats. (**b**) Semiquantitative score of glomerular damage index ($n = 7$). $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with the values obtained from vehicle-treated hHcys rats.

regular chow. Based on these results, we conclude that inhibition of *de novo* ceramide synthesis or blockade of NAD(P)H oxidase does not have effects on glomerular structure and function in rats on a normal diet. In the following mechanism studies, therefore, only four groups of experiments are presented that include normal diet or folate-free diet with or without myriocin or apocynin.

Effects of myriocin and apocynin on Hcys-increased expression of TIMP-1 mRNA and protein levels

By real-time reverse transcription-polymerase chain reaction and Western blot analysis, TIMP-1 mRNA and protein levels in the renal cortex from rats with hHcys were increased by 61.0 and 47.5%, respectively (Figure 4a and b). Both myriocin and apocynin attenuated Hcys-induced increase. The results were summarized in Figure 4c.

Restoration of Hcys-reduced MMP-1 activity by myriocin and apocynin treatments

To examine whether the regulation of MMP-1 activity, an important form of MMPs in the kidney, is associated with ceramide–redox signaling pathway in hHcys rats, MMP-1 activity was measured and the results are summarized in Figure 5. MMP-1 activity in the renal cortex of hHcys rats was markedly reduced to 54% of control group, and myriocin and apocynin treatments can partially restore Hcys-reduced MMP-1 activity to 85 and 81%, respectively.

Quantitation of ceramide levels in the renal cortex of hHcys rats

Using liquid chromatography/mass spectrometry analysis, we identified and quantified six different ceramides (C14, C16, C18, C20, C22, C24). In these renal cortical samples, C16 and C24 ceramides are the most abundant species (98% of total ceramides). As summarized in Figure 6, in hHcys rats renal cortical ceramide levels significantly increased by 58%. When these rats were treated with myriocin, increases in renal cortical ceramide levels were attenuated by 98%. Moreover, there was no significant decrease in ceramide levels in the apocynin-treated hHcys rats. On a regular chow, rats receiving apocynin had no changes in ceramide levels in their kidneys, but rats receiving myriocin exhibited a decreased ceramide level of 22% in their kidneys as compared with control rats (Figure 6).

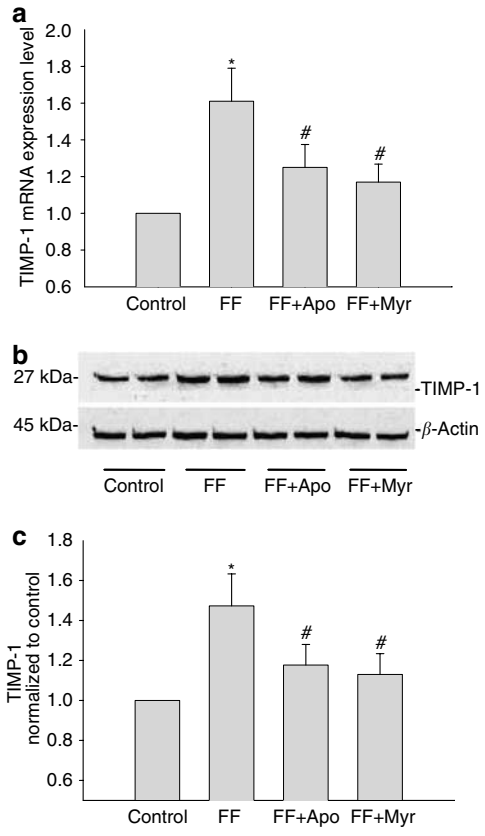

Figure 4 | TIMP-1 mRNA and protein expression levels in the renal cortex from different group of rats on a normal diet, folate-free diet with or without myriocin and apocynin treatment ($n = 7$). (**a**) Summarized data showing TIMP-1 mRNA levels in the renal cortex from these rats. (**b**) Immunoblot for TIMP-1 in the renal cortex from these rats. (**c**) Summarized data showing changes in TIMP-1 protein levels in the renal cortex from these groups of rats. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with the values obtained from vehicle-treated hHcys rats.

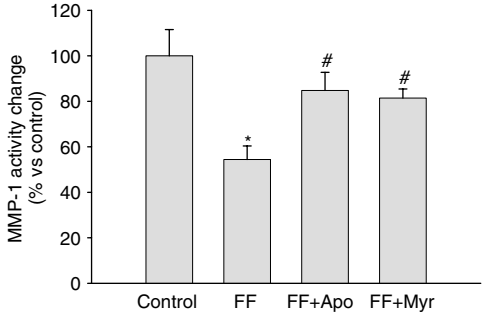

Figure 5 | Effects of *de novo* ceramide synthesis inhibitor myriocin and NAD(P)H oxidase inhibitor apocynin on the MMP-1 activity in the renal cortex from these hHcys rats ($n = 7$). $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with the values obtained from vehicle-treated hHcys rats.

Blockade of enhanced NAD(P)H oxidase activity in hHcys rats by inhibition of *de novo* ceramide synthesis

The activity of NAD(P)H oxidase was determined by fluorescence spectrometric assay. Summarized data showed

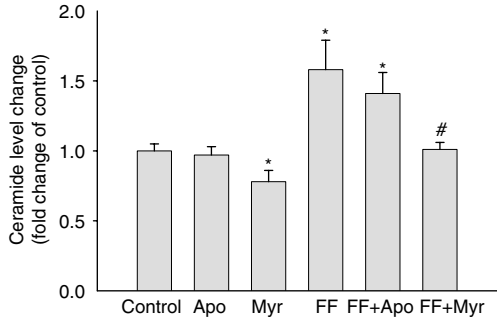

Figure 6 | Effects of *de novo* ceramide synthesis inhibitor myriocin and NAD(P)H oxidase inhibitor apocynin on ceramide production in the renal cortex of hHcys rats ($n = 6$). Values are mean ± s.e. from seven samples each group. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with the values obtained from vehicle-treated hHcys rats.

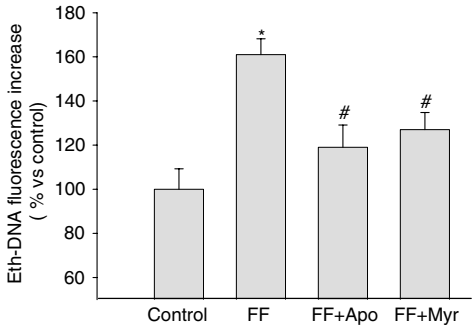

Figure 7 | NAD(P)H oxidase activity in the renal cortex from different groups of rats on a normal diet or folate-free diet with or without myriocin and apocynin treatments ($n = 7$). Summarized data showing the effect of Hcys and inhibitors on NAD(P)H-dependent activity in these renal cortex. Data were presented as percent increases in ethidium fluorescence compared to control. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with the values obtained from vehicle-treated hHcys rats.

that NAD(P)H oxidase activity increased by 61% in the renal cortex of rats fed a folate-free diet compared with control rats. Administration of apocynin and myriocin markedly attenuated hHcys-induced NAD(P)H oxidase activity by 69 and 55% (Figure 7).

Expression of NAD(P)H oxidase subunits in the renal cortex

To explore the mechanism by which myriocin attenuate Hcys-induced NAD(P)H oxidase activity in hHcys rats, we examined expression levels of NAD(P)H oxidase subunits in the renal cortex of these treated rats. The representative subunits $p47^{phox}$, $p67^{phox}$, non-phagocytic NAD(P)H oxidase (NOX)-2, and NOX-4 of NAD(P)H oxidase were detected by Western blot analysis. As shown in Figure 8a, expression of these subunits in the renal cortex was not significantly changed. These results are summarized in Figure 8b.

Rac GTPase activation is involved in ceramide–redox signaling pathway induced by hHcys

As documented in Figure 9a, hHcys produced a significant increase in GTP-bound Rac levels in the renal cortex of hHcys

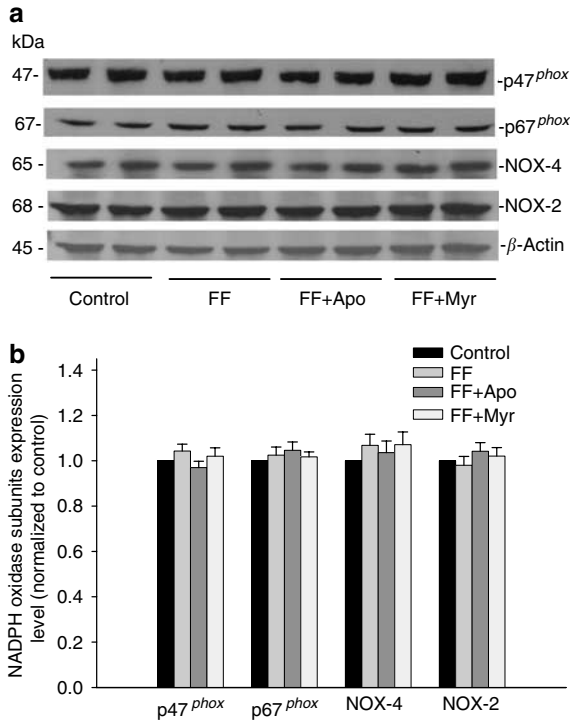

Figure 8 | Expression of NAD(P)H oxidase subunits in the renal cortex from different groups of rats on a normal diet or folate-free diet with or without myriocin and apocynin treatment ($n = 7$). (a) Immunoblot for $p47^{phox}$, $p67^{phox}$, and NOX-2 ($gp91^{phox}$) and NOX-4 in the homogenates from the renal cortex of these rats. (**b**) Summarized data showing changes in $p47^{phox}$, $p67^{phox}$, NOX-2 ($gp91^{phox}$), and NOX-4 in the renal cortex from different groups of rats.

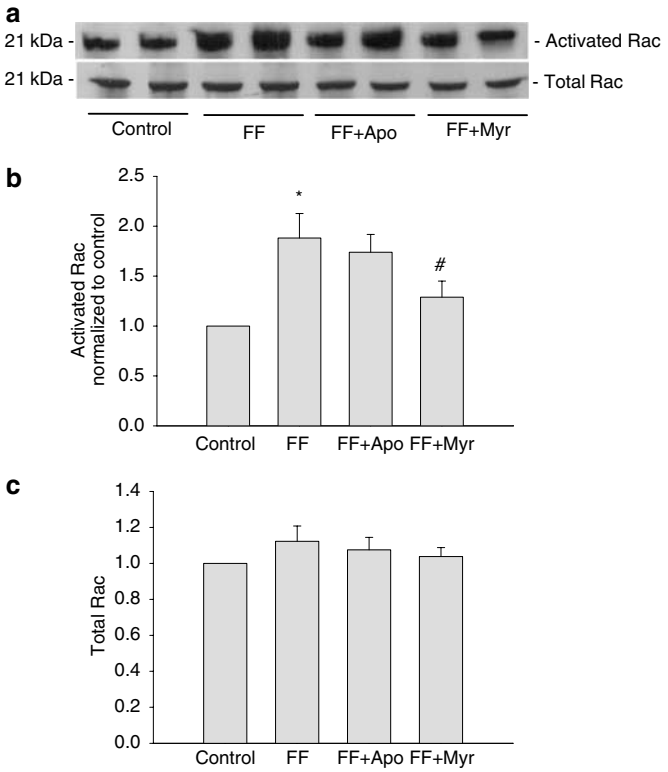

Figure 9 | Rac GTPase activity in the renal cortex from different groups of rats fed on anormal diet or folate-free diet with or without myriocin and apocynin treatment ($n = 7$). (a) Immunoblot for Rac GTPase activity and total Rac GTPase expression levels. (**b**) Summarized data showing changes in Rac GTPase activity and total RaC GTPase expression in renal cortex. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with the values obtained from vehicle-treated hHcys rats.

rats compared with the control group. However, administration of myriocin significantly blocked the Hcys-induced increase in activated Rac levels. The results are summarized in Figure 9b.

DISCUSSION

Recently, we have demonstrated that chronic elevations of plasma Hcys induces glomerulosclerosis and indicated that increased Hcys levels play an important role in the development of glomerulosclerosis. In *in vitro* experiments, Hcys was found to increase $O_2^{\bullet-}$ production via stimulation of *de novo* ceramide synthesis and subsequent enhancement of Rac GTPase activity, which in turn stimulates the formation of TIMP-1 in MG cells, consequently producing the deposition of collagen.[14,15] However, it remains unknown whether this ceramide–redox signaling pathway does contribute to the development of glomerulosclerosis associated with hHcys *in vivo*. In the present study, we performed a series of experiments to investigate the role of this ceramide–redox signaling pathway in the hyperhomocysteinemic glomerular injury in an experimental hHcys animal model produced by feeding rats with a folate-free diet.

hHcys– associated with folate-free diet induced significant glomerular injury

It is well known that Hcys is an intermediate metabolite of methionine metabolism and is metabolized by two pathways: namely, the re-methylation pathway that regenerates methionine by cofactors folate and vitamin B12, and the *trans*-sulfuration pathway that degrades Hcys into cysteine and then taurine by vitamin B6 and other relative coenzymes. Therefore, two major experimental models to produce hHcys are widely used.[20–22] One is to use methionine-rich diet, whereby methionine can be metabolized to produce Hcys, and another is to use folate-free diet. Considering the treatment of hHcys in clinical studies by administration folic acid and other vitamin supplementation to reduce Hcys levels and some concern about possible nonspecific effects of methionine in producing hHcys, the present study employed an animal model to induce hHcys by dietary folate restriction. Our results showed that an 8-week folate-free diet significantly increased plasma Hcys. In these rats, remarkable glomerular damage or sclerosis was observed, as shown by increased injury score of glomeruli and enhanced urinary protein and albumin excretion. In these rats, urinary protein excretion increased much higher than albumin

excretion compared with those of control rats. It is possible that in addition to filtered albumin, some other proteins owing to injury of other tissues may enter into urine, suggesting that renal tissue degeneration may occur in hHcys rats.

It is well known that glomerular accumulation of the ECM is the common pathologic feature of glomerular injury, and alterations in the synthesis and degradation of the ECM are importantly involved in the accumulation of the ECM in the glomerular. Recent studies have demonstrated the pathologic role of matrix-degrading MMPs and their inhibitor TIMPs in glomerular injury.[23] MMPs can break down collagens and thereby importantly regulate the amount of the ECM.[24,25] It has been reported that TIMP-1 plays a key role in the regulation of the ECM accumulation in glomerular injury or sclerotic diseases. Excessive TIMP-1 can markedly reduce the MMPs activity.[26,27] It is known that MMP-1 is an important proteolytic enzyme that degrades collagens.[27,28] In the present study, it was found that both TIMP-1 mRNA expression and protein levels in the renal cortex from hHcys rats were significantly increased and fluorescence resonance energy transfer (FRET) assay demonstrated that the MMP-1 activity was markedly reduced. These results indicated that hHcys associated with folate-free diet induced an abnormality of the ECM in the glomeruli, which may ultimately lead to severe significant glomerular injury.

Role of *de novo* ceramide synthesis in hHcys-induced glomerular injury

In hHcys rats fed a folate-free diet, we tested whether ceramide is involved in hHcys-induced NAD(P)H oxidase activity and consequent glomerular injury. Ceramide, a potent regulator of cell proliferation, activation, and apoptosis, plays an important role in lipoprotein aggregation and may promote foam cell formation in atherosclerosis.[29] It has been implicated in the detrimental actions of many different injury factors in different cells and tissues.[18,29,30] Recent reports have shown that serine palmitoyl-coenzyme A transferase inhibition by myriocin leads to a reduction of plasma sphingomyelin, ceramide production, and atherosclerosis in apolipoprotein E-knockout mice.[31,32] More recently, we reported that *de novo* ceramide synthesis contributes to Hcys-induced activation of NAD(P)H oxidase activity in MG cells.[15] In this regard, the possible pathological relevance of ceramide to glomerular injury induced by Hcys was proposed. The present study explored this possibility in experimental hHcys rats. We utilized myriocin to inhibit the first step of *de novo* ceramide synthesis and to determine the impact of serine palmitoyl-coenzyme A transferase inhibition on lipid metabolism and glomerulosclerosis development in hHcys rats. It was found that renal cortical ceramide levels in hHcys rats significantly increased and that treatment of rats with myriocin markedly reduced ceramide production in the kidneys of these rats. Meanwhile, glomerular injury in myriocin-treated rats was markedly improved as shown by reduced urinary or albuminuria and decreased glomerular damage index. Correspondingly, ECM components changed towards to normal level with decreased TIMP-1 and increased MMP-1 activity. These results provide direct evidence indicating the important role of spingolipids, especially ceramide in hHcys-induced glomerular injury in rats. It should be noted that myriocin may have nonspecific effects in the protection of glomeruli from injury associated with hHcys. However, several lines of evidence support that at the dose used with 8-week treatment myriocin may primarily work on *de novo* ceramide synthesis. First, liquid chromatography/mass spectrometry analysis demonstrated that myriocin significantly inhibited ceramide production in rats with hHcys. Second, in the rats treated with myriocin, increase in NADPH oxidase activity by hHcys was markedly blocked. Based on our previous studies,[19] it is ceramide, rather than other sphingolipid that stimulates NADPH oxidase. This further supports that the action of myriocin is through inhibition of ceramide production. Third, recent studies have indicated that immunomodulation is not responsible for myriocin-induced inhibition of atherogenesis in apolipoprotein E-knockout mice.[32] In addition, although immune cell dysfunction may play some roles in the development of glomerular sclerosis, it is well accepted that this immunopathogenic effect may not be a major mechanism responsible for the development of glomerular sclerosis.

Increased NAD(P)H oxidase activity contributes to hHcys-induced glomerular injury

It is well known that reactive oxygen species decisively contributes to cellular signaling, affecting almost all aspects of cellular function including gene expression, proliferation, migration, cell death, and sclerosis.[33,34] Although multiple enzymes contribute to oxidative stress in different tissues or cells, recent studies have been demonstrated that NAD(P)H oxidase is a major enzyme to produced $O_2^{\bullet-}$ in the kidney under physiological conditions.[35] NAD(P)H oxidase was initially characterized in neutrophils including membrane subunits $p22^{phox}$ and $gp91^{phox}$ and cytoplasmic subunits $p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and Rac GTPase.[36,37] In the past few years, a family of $gp91^{phox}$-like proteins, termed the NOX proteins, has also been discovered which includes NOX1, NOX2 ($gp91^{phox}$), NOX3, NOX4, and NOX5. NOX4 is mainly expressed in the kidney.[38] In recent studies, we have demonstrated that Hcys increases NAD(P)H oxidase-induced $O_2^{\bullet-}$ and thereby stimulates TIMP-1 in renal MG cells, ultimately resulting in the deposition of collagen. The present study demonstrated *in vivo* that NAD(P)H oxidase activity significantly increased in the renal cortex of rats fed a folate-free diet. When these hHcys rats were treated with an NAD(P)H oxidase inhibitor, apocynin, significant beneficial effects to improve various hHcys-induced biochemical or morphological alternations in the glomeruli were observed including decreased urinary protein excretion, improved glomerular damage, and increased MMP-1 activity. Interestingly, analysis of NADPH oxidase activity showed that Hcys-induces increase in NADPH activity was also markedly attenuated in the presence of myriocin, a *de novo* ceramide

synthesis inhibitor. These results provide direct evidence supporting the view that $O_2^{\bullet-}$ production is associated with NADPH oxidase in hHcys rats.

To explore the mechanisms by which Hcys activates NAD(P)H oxidase in hHcys rats, we first examined expression level of NAD(P)H oxidase subunits. Western blot analysis showed that the amount of NAD(P)H oxidase subunits in the renal cortex had no change, no matter whether the rats were treated by myriocin or apocynin, suggesting that increased NAD(P)H oxidase activity is not through increased protein expression levels. Then, we tested whether Rac GTPase, a critical regulator, contributes to increased NAD(P)H oxidase activity in hHcys rats. It was reported that Rac-GTP interacts with the N-terminal of $p67^{phox}$ for the assembly of NAD(P)H oxidase subunits to activate this enzyme.[39,40] In our recent studies, ceramide was found to increase via its *de novo* synthesis by Hcys treatment in rat MG cells. This increased ceramide-activated Rac GTPase and enhanced NAD(P)H oxidase activity. The present study indicated that Rac activity was increased by 88.3% in the kidney of hHcys rats. When myriocin was administrated, Hcys-induced increase in Rac GTPase activity was markedly reduced, but apocynin had no effect. These results further support the view that *de novo* ceramide production is involved in Hcys-induced NAD(P)H oxidase activity by activation of Rac GTPase in the kidney of hHcys rats.

In summary, the present study demonstrates that ceramide-activated NAD(P)H oxidase is responsible for glomerular injury associated with hHcys *in vivo* and that activation of NAD(P)H oxidase is attributed to increased Rac-GTPase activity in the kidney of the hHcys rats. These results suggest a new pathogenic pathway contributing to glomerular injury associated with hHcys, which may direct toward the development of new therapy of end-stage renal diseases related to hHcys.

MATERIALS AND METHODS

Animals

Experiments were performed using Sprague–Dawley rats (200 g, 6 weeks old) purchased from Harlan Inc. (Madison, WI, USA). To speed up the damaging effect of Hcys on the glomeruli, the rats were uninephrectomized. After a 1-week recovery period from uninephrectomy, the rats were maintained on a regular chow or a folate-free diet, which was purchased from Dyets Inc. (Bethlehem, PA, USA) for 8 weeks. In additional groups, *de novo* ceramide synthesis inhibitor, myriocin (0.3 mg/kg/day, intraperitoneally), or NAD(P)H oxidase inhibitor, apocynin (15 mg/kg/day in drinking water), was continuously administrated throughout the period feeding a normal diet or folate-free diet. Myriocin and apocynin doses were chosen according to recent reports[31,32] and our previous dose-dependent experiments. All protocols were approved by Institutional Animal Care and Use Committee of both Medical College of Wisconsin and Virginia Commonwealth University. During recording days, blood and 24-h urine samples were collected. Plasma total Hcys was measured by fluorescence HPLC analysis as described.[41] Urinary protein concentration was determined by BCA assay kit (Bio-Rad, Hercules, CA, USA). Urinary albumin excretion was measured using a rat albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories, Montgomery, TX, USA).

NAD(P)H oxidase assay

A dihydroethidium-based fluorescence spectrometric assay was used to assess $O_2^{\bullet-}$ production from NAD(P)H oxidase.[14,15] Dihydroethidium is oxidized specifically by $O_2^{\bullet-}$ to yield ethidium, which binds DNA and has fluorescence at excitation/emission of 480/610 nm. Briefly, 20 μg proteins were incubated with 100 μM dihydroethidium and 0.5 mg/ml salmon test DNA (binds ethidium to amplify fluorescence signal) in 200 μl phosphate-buffered saline. NADPH (1 mM) was added immediately before recording ethidium fluorescence by a fluorescence microplate reader (FLX800, Bio-Tek, Winooski, VT, USA). The ethidium fluorescence increase was used to represent NAD(P)H oxidase activity.

Western blot analysis

Western blotting was performed as described.[14,15] Briefly, 20 μg proteins were subjected to SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and blocked. The membrane was probed with primary monoclonal antibodies: anti-$p47^{phox}$, anti-$p67^{phox}$, anti-$gp91^{phox}$ (1:1000 dilution; BD Transduction Laboratories, Lexington, KY, USA), anti-TIMP-1 (1:1000 dilution; R&D systems, Minneapolis, MN, USA), or anti-NOX-4 (1:500 dilution, Santa Cruz Biotech, Santa Cruz, CA, USA), overnight at 4°C followed by incubation with horseradish peroxidase-labeled anti-mouse immunoglobulin G or anti-goat immunoglobulin G. The immunoreactive bands were detected by chemiluminescence methods and visualized on Kodak Omat film.

Rac GTPase activation assay

A specific pull-down experiment was performed to determine Rac GTPase activation using an Rac activation assay kit (Upstate, Lake Placid, NY, USA) as described.[15] Briefly, the renal cortex tissue was lysed in Mg^{2+} lysis buffer. After precleaned by glutathione agarose, the pull-down proteins (200 μg/sample) were incubated with 10 μg of PAK-1 PBD (glutathione *S*-transferase fusion protein, corresponding to p21-binding domain (PBD, residues of 67–150) of human PAK-1) agarose for one hour at 4°C to bind Rac-GTP. Then, the pelleted beads were washed three times with Mg^{2+} lysis buffer, resuspended in 25 μl of 2 × Laemmli reducing sample buffer, and boiled for 5 min. The sample mixtures were then loaded onto SDS-polyacrylamide gel electrophoresis gels. The bound active GTP-Rac was analyzed by Western blotting using anti-Rac monoclonal antibody.

RNA extraction and real-time reverse transcription-polymerase chain reaction of TIMP-1

Total RNA was isolated from renal cortex using TRIzol reagent (GIBCO, Life Technologies, Carlsbad, CA, USA) according to the protocol described by the manufacturer. The mRNA levels for TIMP-1 were analyzed by real-time quantitative reverse transcription-polymerase chain reaction using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA, USA) according to the protocol described by the manufacturer. The mRNA level of TIMP-1 was normalized to the 18S mRNA. The specific primers for TIMP-1 based on the core sequence of rat TIMP-1 cDNA (Accession number NM-003254) were 5′-CCA GAA ATC ATC GAG ACC AC-3′ (forward) and 5′-CGG AAA CCT GTG GCA TTT-3′ (reverse).

FRET assay for MMP-1 activity measurement

MMP-1 activity was measured using EnzoLyte™ 520 MMP-1 assay kit from AnaSpec Inc. (San Jose, CA, USA). This kit uses a 5-FAM (fluorophore)- and QXL520™ (quencher)-labeled FRET peptide substrate for continuous measurement of the enzyme activities. In an intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL520™. Upon the cleavage of the FRET peptide by MMP-1, the fluorescence of 5-FAM is recovered, and can be continuously monitored at excitation/emission (490/520 nm). MMP-1 activity was measured according to the protocol described by the manufacturer. The change of enzyme activity of MMP-1 was presented as percent change in relative fluorescence unit vs control.

Liquid chromatography–electrospray ionization tandem–mass spectrometry for quantitation of ceramide

Separation, identification and quantitation of ceramide in renal cortex were performed by liquid chromatography/mass spectrometry. The HPLC is from HP 1100 series equipped with a binary pump, a vacuum degasser, a thermostated column compartment, and an autosampler (Agilent Technologies, Waldbronn, Germany). The HPLC separations were performed at 70°C on an RP C18 Nucleosil AB column (5 μm, 70 mm × 2 mm i.d.) from Macherey Nagel (Düren, Germany). The mobile phase was a gradient mixture formed as described.[42] The tissue lipids were extracted according to previous studies.[15,42] To avoid any loss of lipids, the whole procedure was performed in siliconized glassware. Mass spectrometry detection was carried out using an Ultima triple quadrupole instrument (Micromass, Manchester, UK) operating under MassLynx 3.5 and configured with a Z-spray electrospray ionization source. Source conditions were as described previously.[42]

Morphological examination

The fixed kidneys were paraffin-embedded, and sections were prepared and stained with periodic acid-Schiff stain. Glomeruli were evaluated (scored from 0 to 4) on the basis of the degree of glomerulosclerosis and MG matrix expansion as we described before.[7]

Statistical analysis

Data are presented as mean ± s.e.m. Significant differences between and within multiple groups were examined using analysis of variance for repeated measures, followed by Duncan's multiple-range test. Student's *t*-test was used to evaluate the significant differences between two groups of observations. $P < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS

This study was supported by Grants DK54927, HL070726, and HL57244 from National Institutes of Health.

REFERENCES

1. Bostom AG, Lathrop L. Hyperhomocysteinemia in end-stage renal disease: prevalence, etiology, and potential relationship to arteriosclerotic outcomes. *Kidney Int* 1997; **52**: 10–20.
2. Dennis VW, Nurko S, Robinson K. Hyperhomocysteinemia: detection, risk assessment, and treatment. *Curr Opin Nephrol Hypertens* 1997; **6**: 483–488.
3. Dennis VW, Robinson K. Homocysteinemia and vascular disease in end-stage renal in patients with chronic renal failure. *Kidney Int* 1997; **52**: 495–502.
4. Perna AF, Ingrosso D, Castaldo P *et al.* Homocysteine, a new crucial element in the pathogenesis of uremic cardiovascular complications. *Miner Electrolyte Metab* 1999; **25**: 95–99.
5. Bostom AG, Shemin D, Lapane KL *et al.* Hyperhomocystinemia and traditional cardiovascular disease risk factors in end-stage renal disease patients on dialysis: a case–control study. *Atherosclerosis* 1995; **114**: 93–103.
6. Ducloux D, Motte G, Challier B *et al.* Serum total homocysteine and cardiovascular disease occurrence in chronic, stable renal transplant recipients: a prospective study. *J Am Soc Nephrol* 2000; **11**: 134–137.
7. Li N, Chen YF, Zou AP. Implications of hyperhomocysteinemia in glomerular sclerosis in hypertension. *Hypertension* 2002; **39**: 443–448.
8. McCully KS. *The Homocysteine Revolution: Medicine for the New Millennium*. Keats Publishing: New Canaan, CT, 1997.
9. McCully KS. Chemical pathology of homocysteine. I atherogenesis. II Carcinogenesis and homocysteine thiolacton metabolism. III Cellular function and aging. *Ann Clin Lab Sci* 1993; **23**: 477–493.
10. McCully KS. Atherosclerosis, serum cholesterol and the homocysteine theory: a study of 194 consecutive autopsies. *Am J Med Sci* 1990; **299**: 217–221.
11. De Groote MA, Testerman T, Xu Y *et al.* Homocysteine antagonism of nitric oxide-related cytostasis in *Salmonella typhimurium*. *Science* 1996; **272**: 414–417.
12. Harker LA, Ross R, Slichter SJ, Scott CR. Homocystine-induced arteriosclerosis. The role of endothelial cell injury and platelet response in its genesis. *J Clin Invest* 1976; **58**: 731–741.
13. Kanani PM, Sinkey CA, Browning RL *et al.* Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. *Circulation* 1999; **100**: 1161–1168.
14. Yang ZZ, Zou AP. Homocysteine enhances TIMP-1 expression and cell proliferation associated with NADH oxidase in rat mesangial cells. *Kidney Int* 2003; **63**: 1012–1020.
15. Yi F, Zhang AY, Janscha J *et al.* Homocysteine activate NADH/NADPH oxidase through ceramide-stimulated rac activity in rat mesangial cells. *Kidney Int* 2004; **66**: 1977–1987.
16. Yin T, Sandhu G, Wolfgang CD *et al.* Tissue-specific pattern of stress kinase activation in ischemic/reperfused heart and kidney. *J Biol Chem* 1997; **272**: 19943–19950.
17. Kaushal GP, Singh AB, Shah SV. Identification of caspase (ICE-like proteases) gene family in rat kidney and altered expression in ischemia/reperfusion injury. *Am J Physiol* 1998; **274**: F587–F595.
18. Ueda N, Kaushal GP, Shah SV. Apoptotic mechanisms in acute renal failure. *Am J Med* 2000; **108**: 403–415.
19. Zhang DX, Zou AP, Li PL. Ceramide-induced activation of NADPH oxidase and endothelial dysfunction in small coronary arteries. *Am J Physiol Heart Circ Physiol* 2003; **284**: H605–H612.
20. Duthie SJ, Grant G, Narayanan S. Increased uracil misincorporation in lymphocytes from folate-deficient rats. *Br J Cancer* 2000; **83**: 1532–1537.
21. Miller JW, Nadeau MR, Smith J *et al.* Folate-deficiency-induced homocysteinaemia in rats: disruption of S-adenosylmethionine's co-ordinate regulation of homocysteine metabolism. *Biochem J* 1994; **298**(Part 2): 415–419.
22. Bonaventura D, Tirapelli CR, Haddad R *et al.* Chronic methionine load-induced hyperhomocysteinemia enhances rat carotid responsiveness for angiotensin II. *Pharmacology* 2004; **70**: 91–99.
23. Dbaibo GS, El-Assaad W, Krikorian A *et al.* Ceramide generation by two distinct pathways in tumor necrosis factor alpha-induced cell death. *FEBS Lett* 2001; **503**: 7–12.
24. Tummalapalli CM, Tyagi SC. Responses of vascular smooth muscle cell to extracellular matrix degradation. *J Cell Biochem* 1999; **75**: 515–527.
25. Wallner EI, Yang Q, Peterson DR *et al.* Relevance of extracellular matrix, its receptors, and cell adhesion molecules in mammalian nephrogenesis. *Am J Physiol Renal Physiol* 1998; **275**: F467–F477.
26. Schaefer L, Han X, August C *et al.* Differential regulation of glomerular gelatinase B (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in obese Zucker rats. *Diabetologia* 1997; **40**: 1035–1043.
27. Torres L, Garcia-Trevijiano ER, Rodriguez JA *et al.* Induction of TIMP-1 expression in rat hepatic stellate cells and hepatocytes: a new role for homocysteine in liver fibrosis. *Biochim Biophys Acta* 1999; **1455**: 12–22.
28. Hubchak SC, Runyan CE, Kreisberg JI, Schnaper HW. Cytoskeletal rearrangement and signal transduction in TGF-beta1-stimulated mesangial cell collagen accumulation. *J Am Soc Nephrol* 2003; **14**: 1969–1980.
29. Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol* 1995; **15**: 551–561.

30. Coroneos E, Martinez M, McKenna S, Kester M. Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines. *J Biol Chem* 1995; **270**: 23305–23309.
31. Hojjati MR, Li Z, Zhou H *et al.* Effect of myriocin on plasma sphingolipid metabolism and atherosclerosis in apoE-deficient mice. *J Biol Chem* 2005; **280**: 10284–10289.
32. Park TS, Panek RL, Mueller SB *et al.* Inhibition of sphingomyelin synthesis reduces atherogenesis in apolipoprotein E-knockout mice. *Circulation* 2005; **110**: 34665–34671.
33. Landmesser S, Dikalov SR, Price L *et al.* Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 2003; **111**: 1201–1209.
34. Cai H, Griendling KK, Harrison DG. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci* 2003; **24**: 471–478.
35. Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler Thromb Vasc Biol* 2000; **20**: 2175–2183.
36. Takeya RN, Ueno K, Kami M *et al.* Novel human homologues of p47phox and p67phox participate in activation of superoxide-producing NADPH oxidases. *J Biol Chem* 2003; **278**: 25234–25246.
37. Brandes RP, Kreuzer J. Vascular NADPH oxidases: molecular mechanisms of activation. *Cardiovasc Res* 2005; **65**: 16–27.
38. Geiszt M, Kopp JB, Varnai P, Leto TL. Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci USA* 2000; **97**: 8010–8014.
39. Bokoch GM, Diebold BA. Current molecular models for NADPH oxidase regulation by Rac GTPase. *Blood* 2002; **100**: 2692–2696.
40. Abo A, Pick E, Hall A *et al.* Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature* 1991; **353**: 668–670.
41. Chen YF, Li PL, Zou AP. Effect of hyperhomocysteinemia on plasma or tissue adenosine levels and renal function. *Circulation* 2002; **106**: 1275–1281.
42. Fillet M, Van Heugen JC, Servais AC *et al.* Separation, identification and quantitation of ceramides in human cancer cells by liquid chromatography–electrospray ionisation tandem mass spectrometry. *J Chromatogr A* 2002; **949**: 225–233.