Protein Tyrosine Phosphatase SHP2 Mediates Chronic Insulin-Induced Endothelial Inflammation

Hemant Giri, Ilayaraja Muthuramu, Monalisa Dhar, K. Rathnakumar, Uma Ram, Madhulika Dixit

Objective—Insulin promotes adhesion of leukocytes to the endothelium through increased expression of surface adhesion molecules. We determined whether src-homology domain-2–containing protein tyrosine phosphatase 2 (SHP2), a downstream effector of insulin signaling, is involved in insulin-induced endothelial inflammation.

Methods and Results—In human umbilical vein–derived endothelial cells, treatment with insulin (100 nmol/L) increased Tyr542 phosphorylation, activity, and subsequently expression of SHP2. Increase in SHP2 accompanied a parallel decrease in the availability of the anti-inflammatory molecule, NO. This consequently enhanced the expression of cell adhesion molecules. Decrease in NO index was caused by endothelial NO synthase (eNOS) uncoupling and increased arginase activity. Among the 2 isoforms, insulin treatment induced the expression of arginase II. Inactivation of endogenous SHP2 via NSC87877 [8-hydroxy-7-(6-sulfonapthalen-2-yl)-diazenyl-quinoline-5-sulfonic acid] and its knockdown by small interfering RNA decreased arginase activity by blocking arginase II expression; however, it failed to restore eNOS coupling. Inactivation of SHP2 also abrogated insulin-mediated leukocyte adhesion by blocking the expression of adhesion molecules. Finally, downregulation of endogenous arginase II blocked insulin-mediated endothelial inflammation.


Key Words: chronic-hyperinsulinemia ■ endothelial inflammation ■ SHP2 ■ arginase-II ■ NO

NO prevents atherosclerosis by inhibiting leukocyte adhesion and transcytosis through the endothelium. Constitutive endothelial NO synthase (eNOS) generates NO from L-arginine in endothelial cells. Functional eNOS is a dimer and is regulated through protein–protein interactions, subcellular trafficking, and through phosphorylation of serine, threonine, or tyrosine residues.1 Dysregulation of any of these leads to aberrant eNOS activity in diabetes mellitus and thus causes endothelial dysfunction and consequent vascular complications.2,3 Before developing overt hyperglycemia, patients with diabetes mellitus exhibit insulin resistance and compensatory hyperinsulinemia. Recent studies have demonstrated that chronic hyperinsulinemia induces endothelial inflammation in a p38 mitogen-activated protein kinase (MAPK)–dependent manner4,5 and enhances the sensitivity of endothelial cells to suboptimal concentrations of tumor necrosis factor-α.6 However, the signaling intermediates involved in this cascade are ill characterized.

Src-homology domain-2–containing protein tyrosine phosphatase 2 (SHP2) is a ubiquitously expressed cytosolic protein tyrosine phosphatase.7 It potentiates insulin-induced MAPK activation. It also mediates cytokine-induced nuclear factor-κB and Janus kinase-signal transducer and activator of transcription signaling in immune cells.7,8 In endothelial cells, SHP2 regulates eNOS activity in response to flow and vascular endothelial growth factor through mechanisms involving protein kinase A and Akt, respectively.9,10 The enzyme consists of 2 N-terminal SH2 domains, a catalytic protein tyrosine phosphatase (PTP) domain, and C-terminal tyrosine phosphorylation sites (Tyr542 and Tyr580). Tyr542 and Tyr580 constitute the consensus binding sites (YXXN) for growth factor receptor-bound protein 2 (Grb2) SH2 domain.11,12 In a resting state, SHP2 exists in a folded autoinhibitory conformation and gets activated upon binding of its SH2 domains to phosphotyrosine motifs present in adapter proteins such as Grb2–associated-binding protein 1 (Gab1),13 fibroblast growth factor receptor substrate 2,14 insulin receptor substrate-1 (IRS-1),15 or SH2-domain–containing protein tyrosine phosphatase substrate-1.16 Others have shown that phosphorylation of tail tyrosine residues (Tyr542 and Tyr580) of SHP2 is necessary for growth factor– and cytokine-induced MAPK signaling.17,18 Given that SHP2 is a positive regulator of MAPK signaling and because p38 MAPK mediates hyperinsulinemia–induced endothelial inflammation, we wanted to determine whether SHP2 is involved in this cascade. In the present study, we demonstrate that in endothelial cells, SHP2 is phosphorylated at Tyr542 in response to insulin and upregulates its own expression in a p38 MAPK–dependent manner. Increased SHP2 consequently...
upregulates the expression and activity of arginase II, thereby decreasing the cellular NO to promote leukocyte adhesion.

**Methods**

For a detailed section on Materials and Methods, please refer to the online-only Data Supplement. Experimental procedures involving human tissue samples (umbilical cord or blood) were reviewed and approved by the IIT Madras institutional ethics committee in accordance with Declaration of Helsinki revised in 2000.

**Cell Culture**

Human umbilical vein–derived endothelial cells (HUVECs) were isolated from umbilical cords by digestion with collagenase. HUVECs were cultured maximum up to passage 2. Nuclear localization of SHP2 was assessed via confocal microscopy (Zeiss LSM 710; Carl Zeiss Microscopy GmbH, Munich, Germany) and subcellular fractionation as described in a published study. Changes in mRNA expression were determined by semiquantitative reverse-transcriptase polymerase chain reaction. NO release from endothelial cells was measured via 4,5-diaminofluorescein diacetate imaging. 4,5-diaminofluorescein diacetate is a cell-permeable dye that fluoresces upon binding to NO.

**Peripheral Blood Mononuclear Cell–HUVEC Adhesion Assay**

HUVEC monolayers were treated with endotoxin-free insulin for the mentioned duration before adhesion experiment. PKH26-labeled peripheral blood mononuclear cells were incubated with insulin-treated HUVECs at 37°C and 5% CO2 for 3 hours based on a published study. Cells were then washed with PBS thrice to remove nonadherent leukocytes. Adhesion was assessed as the number of leukocytes adhered to HUVEC monolayer per field view. Each experiment was performed in triplicate, and the results are summarized for a minimum of 3 independent experiments.

**Phosphatase Assay**

Immunoprecipitated enzyme was incubated with 20 mmol/L p-nitrophenol phosphate as substrate for 2 hours at 37°C. The reaction was stopped by addition of 5 mmol/L NaOH, and the amount of p-nitrophenol released was measured at 405 nm.

**Arginase Assay**

Cells were lysed by sonication at 20 kHz for 30 s (10 s/cycle) in a lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and protease inhibitor cocktail with or without selective arginase inhibitor (S)-(2-boronoethyl)-L-cysteine hydrochloride. Arginase activity was measured as described previously.

**Data Analysis**

Data are expressed as mean±SEM, and statistics were performed using Student t test. Values of P<0.05 were considered to be statistically significant.

**Results**

**Insulin Promotes Endothelial Inflammation Even at Lower Concentration**

Hyperinsulinemia observed during pre-diabetes and metabolic syndrome is associated with increased circulating levels of proinflammatory cytokines. Others have shown that chronic hyperinsulinemia enhances endothelial inflammation through increased surface expression of adhesion molecules. We sought to determine which between the 2, the concentration or the duration of insulin treatment, is necessary to impart these proinflammatory effects. Confluent cultures of HUVECs were treated with varying concentrations of insulin either acutely for 30 minutes or chronically for 48 hours in the absence of other inflammatory cytokines before performing adhesion experiments. As shown in Figure IA and IB, although higher concentrations of insulin (50 mmol/L or 100 mmol/L) caused a marginal increase in leukocyte adhesion upon short-term exposure (30 minutes), it was the sustained insulin treatments that were more effective. For chronic exposure of 48 hours, even a lower concentration of 1 mmol/L insulin was effective in enhancing leukocyte adhesion by almost 2.5-fold (Figure 1B). Thus, insulin is proinflammatory on endothelial cells even at lower concentrations, provided the treatment is long-term. These effects of insulin were not attributable to secretion of extracellular inflammatory factors because conditioned media obtained from insulin-treated HUVECs failed to elicit inflammatory effects on insulin-naïve endothelial cells (Figure IA in the online-only Data Supplement). Leukocyte adhesion was partially attenuated upon small interfering RNA (siRNA)–mediated downregulation of insulin receptor-β (Figure IC and Figure IB in the online-only Data Supplement). Leukocyte adhesion was partially attenuated upon small interfering RNA (siRNA)–mediated downregulation of insulin receptor-β (Figure IC and Figure IB in the online-only Data Supplement). Leukocyte adhesion was partially attenuated upon small interfering RNA (siRNA)–mediated downregulation of insulin receptor-β (Figure IC and Figure IB in the online-only Data Supplement). Leukocyte adhesion was partially attenuated upon small interfering RNA (siRNA)–mediated downregulation of insulin receptor-β (Figure IC and Figure IB in the online-only Data Supplement). Leukocyte adhesion was partially attenuated upon small interfering RNA (siRNA)–mediated downregulation of insulin receptor-β (Figure IC and Figure IB in the online-only Data Supplement). Leukocyte adhesion was partially attenuated upon small interfering RNA (siRNA)–mediated downregulation of insulin receptor-β (Figure IC and Figure IB in the online-only Data Supplement). Leukocyte adhesion was partially attenuated upon small interfering RNA (siRNA)–mediated downregulation of insulin receptor-β (Figure IC and Figure IB in the online-only Data Supplement).

**Insulin Treatment Attenuates NO**

Chronic insulin dose-dependently reduced the availability of NO from endothelial cells in response to L-arginine, as seen via 4,5-diaminofluorescein diacetate fluorescence in Figure 1B and 1C in the online-only Data Supplement. In a time-response experiment 100 nmol/L insulin induced p38 MAPK activation with stronger effects seen up to 6 hours (Figure 2A). The activation was sustained even at lower concentrations, although the magnitude of activation was lower. Inhibition of p38 MAPK with SB203580, however, blocked chronic insulin-induced leukocyte adhesion (Figure 2B) and expression of adhesion molecules (Figure IIA in the online-only Data Supplement).
Insulin Treatment Increases Protein Tyrosine Phosphatase SHP2 Activity and Expression

Insulin treatment (100 nmol/L) increased tyrosine phosphorylation of SHP2 at position 542 as early as 10 minutes with continued phosphorylation even at later time points (Figure 3A). Furthermore, insulin increased the protein expression of SHP2 from 6 hours onward (Figure 3B). No measurable differences in cell numbers or total cellular protein content after insulin treatment were detected (data not shown). Both NSC87877 and SB203580 blocked insulin-induced increase in SHP2 protein levels (Figure 3C). NSC87877 binds to the catalytic cleft of SHP2 and inhibits it.27 It also inhibits SHP-1 with similar potency; however, the expression of SHP-1 is restricted to hematopoietic and epithelial cells.28 Knockdown of insulin receptor-β also attenuated insulin-induced expression of SHP2 (Figure 3D). When equal amounts of SHP2 were pulled down via immunoprecipitation, even the overall enzyme activity per molecule of SHP2 was significantly enhanced in response to insulin (Figure 3E).

Within 30 minutes of insulin treatment, SHP2 was localized to the nucleus, and this nuclear retention was seen as long as insulin was present (Figure IIIA and IIIB in the online-only Data Supplement). Because IRS-1 is a major SHP2 interacting adapter molecule, we sought to determine whether it is involved in this pathway. It should be noted that although SHP2 lacks a nuclear localization signal, it is present in Gab1.29 Increased activity of SHP2 was accompanied by increased tyrosine phosphorylation (at Tyr627) of Gab1 (Figure 3F). Tyrosine phosphorylation of Gab1 at Tyr627 mediates its interaction with SHP2 and thus assists in SHP2 activation.13 Overexpression of Gab1 mutant (Gab1YF) incapable of interacting with SHP2 although blocked nuclear localization of SHP2 (Figure IIIC in the online-only Data Supplement), it failed to block insulin-induced leukocyte adhesion (Figure IIID in the online-only Data Supplement).
Data Supplement). Even the knockdown of endogenous Gab1 with siRNA failed to block leukocyte adhesion (Figure IIIIE in the online-only Data Supplement). Hence, both IRS-1 and Gab1 are of minimal consequence in this cascade.

**SHP2 Mediates Inflammatory Effects of Insulin**

We next sought to determine whether upregulated SHP2 is involved in insulin-induced leukocyte adhesion and if it is involved, does it bring about insulin-mediated p38 MAPK activation, reduction in cellular NO, and increase in adhesion molecule expression. Inhibition of SHP2 with NSC87877 attenuated p38 MAPK activation (Figure 4A). Overexpression of Y542F mutant of SHP2 (YF-SHP2) also blocked insulin-induced p38 MAPK activation (Figure 4B). Because maximal increase in SHP2 expression and leukocyte adhesion was seen at 48 hours of insulin treatment in conjunction with maximal decrease in NO at 48 hours, all the subsequent experiments were performed with 48 hours of chronic insulin exposure unless specified otherwise. Both knockdown via siRNA and inhibition by NSC87877 partially restored NO despite chronic

**Figure 3.** Src-homology domain-2–containing protein tyrosine phosphatase 2 (SHP2) is upregulated in response to chronic insulin treatment. **A,** Tyr542 phosphorylation of SHP2 in response to insulin. **B,** Time-dependent increase in SHP2 protein expression in response to 100 nmol/L insulin. **C,** Effect of NSC87877 [8-hydroxy-7-(6-sulfonaphthalen-2-yl)-diazenyl-quinoline-5-sulfonic acid] and SB203580 on SHP2 expression. Cells were pretreated with inhibitors for 2 hours before 6-hour insulin treatment. **D,** Effect of knockdown of endogenous insulin receptor (IR)-β on insulin-induced SHP2 expression. **E,** SHP2 immnuophosphatase assay in response to chronic insulin. Inset shows equal pull down of immunoprecipitated SHP2. **F,** Phosphorylation of Tyr627 of Gab1 in response to 100 nmol/L insulin. Bar graphs summarize data for a minimum of 3 independent experiments (*P≤0.05, **P≤0.01, and ***P≤0.001 vs corresponding control and †P≤0.05 vs insulin).

**Figure 4.** Effect of src-homology domain-2–containing protein tyrosine phosphatase 2 (SHP2) downregulation and YF-SHP2 on p38 mitogen-activated protein kinase (MAPK) activation, NO index, and leukocyte adhesion. **A,** Blockade of insulin-induced p38 MAPK activation by NSC87877. **B,** Representative Western blot for blockade of insulin-induced p38 MAPK activation in ECV304 cells overexpressing YF-SHP2. **C,** NO levels in cells treated with SHP2 small interfering RNA (siRNA) in response to chronic insulin. **D,** Endothelial NO synthase (eNOS) protein levels after insulin treatment in the presence or absence of NSC87877. **E,** eNOS uncoupling in response to chronic insulin in the presence of SHP2 siRNA. **F,** Effect of SHP2 siRNA on insulin-induced adhesion molecule expression. **G,** Adhesion assay after chronic insulin treatment in the presence or absence of SHP2 inhibitor. **H,** Effect of SHP2 siRNA on leukocyte adhesion. Inset is a representative Western blot confirming downregulation of endogenous SHP2 (*P≤0.05 and ***P≤0.001 vs control and †††P≤0.001 vs 100 nmol/L insulin treatment).
Increased Arginase Activity Reduces NO Index and Thus Promotes Endothelial Inflammation

Because SHP2 inhibition restored NO index in an eNOS-independent manner, we next sought to determine the involvement of arginases. Arginases compete with eNOS for their common substrate L-arginine. They have a greater V_{max} and thus limit NO synthesis and NO-dependent vasodilatory functions of the endothelium. Insulin time-dependently increased the cellular arginase activity from 6 hours onward (Figure 5A). This increase was reversed upon knockdown of SHP2 via siRNA (Figure 5B). In addition, (S)-(2-boronoethyl)-L-cysteine, a selective arginase inhibitor, restored the availability of NO (Figure 5C) and prevented the adhesion of leukocytes to insulin-treated HUVECs (Figure 5D). Thus, the decreased NO index is attributable to increased arginase activity and NO uncoupling, of which SHP2 downregulation only reverses arginase activity.

SHP2 Promotes Arginase II Gene Expression

Two isoforms of arginases, arginase I and arginase II, are expressed in human endothelial cells. We failed to detect arginase I expression in resting and insulin-treated HUVECs, although it was detected upon treatment with lipopolysaccharides (Figure VA in the online-only Data Supplement). In contrast, insulin time-dependently increased expression of arginase II (Figure 6A), which could be blocked through downregulation of endogenous SHP2 (Figure 6B) or upon overexpression of YF-SHP2 (Figure 6C). Downregulation of SHP2 via siRNA was confirmed through Western blotting (data not shown). Insulin-mediated increase in arginase activity was also blocked by overexpression of catalytically inactive SHP2 mutant (Figure 6D), whereas overexpression of wild-type SHP2 enhanced the expression of arginase II (Figure VB in the online-only Data Supplement). Finally, downregulation of endogenous arginase II blocked insulin-induced expression of adhesion molecules (Figure VC in the online-only Data Supplement) and leukocyte adhesion (Figure 6E). Thus, increase in SHP2 mediates chronic insulin-induced endothelial inflammation by an eNOS-independent and arginase-II–dependent mechanism.

Discussion

Insulin concentrations ranging from 1 to 10 nmol/L are attainable in fasting and postprandial states of prediabetic individuals. We observed that insulin-mediated leukocyte adhesion on to the endothelium is highly pronounced for sustained as opposed to acute insulin treatment even for lower concentration of insulin. This finding suggests that mere infusions of insulin is not effective in inducing endothelial activation; instead a chronic exposure such as that achieved via chronic infusion of low-dose lipids will have more deleterious effects on vascular inflammation. Thus, the present study highlights the importance of chronic insulin exposure toward endothelial inflammation, a condition that can be envisaged in vivo in insulin-resistant hyperinsulinemic prediabetic patients.

In this study, we report that increased Tyr phosphorylation and expression of SHP2 in response to insulin mediates endothelial inflammation. Unlike previous studies, we did not see any involvement of either IRS-1 or Gab1 in this pathway. Upon receipt of insulin signal, Tyr phosphorylation of SHP2 gets phosphorylated as early as 10 minutes. Two models have been put forth to explain the role of phosphorylation of C-terminal tyrosine residues on SHP2-mediated MAPK pathway. In the adapter model, phosphorylation of Tyr and

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** Effect of chronic insulin treatment on arginase activity. A, Time-dependent increase in cellular arginase activity in response to 100 nmol/L insulin. B, Arginase activity in the presence and absence of SHP2 small-interfering RNA (siRNA). Inset represents the knockdown of endogenous SHP2. C, Effect of arginase inhibitor (S)-(2-boronoethyl)-L-cysteine (BEC) on chronic insulin-mediated NO index. D, Effect of BEC on insulin-mediated leukocyte adhesion (*P≤0.05, **P≤0.01, and ***P≤0.001 vs control, and †P≤0.05, ††P≤0.01, and †††P≤0.001 vs corresponding insulin treatment).
activates SHP2 catalytic activity or it directly influences p38 MAPK pathway by interacting with Grb2, however, remains to be determined in future studies.

Arginases are reported to be upregulated in diabetes mellitus and hypertension, and the use of tyrosine phosphatase inhibitor vanadate decreases their expression in diabetic rats. 

It should also be noted that p38 MAPK and cAMP pathways regulate arginase expression, and both of these are in turn regulated by SHP2. Intriguingly, SHP2 appeared to positively regulate its own gene expression in an insulin receptor-β and p38 MAPK-dependent manner. Pretreatment of cells with both NSC87877 and SB203580 abrogated insulin-induced increase in the expression of SHP2. Based on these observations, we propose that insulin triggers p38 MAPK activation in endothelial cells by mediating phosphorylation of Tyr542 of SHP2. Increased MAPK in turn increases expression of SHP2, thus creating a positive feedback loop. Sustained MAPK activation resulting from increased SHP2 ensures increased expression of arginase II to reduce cellular pool of L-arginine and thus NO. These effects finally culminate into increased expression of cell adhesion molecules and leukocyte adhesion as summarized in Figure 6F. Thus, we propose that SHP2 not only initiates the cascade but also promotes it later upon sustained insulin exposure. It, however, remains to be determined which tyrosine kinase triggers phosphorylation of Tyr542 in response to insulin in endothelial cells.

Upregulated protein tyrosine phosphatase activities are reported in rodent models of diabetes mellitus and in skeletal muscles of insulin-resistant Pima Indians. To the best of our knowledge, the present study identifies upregulation of SHP2 in response to insulin as means of enhancing endothelial inflammation in in vitro settings for the first time. Activating mutations of SHP2 (tyrosine-protein phosphatase non-receptor type 11) have been detected in diseases such as Noonan syndrome, juvenile myelomonocytic leukemia, B-cell acute lymphoblastic leukemia, and acute myeloid leukemia. Increased expression of SHP2 is also observed in Condyloma accuminatum and cervical cancer patients after human papillomavirus infections. Given that activating mutations of SHP2 enhance cytokine sensitivity, it is tempting to speculate that insulin-mediated increase in SHP2 sensitizes vascular endothelium to proinflammatory effects of circulating cytokines during prediabetic stages and hence promotes initiation of atherosclerosis. We also observed Gab1-dependent nuclear localization of SHP2 in response to insulin. However, both Gab1 and nuclear SHP2 are dispensable for insulin-mediated inflammatory effects because neither knockdown of endogenous Gab1 nor overexpression of Gab1YF mutant could block insulin-induced endothelial inflammation. What exactly nuclear SHP2 is required for insulin-induced inflammation, remains to be determined in future studies.

**Figure 6.** Insulin upregulates arginase II expression via src-homology domain-2-containing protein tyrosine phosphatase 2 (SHP2). 

A, Reverse-transcriptase polymerase chain reaction for time-dependent expression of arginase II in response to 100 nmol/L insulin. B, Effect of SHP2 small interfering RNA (siRNA) on insulin-induced arginase II expression. C, Effect of YF-SHP2 overexpression on insulin-induced increase in arginase II expression. D, Effect of catalytically inactive SHP2 mutant (CS-SHP2) on insulin-induced cellular arginase activity. E, Effect of arginase II siRNA on chronic insulin-induced leukocyte adhesion. F, Proposed pathway (*P≤0.05 and ***P≤0.001 vs control, and †P≤0.05 and ††P≤0.01 vs corresponding insulin treatment).
At first, the observation of chronic insulin treatment decreasing availability of NO may seem rather odd given that insulin is reported to activate eNOS.\textsuperscript{50} However, it should be noted that physiological concentrations of insulin for shorter durations activate eNOS\textsuperscript{50} while these observations were made with sustained exposure to insulin. The ability of SHP2 to decrease NO index in response to chronic insulin also seems perplexing given that a previous study has demonstrated it to be necessary for flow-induced NO-dependent vasodilation.\textsuperscript{50} However, in the present study, SHP2 is regulating NO index by increasing the expression of arginase II. Although we observed an increase in eNOS uncoupling with hyperinsulinemia, it was not reversed upon SHP2 knockdown. Thus, the SHP2-mediated decrease in NO in response to chronic insulin is predominantly an arginase-II–dependent and eNOS-independent event. Reasons for increased eNOS uncoupling could be multiple. Because functional eNOS dimer exists in complex with cofactors such as calmodulin, heat shock protein 90, and tetrahydrobiopterin, decrease in total pool of any of these cofactors will result in eNOS uncoupling.\textsuperscript{1} Alternatively, hyperinsulinemia may promote interaction of eNOS with its negative regulators such as caveolin-1, or increased reactive oxygen species may interact with NO and decrease its bioavailability. However, given that insulin is a known antioxidant the latter possibility seems unlikely. What is indeed perplexing is that knockdown of SHP2 alone enhances eNOS uncoupling, the reasons for the same are currently unknown.

Disclosures
None.

References
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Supplemental Figure I
Supplemental Figure II

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![Graph showing NO index normalised to CTL](image)

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![Bar graph showing number of PBMCs adhered per field of view](image)
Supplemental Figure II

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![Control YF](image14) ![Chronic YF](image15)
Supplemental Figure IV
Supplemental Figure V
Supplemental Figure VI
Supplemental Materials

Materials and Method

Materials

Insulin (cell culture tested) was obtained from Roche Diagnostics, Germany. Endothelial Basal Medium (EBM bullet kit) was from Lonza, Wakersville, USA. Fetal Bovine serum of South American origin and Lipofectamine RNAiMax were from GIBCO, Invitrogen, NY, USA. SHP2 antibody for immuno-precipitation and western blot was from R& D system, Inc. (Minneapolis, MN). SHP2 inhibitor (NSC87877) and arginase inhibitor (BEC hydrochloride) were from Calbiochem, Inc. La Jolla, CA. M-MuLV reverse transcriptase and DNA polymerase were from New England Biolabs, UK. All primers, L-Arginine, Histopaque solution, PKH26, p-Nitrophenol phosphate (pNPP), Protein G sepharose and all other lab chemicals were from Sigma Aldrich, St. Louis, MO. Myc tagged wild type (WT) and catalytically inactive (CS) SHP2 constructs in pCDNA3 were a kind gift from Dr. Judith Haendeler (University of Duesseldorf, Germany). Construction of replication deficient recombinant adenovirus expressing Gab1YF mutant is described in previous publication\(^1\).

Cell culture

HUVECs were isolated from umbilical cords by digestion with collagenase as described earlier\(^2\). Cells were cultured in endothelial specific medium along with required growth factors. Isolated cells were seeded in fibronectin coated T-25 flasks and grown to confluence in EBM with 10% FBS. Endothelial cells were characterized by immunofluorescence staining for eNOS, VE-Cadherin and vWF (Data not shown). Uptake of fluorescently labeled acetylated LDL and Ulex-lectin (Sigma) was also assessed. Cells were sub-cultured for experimental conditions either in 6-well or 24 well fibronectin-coated tissue culture dishes.

PKH26 Labeling
Human PBMCs were isolated from whole blood by density gradient centrifugation using Histopaque 1077 (Sigma). The purity of PBMC fraction was assessed to be greater than 85% by detecting the expression of CD45 with FACS (FACS, Calibur, Becton Dickinson Biosciences). Isolated PBMCs were washed thrice with PBS buffer. 2 X 10^7 PBMCs were labeled using PKH26 Red fluorescent cell linker kit from Sigma following manufacturer’s instructions.

**RT-PCR**

Total RNA was isolated from 5 X 10^6 endothelial cells using TRIZOL reagent (from Sigma) following manufacturer’s instructions. After DNase treatment, total RNA (1μg) was reverse transcribed (RT) using M-MuLV RT enzyme and Oligo dT_{12-18} primers in a 20 μL reaction volume. A reaction performed in absence of reverse transcriptase enzyme acted as negative control. Amplified cDNA was subjected to PCR using gene specific primers as listed in Table 1 (Supplemental Materials). RT-PCR was also performed for GADPH as control.

**Immuno-precipitation and Immuno-Blotting**

Cells were washed with ice cold PBS and then lysed with triton X lysis buffer (20mmoleL^{-1} Tris, 100mmoleL^{-1} NaCl, 10% triton X-100, 1 mmolesL^{-1} EDTA, 1 mmoLeL^{-1} sodium orthovanadate, 2.5mmolesL^{-1} sodium pyrophosphate, 0.5% sodium deoxycholate, 1X protease inhibitor from Sigma). For Immuno-precipitation cells were lysed in modified RIPA buffer (50 mmolesL^{-1} Tris-HCl, 150 mmolesL^{-1} NaCl, 0.1%SDS, 0.5% sodium deoxycholate, 1% Triton-X-100, 1 mmoLeL^{-1} EDTA, 1 mmoLeL^{-1} sodium orthovanadate, and 1X protease inhibitor) followed by pre-clearing with Protein G sepharose fast flow (Sigma) for one hour at 4°C. 2 μg of SHP2 antibody was added per 300 μg of total protein and incubated overnight at 4°C in rotating condition. 20 μl of 50% Protein G sepharose was added and incubated for additional 2-3 hours at 4°C in rotating condition. Beads were washed with 20 mmolesL^{-1} Tris, 100 mmolesL^{-1} NaCl, 0.1% Triton X-100 three times prior to performing phosphatase assay as published earlier^3. The assay buffer composition was as
follows: 20 mmol·L⁻¹ Tris (pH 7.4), 100 mmol·L⁻¹ NaCl, 5 mmol·L⁻¹ DTT, 2 mmol·L⁻¹ EDTA and 20 mmol·L⁻¹ pNPP.

**Arginase Assay**

Cell lysates were mixed with equal volume of activation buffer (50 mmol·L⁻¹ Tris·HCl, pH 7.5 and 10 mmol·L⁻¹ MnCl₂) and incubated at 55°C for 10 minutes. The reaction was started by incubating 80 µl of L-arginine (0.5 mole·L⁻¹; pH 9.7) at 37°C for one hour. The reaction was stopped by the acid mixture (H₂SO₄∶H₃PO₄∶H₂O, 1∶3∶7) and urea concentration was determined by adding isonitrosopropiophenone (25 µl, 9% in absolute ethanol), which produces pink colour on heating at 100°C for 45 min. After cooling the sample at room temperature for 10-20 minutes in dark, absorbance was measured spectrophotometrically at 540 nm with a microplate reader (Molecular Devices).

**Confocal Imaging**

Cells were cultured on fibronectin coated glass cover slips. They were treated with or without insulin for indicated time. For confocal imaging cells were washed with ice cold PBS thrice followed by fixation with 4% paraformaldehyde for 20 min and permeabilization with 0.25% Triton-X-100 for 10 min. Blocking was done with 5% FBS. Cells were stained with anti-SHP2 mouse antibody, washed and incubated with anti mouse rhodamine conjugated secondary antibody. Nuclear staining was done with DAPI. Images were taken with Zeiss LSM confocal laser scanning microscope by a technician who was blind for the experimental treatments. For cell fractionation studies, cytoplasmic and nuclear fractions were prepared as described. Following insulin treatment, cells were lysed in lysis buffer containing 10 mmol·L⁻¹ HEPES (pH 7.5), 2 mmol·L⁻¹ MgCl₂, 1 mmol·L⁻¹ EDTA, 10 mmol·L⁻¹ KCl, 1 mmol·L⁻¹ EDTA, 1 mmol·L⁻¹ EGTA, 1 mmol·L⁻¹ dithiothreitol, 10 mmol·L⁻¹ NaF, 0.1 mmol·L⁻¹ Na₃VO₄ and 0.5% Nonidet P-40. Following lysis,
samples were centrifuged at 13,000 rpm and the supernatant was collected as cytosolic fraction. Nuclear fraction in the pellet was washed twice prior to loading on SDS page for western blotting.

**Site directed mutagenesis**

The tyrosine 542 residue of myc tagged WT-SHP2 cloned in pCDNA3.1 was mutated to phenylalanine using the listed primers below as per the Stratagene’s site directed mutagenesis kit in E.coli XL-1. The primers were designed such that they generate an EcoRI restriction site and the site of mutation. The generation of mutation (YF-SHP2) was confirmed via sequencing prior to using the plasmid for experiments.

**siRNA and plasmid Transfections**

HUVECs were transfected with 200 nmolesL⁻¹ of control and SHP2 siRNA (ON –TARGET plus-siRNA mix consisting of four different siRNA sequences against SHP2 for effective down regulation from Dharmacon) or with 20 nmolesL⁻¹ of control and Insulin receptor esiRNA (Sigma) along with Lipofectamine RNAiMax from Invitrogen as transfection reagent as per the manufacturer’s instructions. After 48 hours the cells were transferred to medium containing 2% FBS and 24 hours thereafter the adhesion experiments were performed with PKH26 labeled PMBCs as described. The specific knockdown of endogenous SHP2 was confirmed via western blotting. For transient transfections of WT or YF-SHP2 constructs, endothelial cells or endothelial cell line ECV304 were grown till 70-80% confluency. They were transfected with 6µg of plasmids along with Lipofectamine from Invitrogen as per manufacturer’s instructions. After 48 hours of transfection, cells were serum starved for 6 hours followed by treatment with 100 nmolesL⁻¹ for 30 minutes (for p38MAPK blots) and 6 hours (for arginase II RT-PCRs)

**NO measurements via DAF2DA Imaging**

Cells were seeded with or without 100nmolesL⁻¹ insulin for 48 hours. After 4 to 6 hours of serum starvation they were co-incubated with 1mmoleL⁻¹ of L-arginine as substrate for NO synthase
activity of eNOS and with 10µmolesL⁻¹ of Diaminofluorescein –2 Diacetate (DAF2-DA) for measuring release of NO. Image analysis was done by ProgResCapturePro 2.7(Olympus).
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<td>5'CTTAATATTGGATTTGCCTCTGCTTTC</td>
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Table I: List of primer sequences used in the study.
Figure Legends for Supplementary Figures:

Supplementary Figure I:

(A) Effect of conditioned media on insulin naïve HUVECs with respect to leukocyte adhesion. Bar graph summarizes data for three independent experiments, with each experiment done in triplicates. (B) Bar graph summarizing effect of IR-β knockdown on chronic insulin induced leukocyte adhesion. (C) Bar graph summarizing RT-PCR data for expression of E-selectin (D) ICAM-1 (E) VCAM-1 and (F) PECAM-1 in response to 100nmolesL⁻¹ insulin for different time points. (**p≤0.01 and ***p≤0.001 versus control and †††p≤0.001 versus insulin chronic insulin treatment)

Supplementary Figure II:

(A) Effect of SB203580 on chronic insulin induced expression of adhesion molecules. (B) Dose dependent decrease in cellular NO upon chronic treatment for 48 hours with insulin via DAF-2DA assay. (C) Bar graph summarizing data for three independent experiments (D) Bar graph representing adhesion of leukocytes to chronically treated endothelial cells in presence or absence of NO donor DETANO (50µmolesL⁻¹). (*p≤0.05, **p≤0.01 and ***p≤0.001 versus control and †††p≤0.001 versus insulin chronic insulin treatment

Supplementary Figure III:

(A) Nuclear localization of SHP2 in response to insulin. (B) Nuclear localization of SHP2 in response to acute (30 min) and chronic (48 hours) insulin. (C) Effect of over-expression of Gab1YF mutant on insulin induced nuclear localization of SHP2. (D) Effect of Gab1YF over-expression on insulin induced leukocyte adhesion. (E) Effect of Gab1 knockdown via siRNA on
insulin induced leukocyte adhesion. Bar graphs summarize data for a minimum of 3 independent experiments. (**p≤0.01 and ***p≤0.001 versus control).

**Supplementary Figure IV:**

(A) Bar graph summarizing reversal of NO index upon down-regulation of endogenous SHP2 for 3 independent experiments. (B) Chronic insulin mediated cellular NO in presence and absence of NSC87877. Bar graphs summarize data for three independent experiments. (C) Effect of SHP2 down-regulation on insulin mediated increase in expression of E-selectin and ICAM-1. (D) Effect of SHP2 inhibition following chronic insulin treatment on adhesion molecule expression. (E) Bar graph summarizing leukocyte adhesion to insulin treated HUVECs in presence and absence of NSC87877 (F) Representative picture depicting insulin mediated leukocyte adhesion to HUVECs upon down-regulation of endogenous SHP2. (**p≤0.001 versus control and †††p≤0.001 versus corresponding insulin treatment).

**Supplementary Figure V:**

(A) Effect of LPS (10ng/ml) and chronic insulin treatment (100nmolesL⁻¹) on expression of arginase II in HUVECs via RT-PCR. (B) Effect of over-expression of wild type SHP2 (WT-SHP2) and CS-SHP2 on gene-expression of arginase II. (C) Effect of arginaseII down-regulation on insulin induced expression of adhesion molecules.

**Supplementary Figure VI:**

Sequence analysis of WT and Y542F SHP2 constructs. * denotes sites of mutation.
Giri, SHP2 in endothelial inflammation.

Reference List


