The vascular endothelial nitric oxide (NO) produced from endothelial NO-synthase (eNOS) is an important regulator of vascular tone and blood homeostasis. NO protects against vascular occlusion through multiple functions including smooth muscle relaxation, inhibition of thrombosis and inflammatory responses. In addition, endothelial cells also produce factors that are importantly involved in blood homeostasis. Among these factors, plasminogen-activator inhibitor-1 (PAI-1), a principal inhibitor of fibrinolysis, has been shown to play a critical role in ischemic cardiovascular events related to atherothrombosis.

Substantial studies provide compelling evidence demonstrating that eNOS uncoupling, that is, generation of superoxide instead of NO from the enzyme, is an important mechanism of endothelial dysfunction under (physio) pathological conditions such as age-related vascular disease, atherosclerosis and diabetes melitus. Importantly, increased PAI-1 expression and elevated plasma PAI-1 levels are also observed in these conditions, which may contribute to pro-thrombotic states and increased risk of ischemic heart disease. Indeed, excess PAI-1 in human atherosclerotic plaques was found; this is exaggerated in type II diabetic patients. A number of inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin-1, and transforming growth factor-β, and high glucose, which are implicated in vascular dysfunctions under pathological conditions and aging, have been found to stimulate PAI-1 production in various cell types including endothelial cells.

In recent years there has been increasing interest in functions of the hexosamine biosynthetic pathway (HBP) in increased plasminogen activator inhibitor-1 (PAI-1), and endothelial nitric oxide synthase (eNOS) dysfunction in diabetes. Glucosamine (GlcN) that directly activates HBP is a dietary supplement and is clinically used to treat osteoarthritis despite uncertain efficacy and adverse cardiovascular effects observed in animal models. p38 mitogen-activated protein kinase (p38mapk) has been shown to be involved in HBP-mediated biological processes. The aim of the present study was to investigate the role of p38mapk in GlcN-induced endothelial PAI-1 expression and eNOS dysfunction.

Methods and Results: In cultured human endothelial cells, GlcN time- and concentration-dependently increased PAI-1 protein level that was further enhanced by tumor necrosis factor (TNF)-α, which was accompanied by a transient synergistic activation of p38mapk. The stimulation of PAI-1 by GlcN alone or by GlcN and TNF-α in combination was inhibited by the specific inhibitor of p38mapk, but not that of JNK or ERK1/2. Moreover, in isolated mouse aortas, GlcN caused eNOS uncoupling resulting in enhanced superoxide and decreased NO production, as well as impaired endothelium-dependent relaxations, which were also fully prevented by the p38mapk inhibitor.

Conclusions: HBP activated by GlcN increases PAI-1 expression and eNOS uncoupling depending on p38mapk, which not only explains hyperglycemic vascular complications, but also may bring into question the clinical use of GlcN. The present results, support currently ongoing clinical application of p38mapk inhibitor in patients with cardiovascular disease. (Circ J 2012; 76: 2015 – 2022)

Key Words: Cardiovascular disease; Endothelial dysfunction; Nitric oxide; Oxidative stress; Risk factor
tional roles of the hexosamine biosynthetic pathway (HBP) in regulation of cardiovascular functions. The HBP flux is activated in hyperglycemic conditions, in which HBP is diverted from glycolysis, whereby fructose-6-phosphate, which is derived from glucose-6-phosphate, is converted to glucosamine-6-phosphate by the rate-limiting enzyme 1-glutamine:fructose-6-phosphate amidotransferase (GFAT) in the presence of glutamine. Glucosamine-6-phosphate is further converted to N-acetylglucosamine-6-phosphate (GlcNAc-6-p) and finally to UDP-GlcNAc, which serves as the monosaccharide donor for O-linked attachment of O-GlcNAc to serine/threonine residues of cellular proteins, leading to post-translational modifications and functional alterations of the proteins. The O-GlcNAc modification of cellular proteins, that is, O-GlcNAcylation, is determined by two enzymes: O-GlcNAc transferase (OGT), which adds, and O-GlcNAcase, which removes, O-GlcNAc to and from proteins, respectively.\textsuperscript{18}

Interestingly, the HBP is not only activated in hyperglycemic conditions,\textsuperscript{17,18} but is also activated in hypoglycemic conditions,\textsuperscript{19} in aging, heart ischemia, and in response to the vasoconstrictor endothelin-1,\textsuperscript{20,21} and to many other stressors including heat shock, UV radiation, ethanol, hypoxia, oxidative, and osmotic stress.\textsuperscript{22} The HBP is therefore considered as a common stress signal in cells subjected to various stressors.\textsuperscript{24,26} The functions under these conditions seem not entirely clear. Enhanced HBP flux can be achieved pharmacologically by glucosamine (GlcN), which bypasses GFAT and enters the HBP by formation of glucosamine-6-phosphate in the cells. GlcN is used as a dietary supplement and clinically used as a supplement to treat osteoarthritis despite uncertain efficacy.\textsuperscript{23}

Moreover, adverse effects on the cardiovascular system have also been reported. Studies demonstrate that activation of the HBP under diabetic and hyperglycemic conditions results from oxidative stress, leading to O-GlcNAcylation of eNOS and inhibition of eNOS enzymatic activity.\textsuperscript{24,25} O-GlcNAcylation of Sp1 transcription factor enhances PAI-1 expression in endothelial cells.\textsuperscript{26} Importantly, animal experiments show that treatment of low density lipoprotein receptor (LDLR) or apolipoprotein E (ApoE) mice with GlcN accelerates atherosclerosis.\textsuperscript{27-29}

Recent studies have indicated that the protein kinase p38 mitogen-activated protein kinase (p38 mapk) is involved in the HBP. It has been shown that OGT activity in the HBP is decreased in hyperglycemic conditions, and inhibition of eNOS enzymatic activity, leading to oxidative stress, leading to O-GlcNAcylation of eNOS and inhibition of eNOS enzymatic activity,\textsuperscript{24,25} and O-GlcNAcylation of Sp1 transcription factor enhances PAI-1 expression in endothelial cells.\textsuperscript{26} Importantly, animal experiments show that treatment of low density lipoprotein receptor (LDLR) or apolipoprotein E (ApoE) mice with GlcN accelerates atherosclerosis.\textsuperscript{27-29}

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Methods

Materials

RPMI-1640 was purchased from Aminmed (Muttenz, Switzerland); Dulbecco Modified Eagle Medium (DMEM)-low glucose, Trypsin-EDTA and D-glucosamine hydrochloride were purchased from Sigma (Buchs, Switzerland); p38 mapk inhibitor SB203580 (4-[4-Fluorophenyl]-2-[4-methylsulfanylphenyl]-5-[4-pyridyl]1H-imidazole) and MEK inhibitor PD98059 (2′-Amino-3′-methylxoyllavone) were purchased from Calbiochem (Lucerne, Switzerland); JNK inhibitor SP600125 (Anthra[1,9-cd]pyrazol-6(2H)-one 1,9-pyrazoloanthrone) was from ANAWA Trading SA (Wangen, Switzerland); Bio-Rad DCTM protein assay kit was from Bio-Rad Laboratories (Basel, Switzerland); MG-132 was from Alexis Enzo Life Sciences (Lausen, Switzerland); rabbit anti-T202/Y204-p44/p42\textsuperscript{ERK}, rabbit anti-phospho-c-Jun-S63, rabbit anti-CREB-S133, rabbit anti-IkB, rabbit anti-phospho-p38 mapk (pThr-180/pTyr-182) and mouse anti-p38 mapk antibodies were from Cell Signaling Technology (Allschwil, Switzerland); anti-plasminogen activator inhibitor-1 antibody (anti-PAI-1, H135) was purchased from Santa Cruz Biotechnol (Basel, Switzerland); anti-α-tubulin antibody was from Sigma (Buchs, Switzerland); anti-mouse and anti-rabbit IgG (H+L) alkaline phosphatase (AP) conjugate antibody and Western Blue\textsuperscript{TM} (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium) stabilized substrate for AP from Promega (Wallisellen, Switzerland). Alexa Fluor680-conjugated anti-mouse IgG (A21057), dihydroethidium (DHE) were from Molecular Probes/Invitrogen (Lucerne, Switzerland), and IRDye800-conjugated anti-rabbit IgG (926–32211) was from LI-COR Biosciences (Bad Homburg, Germany); the membrane-permeable 4,5-diaminofluoresceine acetate (DAF-2DA) was from VWR international SA (Dietikon, Switzerland); endothelial cell growth supplement pack was from PromoCell (Allschwil, Switzerland) and all cell culture media and materials were purchased from Gibco BRL (Lucerne, Switzerland).

Cultivation of Human Umbilical Vein Endothelial Cells (HUVECs)

Endothelial cells were isolated from human umbilical veins and cultured as previously described.\textsuperscript{32} Cells at passage 2–4 were used. For the experiments, cells were cultured in DMEM containing 5.5 mmol/L of glucose and 0.2% BSA overnight prior to stimulation with GlcN alone or in combination with TNF-α (1 ng/ml) at different time points or concentrations of GlcN as indicated in the figures.

Immunoblotting for PAI-1 Expression and Activation of p38 mapk

Cell lysate preparation, SDS-PAGE, and transfer of SDS gels to an Immobilon-membrane were performed as previously described.\textsuperscript{33} Briefly, the membranes were incubated with the rabbit anti-PAI-1 antibody at a dilution of 1:200 overnight at 4°C with continuous agitation after being blocked with blocking buffer. For analysis of protein kinases, the quiescent endothelial cells were incubated with 5 mmol/L of GlcN for the indicated time and subjected to immunoblotting analysis with the antibody against phospho-p38 mapk (Thr-180/Tyr185) as well as total p38 mapk.\textsuperscript{34,35} After washing, the membranes were incubated for 2 h either with an AP-conjugated anti-rabbit secondary antibody and signals were detected using Western Blue\textsuperscript{TM} stabilized substrate, or incubated with a corresponding anti-mouse (Alexa fluor 680 conjugated) or anti-rabbit (IRDye 800 conjugated) secondary antibody, and signals were visualized using Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification of the signals was performed using NIH Image 1.62 software. To normalize the PAI-1 protein level, the membrane was probed with a mouse anti-α-tubulin antibody. PAI-1 level was quantified as the ratio of PAI-1/α-tubulin, and activation of p38 mapk was presented as the ratio of phospho-p38 mapk to total p38 mapk.

Endothelium-Dependent and -Independent Responses in Isolated Mouse Aortas

For the in vitro experiments with isolated blood vessels, C57BL/6J wild-type male mice (3–5 months) fed on chow diet ad libitum were anesthetized with xylazine (10 mg/kg body weight, i.p.) and ketamin (100 mg/kg body weight, i.p.) and killed. The thoracic aortas were removed, placed into cold (4°C) Krebs bicarbonate solution, and dissected free from fat.
and adhering perivascular tissue, and subjected to organ chamber experiments and en face staining as previously described. All the animal work was approved by the Ethics Committee of Veterinary Office of Fribourg, Switzerland and was performed in compliance with guidelines on animal experimentation at University of Fribourg.

The aortas with endothelium were cut into rings (3 mm in length) and suspended in a modified Krebs-Ringer bicarbonate solution aerated with 95% O₂ and 5% CO₂ at 37°C in a Multi-Myograph System (Model 610 M, Danish Myo Technology, Denmark) in parallel. The aortic rings were allowed to equilibrate for 30 min and progressively stretched to a passive tension of 5 mN, which gives the optimal length-tension combination. To test the endothelium-dependent or -independent relaxations, one ring was pretreated with p38 mapk inhibitor (SB203580, 10 μmol/L) or with tiron (10 mmol/L) for 30 min. The aortic rings were then incubated with 5 mmol/L of GlcN for 1 h. Norepinephrine (0.3 μmol/L) was then added to the blood vessels and allowed to induce a contraction. After the norepinephrine-induced contraction reached a plateau, cumulative concentration-response to acetylcholine (ACh; 1 nmol/L–10 μmol/L) or to the NO donor sodium nitroprusside (SNP; 0.1 nmol/L–3 μmol/L) was measured in each aortic ring.

En Face Confocal Detection of O₂⁻ and NO in Mouse Aortas

NO and superoxide production in the absence or presence of GlcN and/or inhibitors (p38 mapk inhibitor SB203580 or eNOS inhibitor Nω-nitro-l-arginine methyl ester (L-NAME)) was assessed with DAF-2DA and DHE staining, respectively, as described. Briefly, mouse aortas cleaned of perivascular tissues were equilibrated for 30 min in Krebs buffer at 37°C aerated with 95% O₂ and 5% CO₂. After equilibration, SB203580 (10 μmol/L) was added in one ring for 30 min, and GlcN (5 mmol/L) was added to respective blood vessels for 1 h. DAF-2DA (5 μmol/L) or DHE (5 μmol/L) was then added for 30 min. For the functional eNOS uncoupling experiment, the aortas were treated as already described, except that L-NAME (1 mmol/L) was added to the blood vessels for 30 min before GlcN treatment, followed by staining with DAF-2DA (5 μmol/L) or DHE (5 μmol/L) for 30 min. The aortas were then washed three times and fixed in 4% paraformaldehyde for 15 min followed by counterstaining with DAPI (300 nmol/L, 2 min). After washing with PBS, the aortas were carefully cut longitudinally and mounted en face (face down) on a slide and then covered with cover slip for endothelial layer imaging. Vectashield mounting medium was used to preserve the fluorescence. Fluorescence was analyzed using a Leica DM6000 confocal microscope within hours after preparation. Fluorescence from DAF-2DA staining was produced with a 488-nm argon laser with emission detection at 500–535 nm, whereas fluorescence from DHE was detected at 590 nm emission. Z-scanning was done for each sample. After the signal on the top (endothelial layer on the lumen border) of the sample was observed, the images were collected. Three consecutive images per field, acquired through the full thickness of endothelial signal, were recorded for analysis. At least 3 different fields per sample were evaluated. The images from DAF-2DA, DHE and DAPI staining were quantified with Image J.
software and results are presented as the ratio of DAF-2DA or DHE to DAPI.

Statistical Analysis

Data are presented as mean±SEM. The statistical analysis of difference between control and test groups was performed using one-way ANOVA with Bonferroni post test. P<0.05 was considered to be statistically significant.

Results

GlcN Enhances PAI-1 Protein Level in Endothelial Cells: Potentiated by TNF-α

Incubation of endothelial cells with GlcN at concentrations ranging from 0.01 to 10 mmol/L over 24h concentration-dependently enhanced PAI-1 protein level (Figure 1A). Moreover, time course experiment showed that the PAI-1 expression stimulated by GlcN increased significantly at 2 h by 3.9-fold, reached a maximum effect at 8 h, and was maintained up to 24 h (Figure 1B; 4.4-fold, P<0.001 to 0.05 vs. non-stimulated cells). TNF-α at a low concentration (1 ng/ml, 24 h) did not induce PAI-1 expression (Figure 1C), but significantly enhanced the stimulating effect of GlcN (5 mmol/L) on PAI-1 expression (Figure 1C, P<0.001).

Role of p38 mapk in PAI-1 Expression in Response to GlcN and TNF-α

The mechanisms of GlcN-induced PAI-1 expression in the endothelial cells were further analyzed. Treatment of the cells with MEK inhibitor PD98059 (50 μmol/L) or JNK inhibitor SP600125 (5 μmol/L), which is sufficient to inhibit the MEK or JNK pathway, respectively, in the cells stimulated with TNF-α (10 ng/ml, 15 min; Figure S1), did not affect GlcN’s effect on PAI-1 expression, whereas the p38 mapk inhibitor SB203580 (10 μmol/L) which efficiently inhibits p38 mapk signaling (Figure S1), abolished the GlcN-mediated increase in PAI-1 expression in the endothelial cells (Figure 2; P<0.05 vs. GlcN), demonstrating the involvement of p38 mapk in PAI-1 expression stimulated by GlcN. Similarly, the enhancing effect of TNF-α (1 ng/ml, 24 h) on PAI-1 expression in response to GlcN (5 mmol/L) was also reduced by the p38 mapk inhibitor SB203580 (10 μmol/L, Figure 2; P<0.001 vs. GlcN plus TNF-α), whereas inhibitors of MEK or JNK had no significant effects (Figure 2). The results demonstrate that PAI-1 expression either stimulated by GlcN alone or potentiated by TNF-α is dependent on p38 mapk. Notably, GlcN or TNF-α (1 ng/ml) alone induced a transient activation of p38 mapk in the endothelial cells over 60 min (Figure 3).
synergistic stimulation of p38 mapk activation by GlcN and TNF-α was observed at 15 min (Figure 3).

**Inhibition of p38 mapk Prevents Endothelial Dysfunction Mediated by GlcN**

In mouse aortic rings with endothelium contracted with nor-epinephrine (0.3 μmol/L), the endothelium-dependent relaxations in response to acetylcholine (1 μmol/L–10 μmol/L) were inhibited by pre-incubation of the aortic rings by GlcN (5 mmol/L) for 1 h (Figure 4; P<0.05 vs. GlcN treatment). The inhibitory effect of GlcN on the endothelium-dependent relaxations was fully prevented by the p38 mapk inhibitor SB203580 (10 μmol/L) and only partly prevented by the reactive oxygen species (ROS) scavenger tiron (10 mmol/L) at a concentration that has been shown to fully inhibit ROS generation in endothelial cells. The endothelium-independent relaxations in response to the NO-donor sodium nitroprusside (SNP) were not affected under these conditions (Figure 4).

**Inhibition of p38 mapk Prevents eNOS Uncoupling Induced by GlcN**

To further investigate the mechanism by which p38 mapk inhibitor prevents endothelial dysfunction caused by GlcN, we examined the effect of GlcN and p38 mapk inhibitor on eNOS uncoupling. Mouse aortas were pretreated with p38 mapk inhibitor SB203580 (10 μmol/L) for 30 min, followed by GlcN treatment (5 mmol/L) for 1 h as described; O$_2^−$ production and NO production were then determined on DHE and DAF-2DA staining, respectively. GlcN increased O$_2^−$ and decreased NO production, and these effects of GlcN were prevented by the p38 mapk inhibitor SB203580 (Figure 5A). The increase in O$_2^−$ production by GlcN was abolished by the eNOS inhibitor L-NAME (1 mmol/L, Figure 5B). Taken together, these results demonstrate that GlcN causes eNOS uncoupling through the p38 mapk pathway.

**Discussion**

Endothelial dysfunction and increased PAI-1 levels are common under various pathological conditions such as diabetes and atherosclerosis. Inflammation and oxidative stress are the main mechanisms of endothelial dysfunction and PAI-1 expression under these disease conditions. Previous studies have shown that the pro-inflammatory cytokine TNF-α either alone or together with high glucose has detrimental effects on endothelial cells, such as oxidative stress, impairment of endothelial-dependent relaxation, and augmentation of endothelial adhesion molecule expression for leukocytes. There is substantial evidence demonstrating that the HBP mediates many deleterious effects of diabetes, including insulin resistance, oxidative stress, enhanced PAI-1 expression, and endothelial dysfunction. As a consequence, enhanced HBP flux has been shown to accelerate atherogenesis in LDLR−/− and ApoE−/− mice. In line with these studies, the present study further demonstrates a synergistic effect of TNF-α and GlcN on endothelial PAI-1 expression. This effect of TNF-α and GlcN is dependent on the p38 mapk pathway, because inhibition of p38 mapk but not MEK or JNK reduced PAI-1 expression. Indeed, activation of p38 mapk by the HBP has been reported in mesangial cells, leading to PAI-1 gene expression. In the present study we detected a transient activation of p38 mapk by GlcN, which was potentiated...
by a low concentration of TNF-α at 15 min of stimulation. This transient potentiation of p38 mapk by GlcN and TNF-α was translated to a synergistic increase in PAI-1 expression. Because approximately 80% of the synergistic stimulation of PAI-1 expression by GlcN and TNF-α is inhibited by the p38 mapk inhibitor, another pathway(s) activated by GlcN and TNF-α may also be involved in endothelial PAI-1 expression.

There are studies showing that p38 mapk activates OGT in the HBP, leading to enhanced O-GlcNAcylation of proteins. It is unlikely, however, that in the present experimental setting, TNF-α enhances GlcN-induced PAI-1 expression through augmentation of increased OGT activity, because our previous study showed no further increase in O-GlcNAcylation of cellular proteins in endothelial cells exposed to TNF-α, although O-GlcNAcylation of certain proteins could not be fully excluded.

One of the important novel findings of the present study is that activation of the HBP by GlcN causes eNOS uncoupling through p38 mapk, thereby impairing endothelium-dependent relaxations in mouse aortas. The detrimental effect of the HBP

**Figure 5.** Inhibition of p38 mitogen-activated protein kinase (p38 mapk) prevents glucosamine (GlcN)-induced endothelial nitric oxide synthase uncoupling. Confocal microscopic en face detection of endothelial NO production and reactive oxygen species generation by DAF-2DA and DHE staining, respectively, followed by counterstaining with DAPI for endothelial nuclei in the intact thoracic aorta segments from C57BL mice. (A) Aortas were treated with GlcN in the absence or presence of p38 mapk inhibitor SB203580 as described in Figure 4. (B) Aortas were treated with GlcN (5 mmol/L, 1 h) in the absence or presence of Nω-nitro-L-arginine methyl ester (L-NAME) (1 mmol/L) prior to en face staining. Quantification of the fluorescence intensity normalized by DAPI is presented in the corresponding right panels. Scale bar, 100 μm. n=4, *P<0.05 and **P<0.01 between the indicated groups.
on endothelial function has been reported in numerous previ-
ous studies that demonstrated that enhanced HBP flux impairs
endothelial function through post-translational O-GlcNAcy-
a tion of Akt, an upstream kinase for eNOS activation.\textsuperscript{41} In
the present study we described a further mechanism of endo-
thelial dysfunction caused by the HBP. Our previous study showed
that activation of the HBP by GlcN enhances superoxide pro-
duction in cultured endothelial cells.\textsuperscript{42} The pro-oxidative ef-
effect of GlcN has also been shown in renal mesangial cells,
pancreas β-cells.\textsuperscript{43,44} The present study showed that the en-
hanced O$_2^{-}$ generation by GlcN in endothelial cells is attrib-
uted to eNOS uncoupling, because the increased O$_2^{-}$ gener-
ation by GlcN is abolished by the eNOS inhibitor, L-NAME.
Importantly, in situ production of O$_2^{-}$ in isolated mouse aortas
caused by GlcN is inhibited and NO production restored by
p38 mapk inhibitor, which is paralleled with restoration of
endothelium-dependent relaxation in isolated mouse aortas.
These results demonstrate that the HBP causes eNOS uncou-
pling through p38 mapk. It should be noted that the impair-
ment of endothelium-dependent relaxations is only partly
prevented by the ROS scavenger tiron at a concentration of
10 mmol/L, which was shown to fully inhibit ROS generation
in endothelial cells.\textsuperscript{45} This indicates that besides eNOS uncou-
pling, other mechanisms that are also activated by p38 mapk,
are involved in GlcN-induced endothelial dysfunction. The
results suggest that inhibition of p38 mapk signaling repre-
sents a more powerful means of improvement of endothelial
dysfunction than inhibition of oxidative stress.

These results shed new light on the mechanism of endo-
thelial dysfunction caused by the HBP, which may contribute
to the accelerated atherosclerosis in diabetics, as observed in
ApoE$^{-/-}$ mice treated with GlcN.\textsuperscript{27,28} The mechanism of eNOS
uncoupling mediated by p38 mapk in response to GlcN re-
gains unknown at this stage and warrants further investiga-
tion. Nevertheless, the present results further illustrate that
p38 mapk might be a potential target for treatment of endo-
thelial dysfunction under various pathological conditions. Indeed,
the role of p38 mapk in endothelial dysfunction has been re-
cently demonstrated in vitro as well as in vivo. Exposure of
endothelial progenitor cells isolated from healthy adult sub-
jects to uremic solute p-cresol to mimic uremic condition in
patients, enhances p38 mapk activity and impairs the angio-
dysfunction caused by the HBP, which may contribute to
the cardiovascular system.\textsuperscript{46} This concentration is clearly
lower than that used in the present study in vitro. The fact that
0.1–1 mmol/L of GlcN in vitro can affect endothelial function
in a very short time (\textbf{Figure 1}), and that patients are treated
chronically for years on a daily basis, suggest that there are
still some concerns about the possible adverse effects of the
clinical use of GlcN, which should be clarified in future long-
term clinical trials, as suggested by some authors.\textsuperscript{29} Second,
the HBP pathway is involved in endothelial dysfunction and
glucoxotoxicity in diabetes mellitus. The present findings fur-
ther support the role of the HBP pathway in diabetic vascular
complications. And third, p38 mapk is involved in multiple cellular
abnormalities including metabolic stress, inflammatory re-
sponses, endothelial PAI-1 expression, and endothelial dys-
fuction; the present study reinforces the ongoing clinical
trials, which are designed to investigate clinical outcomes in
patients with cardiovascular diseases treated with p38 mapk
inhibitors (Clinical Trial Nr. ClinicalTrials.gov Identifier:
NVT00570752 and NCT00910962).

\section*{Acknowledgments}
This work is supported by Swiss National Science Foundation (310000-
120435/1), the Swiss Heart Foundation. Z.W. and Y.X. are supported by
Chinese Scholarship Council.

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Supplementary Files

Supplementary File 1

Figure S1. Efficacy and specificity of signaling pathway inhibitors in endothelial cells: immunoblotting showing efficacy and specificity of different inhibitors on respective signaling pathways activated by tumor necrosis factor (TNF)-α (10 ng/ml, 15 min) in human endothelial cells.

Please find supplementary file(s) at: http://dx.doi.org/10.1253/circj.CJ-12-0016