ORIGINAL ARTICLE

Importance of Riboflavin Kinase in the Pathogenesis of Stroke

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Keywords

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Introduction

Stroke is the second leading cause of death and the principal cause of serious disability among older adults. Despite progress made in understanding the pathophysiology of stroke, no method available has proved efficacious for the prevention and treatment of ischemic stroke. Further studies are required to investigate potential risk factors for the occurrence of stroke and measures for primary and secondary prevention.

Hypertension has been considered to be the most consistent and powerful predictor of stroke and explains 60% of the population-attributable risk of stroke [1]. But hypertension is not the only risk factor for stroke [2,3]. Neither all patients with stroke had the history of hypertension nor do all patients with hypertension suffer from stroke. This reminds us to wonder: whether there are some other risk factors beyond hypertension that may also contribute to the occurrence of stroke.

The spontaneously hypertensive rat (SHR) is a classic and the most widely studied animal model for the study of human essential hypertension. Several similarities between human primary hypertension and hypertension in SHR have been identified in both the pathophysiology and the clinical course of the hypertensive disease [4]. The stroke-prone spontaneously hypertensive rat (SHRSP) is the most frequently used experimental model for human stroke [5]. SHRSP is preferential mating from SHR and endogamy through generations. Almost all

SUMMARY

Aims: To explore risk factors for stroke independent of hypertension and the relationship between riboflavin kinase (RFK) and stroke. **Methods:** Gene expression profiling in the brains of spontaneously hypertensive rats (SHR) and stroke-prone spontaneously hypertensive rats (SHRSP) was comparatively analyzed by gene chips. The differentially expressed gene RFK was further verified by q-PCR and Western blot. The protective role of RFK-regulated flavins (including riboflavin, flavin mononucleotide, and flavin adenine dinucleotide) in stroke was observed in middle cerebral artery occlusion (MCAO) mice. Influence of flavins on apoptosis and death in oxygen and glucose deprivation (OGD)-treated neurons was examined by flow cytometry. Bax and Bcl-2 proteins were detected by Western blot. **Results:** Of the 76 differentially expressed genes, 41 genes were upregulated, and 35 genes were downregulated in SHRSP as compared with SHR. RFK was significantly downregulated in SHRSP. Flavins markedly decreased infarct area in MCAO mice, inhibited apoptosis and death in OGD-treated neurons, and decreased Bax protein expression. **Conclusions:** Physiological downregulation of RFK may be a new potential risk factor for stroke, which probably affects the absorbance and utility of riboflavin and further destroys the protective effect of flavins on stroke. RFK might act as a therapeutic target for stroke.

adult SHRSP spontaneously suffered from stroke and died of stroke, and therefore, it is regarded as an ideal animal model for stroke study.

Both SHR and SHRSP are hypertensive animals, although SHRSP is more susceptible to stroke as compared with SHR [6]. Therefore, we planned to compare the gene expression profiling of SHRSP with that of SHR to investigate risk factors contributing to stroke susceptibility independent of hypertension.

The Gene Ontology (GO) project is a major collaborative bioinformatics classification that aims to standardize the presentation of gene and gene product attributes across species. The project provides a controlled vocabulary of terms for describing gene product characteristics, supports gene product annotation data from GO Consortium members, and develops tools to assess and process these data. GO analysis [7] includes three independent ontology: biological processes, molecular function, and cellular component, making it possible to annotate altered genes biologically. We supposed that GO-based mapping of gene expression changes could provide a better understanding about different biological events between SHRSP and SHR.

The aim of this study is to find some new risk factors for stroke. SHR and SHRSP were used to exclude the influence of hypertension. Differently expressed genes were further extracted by GO categories analysis. Finally, the correlation between the differently expressed gene riboflavin kinase (RFK) and stroke was further evaluated.

Materials and Methods

Microarray Analysis and Data Analysis

The gene expression profiling between SHRSP and SHR was compared using Affymetrix GeneChip (Affymetrix rat genome 230 2.0 array) expression analysis. All animals used in our study were humanely cared for according to the Institutional Animal Care Guidelines, and the experimental design was approved by the Local Institutional Committee of the Second Military Medical University in Shanghai, China. Five-month-old male SHR and SHRSP were supplied by the animal center of the said university. Brain tissues were homogenized, and total RNA was extracted using the maxi RNeasy kit according to the manufacturer's protocol (Qiagen, Shanghai, China). Biotinylated amplified target cRNA was prepared and hybridized to the Affymetrix Rat genome 230 2.0 array set (a total of 31,000 probe sets) as described by Affymetrix. After hybridization, microarray chips were washed, stained, and scanned. Raw data analysis was performed using software GCOS 1.4. For each of the raw data probe sets, a perfect match versus mismatch value was calculated, and the data were compiled into one value for each probe set. Statistical tools available from Genespring 1 GX 10 (Silicon Genetics, Redwood City, CA, USA) were used to normalize and filter the chip data and identify differentially expressed probe sets among SHRSP and SHR. Genes were defined as differentially expressed if the fold change was at least 2-fold and statistically significant.

GO Categories Analysis

To identify differentially expressed GO terms in the biological process categories, GO categories analysis was performed using the GeneSpring (Silicon Genetics, Santa Clara, CA, USA) GO browser tool, which iterates overall GO terms. $P < 0.05$ was considered statistically significant.

Middle Cerebral Artery Occlusion

Adult male C57BL mice aged 10 weeks (Sino-British SIPPR/BK Lab Animal Ltd., Shanghai, China) were subjected to permanent MCAO. Before MCAO, mice ($n = 9$) were intraperitoneally administered with: (1) saline for sham operation group; (2) 3.5 mmol/kg riboflavin (R9504; Sigma-Aldrich, St. Louis, MO, USA); (3) 3.5 mmol/kg flavin mononucleotide (FMN, Sigma, F6625); or (4) 3.5 mmol/kg flavin adenine dinucleotide (FAD, Sigma, F8384) for 7 consecutive days. MCAO was performed as described in a previous report [8]. Small cortical infarcts were induced by irreversible transtemporal MCAO distal to the lenticulostriate artery. Mice were anesthetized with 15% chloral hydrate. After a 1-cm skin incision was made between the right eye and ear, a burr hole was drilled through the temporal skull. The dura mater was removed, and the middle cerebral artery was permanently occluded using a bipolar electrocoagulation forceps. Sham operation was performed as described above except for coagulation of the middle cerebral artery. After 24-h MCAO, C57BL mice were analgesically sacrificed to obtain the brain. Serial coronal

sections (1 mm apart) were stained with 2,3,5-triphenyltetrazolium chloride monohydrate (TTC; Sigma-Aldrich, St. Louis, MO, USA) for assessing the infarct area [9]. Sections were digitized, and the infarct area and noninfarct tissues were calculated by the software Image J [10].

Primary Cultured Neurons and Treatment

Primary cultured neurons from cortices derived from embryonic day 16–18 SHRSP pups were prepared according to previously established protocols [11] with minor modifications. Briefly, neurons were isolated from the cerebral cortex of fetal SHRSP. The cortex was dissected under a microscope. After removing the meninges, the cortex was digested with 0.25% trypsin (GIBCO). Cells were cultured on poly-D-lysine-coated plates at a density of 1×10^6 cells/mL in medium containing neurobasal medium (GIBCO) supplemented with 2% B27, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.5 mM glutamine (GIBCO) and incubated at 37°C with 5% CO₂. The medium was changed twice a week. All experiments were performed 1 week postisolation.

The cells were respectively treated as follows: (1) saline; (2) riboflavin; (3) FMN; and (4) FAD. The apoptosis and death rate of neurons induced by oxygen and glucose deprivation (OGD) were determined by flow cytometry. The protein expression of Bax and Bcl-2 in these neurons was determined by Western blot.

Oxygen and Glucose Deprivation

To eliminate the influence of riboflavin supplement (0.4 mg/L) in commercial neurobasal medium, Krebs' solution (126 mM NaCl, 2.5 mM KCl, 2.4 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃) was used as substitute medium during experiment.

On the day of the experiment, the neurobasal medium was exchanged for high-glucose Krebs' solution (with 11 mM Glucose) with indicated doses of flavin (riboflavin, FMN, or FAD) in the incubator under 5% CO₂ and 95% air at 37°C for 2 h. To model ischemia-like conditions *in vitro*, cultured neurons were then exposed to the OGD condition by transfer to glucose-free Krebs' solution with indicated doses of flavin in a box gassed with 94% N₂, 5% CO₂, and 1% O₂ at 37°C for 1.5 h. Neuronal viability and protein expression were measured after OGD. Control cells were transferred in the same way without adding flavin.

Flow Cytometry

Oxygen and glucose deprivation-induced apoptosis and death of cultured neurons were evaluated by a commercial Annexin V-FITC detection kit with Propidium iodide (Keygen, Nanjing, China). After OGD, neurons were digested with 0.25% trypsin (GIBCO), collected, and processed to single-cell suspension. The neurons were then stained following the manufacturer's protocols. Flow cytometry was performed on a Beckton Dickinson FAC-Scan and analyzed by CellQuest Pro software (BD, Franklin Lakes, NJ, USA).

Real-Time Polymerase Chain Reaction

The mRNA level of RFK in the brain was quantitated by real-time PCR [12,13]. Total RNA was extracted from the brains by Trizol (Invitrogen, Carlsbad, CA, USA), and 2 μ g RNA was reverse-transcribed to cDNA using RNA reverse transcriptase (Promega, Madison, WI, USA). The quantitative real-time PCR was performed in an Opticon Monitor 3 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) on an ABI 7500 PCR instrument (Applied Biosystems, Carlsbad, CA, USA) with the SYBR Premix Ex Taq™ Mixture (Takara, Otsu, Japan) following the manufacturer's instructions. The primers for RFK were as follows: 5'-GTGTCCACTGGCATCTATTACG-3' (sense) and 5'-CAGGTC-TGAGGTAGCCAACAAT-3' (antisense); for GAPDH were as follows: 5'-AGACCTCTATGCCAACACAGTGC-3' (sense) and 5'-GAGCCACCAATCCACACAGAGT-3' (antisense). GAPDH was used as internal control. The real-time PCR was performed with transcription efficiency >90% [14]. The result was analyzed using the $2^{-\Delta\Delta C_t}$ method.

Western Blot

Western blot analysis was performed as described in a previous report [11] with minor modifications [15,16]. Total protein was immediately extracted and run on 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The proteins were electrotransferred to nitrocellulose membranes, incubated with the primary antibody for 2.5 h, and then incubated with corresponding secondary antibodies. The images were captured and analyzed by the Odyssey infrared fluorescence imaging system (Li-Cor Bioscience, Lincoln, NE, USA). The adoption of this system allowed for superior sensitivity, linearity, and reproducible quantitation compared with formerly used X-ray film-based technologies for WB.

Statistics

Data are expressed as mean $\pm$ SD. Comparisons among groups were made by analysis of variance (ANOVA) followed by Dunnett's *t*-test. $P < 0.05$ was considered statistically significant.

Table 1 Differential Gene Ontology (GO) with statistical significance related to riboflavin

GO ID	GO term	Genes in term	<i>P</i> value
GO:0009231	Riboflavin biosynthetic process	3	0.000
GO:0006771	Riboflavin metabolic process	3	0.000
GO:0008531	Riboflavin kinase activity	3	0.000
GO:0042726	Riboflavin and derivative metabolic process	3	0.000
GO:0042727	Riboflavin and derivative biosynthetic process	3	0.000

"Genes in term" represents the number of altered genes in the GO term. *P* values <0.05 were considered statistically significant. GO terms are ranked by *P* values.

Table 2 Genes in the differential Gene Ontology (GO) category related to riboflavin

Unigene	Gene symbol	Fold change	Regulation	<i>P</i> value
13360	Riboflavin kinase	1.32	Down	0.003
147523	Nmnat1	2.14	Down	0.024
202968	Alb	3.07	Down	0.042
19716	LOC499330	1.35	Up	0.023
91245	Gad1	1.95	Up	0.014
6526	Cortl/kif1b	3.10	Up	0.003

Down: downregulation in spontaneously hypertensive rats (SHR)-SP compared with control. Up: upregulation in SHR-SP compared with control.

Results

Gene Expression Profiling

Genome microarrays showed very significant differences in gene expression profiling between SHRSP and SHR (Supplementary Figure S1). A total of 76 probe sets were identified with significant differential expression levels between SHRSP and SHR. Of the 76 differentially expressed probe sets, 41 genes were upregulated,

Figure 1 (A) Real-time PCR showed that riboflavin kinase (RFK) mRNA level in the brain of stroke-prone spontaneously hypertensive rats (SHRSP) was lower than that of spontaneously hypertensive rats (SHR) ($n = 8$, $**P < 0.01$). (B) Western blot showed that RFK protein level in the brain of SHRSP was lower than that of SHR ($n = 3$, $**P < 0.01$). Data are presented as mean $\pm$ SD.

Figure 2 Flavin (riboflavin, FMN, or FAD) treatment decreased the middle cerebral artery occlusion (MCAO)-induced infarct area in C57BL. The mice received flavins (3.5 mmol/kg; intraperitoneal injection), respectively, for 7 consecutive days before MCAO. At 24 h after MCAO, mice were anesthetized and the brains were immediately stained with triphenyltetrazolium chloride monohydrate (TTC) ($n = 9$, $**P < 0.01$).

and 35 genes were downregulated in SHRSP as compared with SHR. (Supplementary Table S1.)

Feature Extraction of Altered Genes by GO

Genes that were differentially expressed between SHRSP and SHR were subjected to functional clustering according to their GO classification (in the biological process category). It was found that of the top 10 GO categories altered in SHRSP compared with SHR, 5 GO categories related to “riboflavin” (Table 1). A further evaluation of the altered gene list (Table 2) in these GO categories

revealed that RFK gene in SHRSP decreased significantly to about 60% as compared with SHR ($P = 0.003$, fold change = 1.32).

Verification of RFK Alteration

To further confirm RFK alteration, the RFK expression in the healthy adult rat brain of SHRSP and SHR was measured by real-time PCR and Western blot. The result showed that the level of RFK mRNA and protein expression in the brain of SHRSP was 27% (Figure 1A) and 46% (Figure 1B) less than that of SHR, respectively.

Figure 3 Flavin (riboflavin, FMN, or FAD) treatment inhibited the apoptosis rate and death rate of neurons induced by oxygen and glucose deprivation (OGD). Neurons were treated with different concentrations of flavin, respectively, in air and high glucose for 2 h and then in OGD for 1.5 h. After OGD, the apoptosis rate and death rate of neurons were immediately determined by flow cytometry. Data are presented as mean $\pm$ SD ($n = 3$, $*P < 0.05$, $**P < 0.01$). (A) Images captured by flow cytometry. (B) Apoptosis rates. (C) Death rates.

Flavin Treatment Decreases the Infarction Area in MCAO Mice

Flavins, include riboflavin, FMN, and FAD, play an important role in respiratory chain. FMN and FAD are the active forms of riboflavin *in vivo*. RFK is an enzyme that catalyzes the phosphorylation of riboflavin to form FMN, which is an obligatory step in riboflavin utilization and flavin cofactor synthesis. Considering that the altered GO categories are closely related to “riboflavin,” we presumed that decreased RFK may result in the decrease of FMN and FAD, finally leading to more susceptibility of SHRSP to stroke as compared with SHR.

To evaluate the protective role of RFK, the effect of flavins on infarct areas was observed in MCAO mice. Quantitative assessment of TTC-stained sections showed that flavin treatment significantly decreased the infarct area at 24 h after MCAO operation as compared with the sham operation group (Figure 2). There was no significant difference between the flavin treatment groups.

Flavin Treatment Inhibited OGD-induced Neuronal Apoptosis and Death *in Vitro*

To explore the neuroprotective effect of flavins, the effect of flavins on apoptosis and death in OGD-induced neurons was observed. The results showed that 0.5 μM flavin significantly inhibited OGD-induced neuronal apoptosis and death (Figure 3).

Inhibition of Bax Expression is Involved in Flavin-induced Neuroprotection

To further explore the mechanism of neuroprotective effect of flavins, the effect of flavins on the expression of apoptosis-triggering enzyme Bax and antiapoptosis protein Bcl-2 in OGD-treated neurons was observed. As shown in Figure 4, riboflavin and FMN significantly decreased Bax protein expression, which was most obvious at the doses of 1.0–1.5 μM (Figure 4A,B). FAD treatment did not influence the Bax protein expression. Bcl-2 was not obviously affected by flavin in OGD-treated neurons.

Discussion

Using a comparative genome analysis, we screened out 76 differentially expressed genes in SHRSP as compared with SHR. To the best of our knowledge, this is the first study to compare the gene profiling between SHR and SHRSP by excluding hypertension from the risk factors for stroke.

The results of GO categories analysis showed that downregulation of RFK made SHRSP more susceptible to stroke as compared with SHR. RFK, also known as flavokinase, is an enzyme that catalyzes the phosphorylation of riboflavin (VB_2) to generate FMN, which is adenylated by FAD synthetase to generate FAD [17]. RFK is a physiological regulator and may involve in the pathogenesis of stroke. To the best of our knowledge, this is the first study to suggest a relationship between RFK and stroke.

Riboflavin is a water-soluble vitamin existing in a wide variety of foods. A small amount of riboflavin is present in foods as free riboflavin, which is an isoalloxazine ring bound to a ribitol side

Figure 4 Riboflavin or FMN treatment inhibited the expression of Bax in neurons induced by oxygen and glucose deprivation (OGD). Neurons were treated with flavin (riboflavin, FMN, and FAD), respectively, in air and high glucose for 2 h and then in OGD for 1.5 h. Protein was immediately extracted after OGD. Bax and Bcl-2 were determined by Western blot. Data are presented as mean $\pm$ SD ($n = 3$, $*P < 0.05$, $**P < 0.01$). (A) Images of the expression level of Bax and Bcl-2 protein. (B) Relative expression of Bax in neurons treated with flavins. (C) Relative expression of Bcl-2 in neurons treated with flavins.

chain mostly presenting as a derivative FAD, and a smaller amount occurs as the monophosphorylated form, FMN [18]. The absorption of riboflavin takes place predominantly in the proximal small intestine through an active, carrier-mediated, and saturable transport process [19]. Riboflavin enters the plasma from the small intestine as free from or as FMN. Thus, the downregulation of RFK inhibits the transform of free riboflavin to FMN and FAD and further influences the absorption and utilization of riboflavin *in vivo*. It is said the low level of RFK will results in deficiency of riboflavin [20], which may play a protective role against stroke.

Considering this, a further study on the relationship between flavins and stroke should be performed.

Recent interest on the relationship of riboflavin and stroke is focused on the role that riboflavin plays in determining circulating concentrations of homocysteine, a risk factor for cardiovascular disease [21]. A high proportion of acute stroke patients were biochemically deficient of riboflavin immediately after infarction [20]. Riboflavin has been reported to reduce ischemic brain injury in focal cerebral ischemia [22]. Our study showed that flavin treatment significantly decreases the brain infarct area induced by MCAO as compared with the sham operation group, indicated that flavin inhibits the progression of stroke and protected the brain tissue against ischemia.

To explore the mechanism underlying the protective effect of RFK, we tried to find out the relationship between RFK and ischemia-induced apoptosis. Apoptosis and necrosis are suggested to be the two poles of a continuum of cellular death [23]. Apoptosis after cerebral ischemia is responsible for an important portion of ischemic brain damage [24], and that inhibitors of this cascade would harbor therapeutic, neuroprotective potential [25]. RFK has been reported to be involved in death receptor 4 and 5 that activate Nox1 NADPH oxidase to induce reactive oxygen species-mediated apoptotic cell death [26].

Mitochondria play an important role in the process of apoptosis [27]. The Bcl-2 protein family is a principal regulator of mitochondrial membrane integrity and function. Bax represents the proapoptotic protein, and Bcl-2 represents the antiapoptotic protein. In response to death signals, Bax translocates from the cytosol to the mitochondria, resulting in abnormally permeability of the outer membrane of mitochondria and the release of cytochrome c

from the intermembrane space [28]. This proapoptotic protein translocation is controlled by the family of Bcl-2 protein [29]. The results of the present study suggest that Bax, but not Bcl-2, may participate in protective ischemic preconditioning of riboflavin and FMN induced by OGD. The protective action of RFK might be due to increased utilization of flavins and inhibition on the progress of neuronal apoptosis *in vivo*, suggesting that RFK may act as a candidate therapeutic target.

In conclusion, of the 76 differentially expressed genes between SHRSP and SHR, 41 genes were upregulated, and 35 genes were downregulated in the brain of SHRSP as compared with SHR. The downregulation of RFK might be the reason why SHRSP is more susceptible to stroke than SHR. This was supported by the result that flavin treatment significantly decreased the MCAO-induced infarct area in mice, and OGD induced apoptosis and death in primary cultured neurons. The mechanism underlying the antiapoptosis effect of flavins is mediated by depressing the expression of Bax protein to inhibit mitochondrial apoptosis. If the relationship between RFK and stroke could be confirmed and the detailed mechanism could be clarified in more future studies, it will provide a novel therapeutic strategy for stroke.

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Conflict of Interest

The authors declare no conflict of interest.

References

- Lawes CM, Vander HS, Law MR, Elliott P, MacMahon S, Rodgers A. Blood pressure and the global burden of disease 2000. Part II: estimates of attributable burden. *J Hypertens* 2006;**24**:423–430.
- Mandić M, Rancić N. Risk factors for stroke. *Med Pregl* 2011;**64**:600–605.
- Endres M, Heuschmann PU, Laufs U, Hakim AM. Primary prevention of stroke: blood pressure, lipids, and heart failure. *Eur Heart J* 2011;**32**:545–552.
- Púzserová A, Kopincová J, Bernátová I. Endothelial (dys) function in the experimental model of primary hypertension. *Cesk Fysiol* 2010;**59**:4–14.
- McBride MW, Carr FJ, Graham D, et al. Microarray analysis of rat chromosome 2 congenic strains. *Hypertension* 2003;**41**:847–853.
- Zhang W, Liu AJ, Yi-Ming W, Liu JG, Shen FM, Su DF. Pressor and non-pressor effects of sodium loading on stroke in stroke-prone spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol* 2008;**35**:83–88.
- Ashburner J, Friston KJ. Voxel-based morphometry—the methods. *Neuroimage* 2000;**11**:805–821.
- Zhu W, Fan Y, Frenzel T, et al. Insulin growth factor-1 gene transfer enhances neurovascular remodeling and improves long-term stroke outcome in mice. *Stroke* 2008;**39**:1254–1261.
- Chen W, Hartman R, Ayer R, et al. Matrix metalloproteinases inhibition provides neuroprotection against hypoxia-ischemia in the developing brain. *J Neurochem* 2009;**111**:726–736.
- Swanson RA, Morton MT, Tsao WG, Savalos RA, Davidson C, Sharp FR. A semiautomated method for measuring brain infarct volume. *J Cereb Blood Flow Metab* 1990;**10**:290–293.
- Qiu J, Xu J, Zheng Y, et al. High-mobility group box 1 promotes metalloproteinase-9 upregulation through toll-like receptor 4 after cerebral ischemia. *Stroke* 2010;**41**:2077–2082.
- Bjornstad JL, Sjaastad I, Nygard S, et al. Collagen isoform shift during the early phase of reverse left ventricular remodelling after relief of pressure overload. *Eur Heart J* 2011;**32**:236–245.
- Llach A, Molina CE, Prat-Vidal C, et al. Abnormal calcium handling in atrial fibrillation is linked to up-regulation of adenosine A2A receptors. *Eur Heart J* 2011;**32**:721–729.
- Wang P, Yang FJ, Du H, et al. Involvement of Leptin Receptor Long Isoform (LepRb)-STAT3 signaling pathway in brain fat mass- and obesity-associated (FTO) downregulation during energy restriction. *Mol Med* 2011;**17**:523–532.
- Ma H, Guo R, Yu L, Zhang Y, Ren J. Aldehyde dehydrogenase 2 (ALDH2) rescues myocardial ischemia/reperfusion injury: role of autophagy paradox and toxic aldehyde. *Eur Heart J* 2011;**32**:1025–1038.
- Brokopp CE, Schoenauer R, Richards P, et al. Fibroblast activation protein is induced by inflammation and degrades type I collagen in thin-cap fibroatheromata. *Eur Heart J* 2011;**32**:2713–2722.
- Hirano G, Izumi H, Yasuniwa Y, et al. Involvement of riboflavin kinase expression in cellular sensitivity against cisplatin. *Int J Oncol* 2011;**38**:893–902.
- Powers HJ. Riboflavin (vitamin B-2) and health. *Am J Clin Nutr* 2003;**77**:1352–1360.
- Jusko WJ, Levy G. Absorption, metabolism and excretion of riboflavin 5'-phosphate in man. *J Pharm Sci* 1967;**56**:58–62.
- Gariballa S, Ullegaddi R. Riboflavin status in acute ischaemic stroke. *Eur J Clin Nutr* 2007;**61**:1237–1240.
- Ward M, Wilson CP, Strain JJ, Horigan G, Scott JM, McNulty H. B-vitamins, methylenetetrahydrofolate reductase (MTHFR) and hypertension. *Int J Vitam Nutr Res* 2011;**81**:240–244.
- Betz AL, Ren XD, Ennis SR, Hultquist DE. Riboflavin reduces edema in focal cerebral ischemia. *Acta Neurochir Suppl (Wien)* 1994;**60**:314–317.
- McManus JP, Buchan AM. Apoptosis after experimental stroke: fact or fashion? *J Neurotrauma* 2000;**17**:899–914.
- Sairanen T, Karjalainen-Lindsberg ML, Paetau A, Ijäs P, Lindsberg PJ. Apoptosis dominant in the periinfarct area of human ischaemic stroke—a possible target of antiapoptotic treatments. *Brain* 2006;**129**:189–199.
- Nicotera P, Lipton SA. Excitotoxins in neuronal apoptosis and necrosis. *J Cereb Blood Flow Metab* 1999;**19**:583–591.
- Yazdanpanah B, Wiegmann K, Tchikov V, et al. Riboflavin kinase couples TNF receptor 1 to NADPH oxidase. *Nature* 2009;**460**:1159–1163.
- Verdin E, Hirschey MD, Finley LW, Haigis MC. Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling. *Trends Biochem Sci* 2010;**35**:669–675.
- Kroemer G. Mitochondrial control of apoptosis: an introduction. *Biochem Biophys Res Commun* 2003;**304**:433–435.
- Kuwana T, Mackey MR, Perkins G, Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* 2002;**111**:331–342.

Supporting Information

The following supplementary material is available for this article:

Figure S1. Cluster diagram.

Table S1. Genes differentially expressed between SHRSP and SHR.

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