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 effecter 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 Tyr^{542} 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.

Conclusion—SHP2 mediates chronic insulin-induced endothelial inflammation by limiting the production of NO in an eNOS–independent and arginase-II–dependent manner. **(*Arterioscler Thromb Vasc Biol.* 2012;32:1943-1950.)**

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 manner[4,5] 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 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 (Tyr^{542} and Tyr^{580}). Tyr^{542} and Tyr^{580} constitute the consensus binding sites (YXNX) 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 (Tyr^{542} and Tyr^{580}) of SHP2 is necessary for growth factor– and cytokine-induced MAPK signaling.[17–19] 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 Tyr^{542} in response to insulin and upregulates its own expression in a p38 MAPK–dependent manner. Increased SHP2 consequently

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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.[20] 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.[21] 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% CO_2 for 3 hours based on a published study.[22] 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 nmol/L NaOH, and the amount of p-nitrophenol released was measured at 405 nm.[23]

Arginase Assay

Cells were lyzed 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.[24]

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.[25,26] Others have shown that chronic hyperinsulinemia enhances endothelial inflammation through increased surface expression of adhesion molecules.[4,5] 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 1A and 1B, although higher concentrations of insulin (50 nmol/L or 100 nmol/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 nmol/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-naive 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 1C and Figure IB in the online-only Data Supplement). We next determined the effect of insulin on time-dependent expression of adhesion molecules via reverse-transcriptase polymerase chain reaction (Figure 1D). Because for both acute and chronic treatment maximal effects were seen with higher concentration, we used 100 nmol/L insulin for gene expression analysis. A biphasic response was seen for E-selectin with immediate expression at 30 minutes followed by sustained expression from 6 hours onward. In contrast, expression of intercellular cell adhesion molecule-1, vascular cell ahesion molecule-1, and platelet endothelial cell adhesion molecule-1 remained significantly high from 3 hours onward. The bar graphs for the same are represented in Figure IC to IF in the online-only Data Supplement. Increase in protein expression for E-selectin and intercellular cell adhesion molecule-1 in response to chronic insulin was also confirmed (Figure 1E). It should be noted that the expression of adhesion molecules was maximal for chronic exposures of insulin (ie, for exposure ≥12 hours). In addition, insulin induced p38 MAPK activation with stronger effects seen up to 6 hours (Figure 2A). The activation was sustained even at later time points, 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 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 IIB and IIC in the online-only Data Supplement. In addition, in a time-response experiment 100 nmol/L insulin decreased NO levels from 3 hours onward (Figure 2C). Intriguingly, the decrease in NO was not attributable to defective activation of Akt, neither was it attributable to defective phosphorylation of eNOS at its activating Ser^{1177} residue (Figure 2D). However, the monomer (M) to dimer (D) ratio of

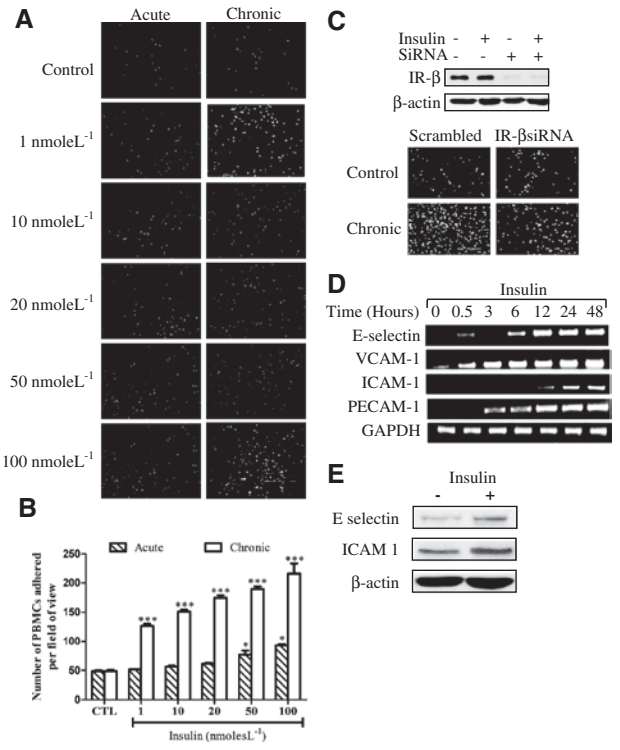

Figure 1. Endothelial inflammation in response to insulin. **A**, Representative picture depicting adherence of PKH26-labeled leukocytes to insulin-treated human umbilical vein–derived endothelial cell (HUVEC) monolayer. **B**, Bar graph summarizing data for 5 independent experiments. **C**, Effect of insulin receptor (IR)-β knockdown on chronic insulin-induced leukocyte adhesion. **D**, Reverse-transcriptase polymerase chain reaction for adhesion molecules and (**E**) representative Western blot for protein expression of E-selectin and intercellular cell adhesion molecule-1 (ICAM-1) (*P≤0.05 and ***P≤0.001 vs corresponding control).

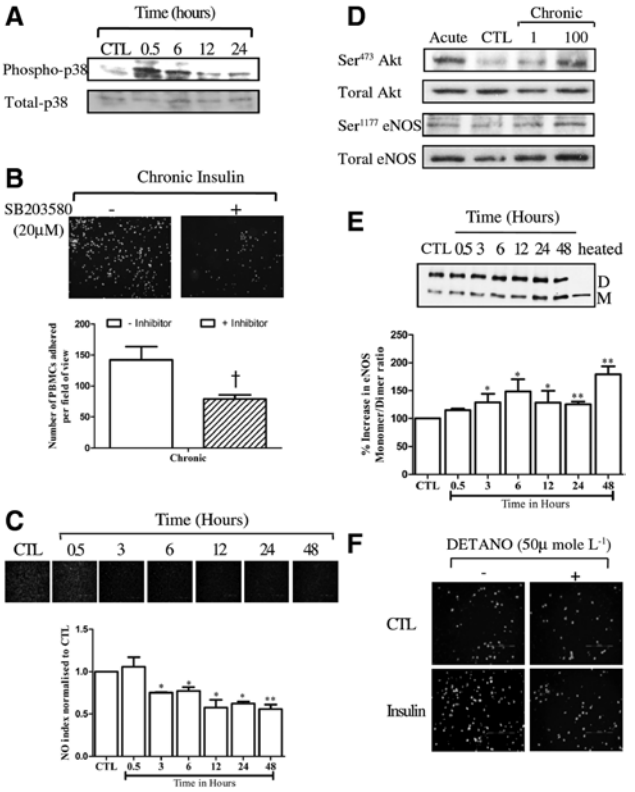

Figure 2. Leukocyte adhesion is attributable to increased p38 mitogen-activated protein kinase (MAPK) and decreased NO. **A**, Time-dependent activation of p38 MAPK in response to 100 nmol/L insulin. **B**, Effect of SB203580 on chronicinsulin-mediated leukocyte adhesion. **C**, Time-dependent decrease in NO levels upon exposure to 100 nmol/L insulin. **D**, Representative Western blot for Ser^{473} Akt and Ser^{1177} endothelial NO synthase (eNOS). **E**, eNOS uncoupling in response to insulin. D indicates dimer; M, monomer. **F**, Effect of diethylenetriamine nitric oxide (DETANO) on leukocyte adhesion. Bar graphs summarize data for a minimum of 3 independent experiments (*P≤0.05 and **P≤ 0.01 vs corresponding control and †P≤0.05 vs insulin).

eNOS increased from 3 hours onward with maximal increase at 48 hours (Figure 2E), thereby suggesting eNOS uncoupling. Upon treatment with exogenous NO donor diethylenetriamine nitric oxide, inflammatory effects of insulin were abrogated (Figure 2F and Figure IID 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 cascade. Neither acute nor chronic insulin treatment induced tyrosine phosphorylation of IRS-1 (data not shown). It also failed to mediate any interaction of SHP2 with IRS-1 (data not shown). We then determined whether adapter protein Gab1, which is also a coactivator of SHP2, 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 Tyr^{627}) of Gab1 (Figure 3F). Tyrosine phosphorylation of Gab1 at Tyr^{627} 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

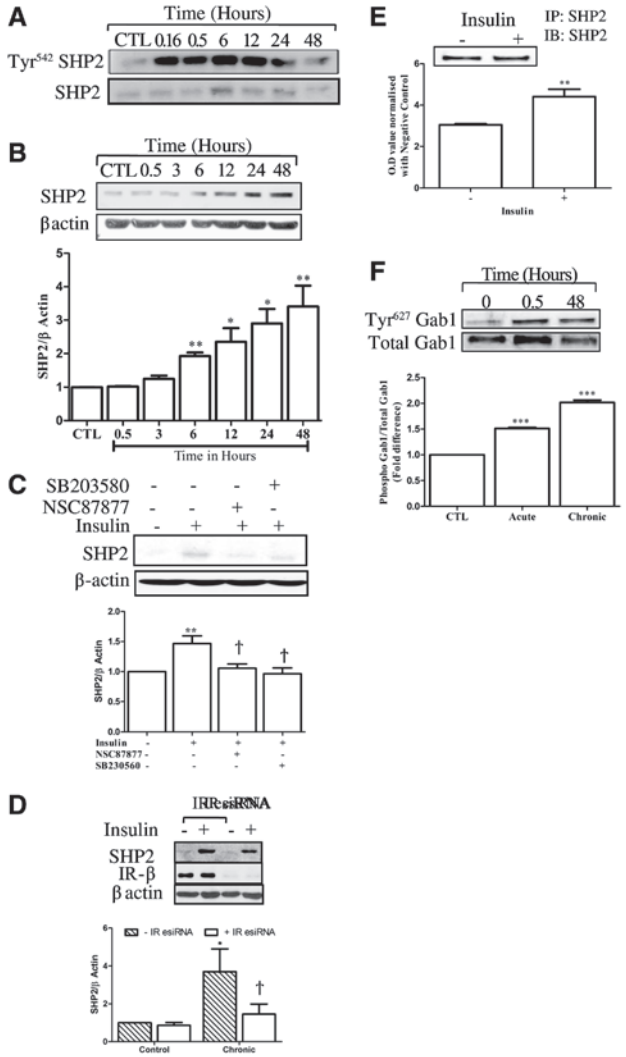

Figure 3. Src-homology domain-2–containing protein tyrosine phosphatase 2 (SHP2) is upregulated in response to chronic insulin treatment. **A**, Tyr^{542} phosphorylation of SHP2 in response to insulin. **B**, Time-dependent increase in SHP2 protein expression in response to 100 nmol/L insulin. **C**, Effect of NSC87877 [8-hydroxy-7-(6-sulfonapthalen-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 Tyr^{627} 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).

Data Supplement). Even the knockdown of endogenous Gab1 with siRNA failed to block leukocyte adhesion (Figure IIIE 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

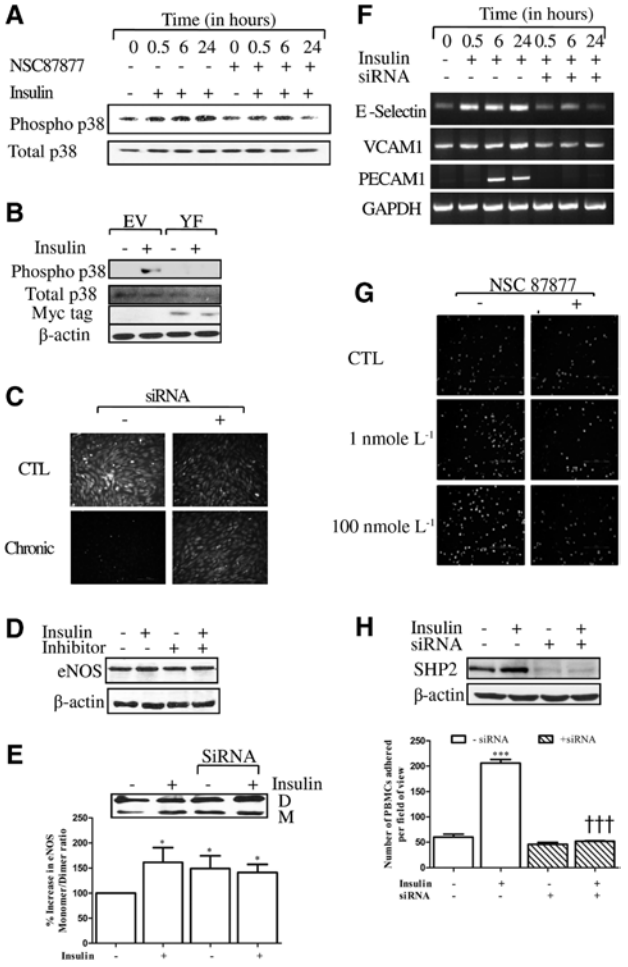

Figure 4. Effect of src-homology domain-2–containing protein tyrosine phosphatase 2 (SHP2) downregulation and YF-SHP2 on p38 mitogen-activated protein kinase (MAPK) activation, NO index, and leukocyte adhesion. **A**, Blockade of insulin-induced p38 MAPK activation by NSC87877 [8-hydroxy-7-(6-sulfonapthalen-2-yl)-diazenyl-quinoline-5-sulfonic acid]. **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).

insulin treatment (Figure 4C and Figure IVA and IVB in the online-only Data Supplement). Reversal of NO index was not attributable to any changes in protein levels of eNOS (Figure 4D) neither was it attributable to reversal of eNOS uncoupling (Figure 4E). Expression of adhesion molecules in response to chronic insulin was blocked by SHP2 downregulation (Figure 4F and Figure IVC in the online-only Data Supplement) as well as by NSC87877 (Figure IVD). Furthermore, adhesion of leukocytes to chronically treated HUVECs (1 nmol/L or 100 nmol/L insulin) was significantly attenuated by NSC87877 and SHP2 siRNA (Figure 4G and 4H and Figure IVE and IVF in the online-only Data Supplement). Thus, irrespective of the concentration of insulin used, the chronic insulin-mediated inflammatory responses are abrogated by blocking SHP2.

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.[30] 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 eNOS 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.[30] 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.

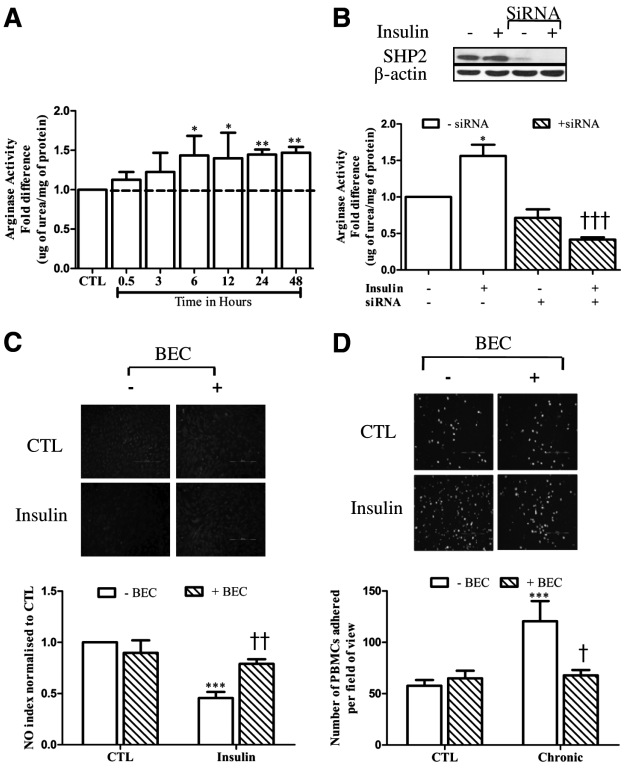

Figure 5. Effect of chronic insulin treatment on arginase activity. **A**, Time-dependent increase in cellular arginase activity in response to 100 nmol/L insulin. **B**, Arginase activity in the presence and absence of SHP2 small-interfering RNA (siRNA). Inset represents the knockdown of endogenous 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).

Discussion

Insulin concentrations ranging from 1 to 10 nmol/L are attainable in fasting and postprandial states of prediabetic individuals.[31,32] 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[33]; instead a chronic exposure such as that achieved via chronic infusion of low-dose lipids[34] 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^{542} 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^{542} 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^{542} and

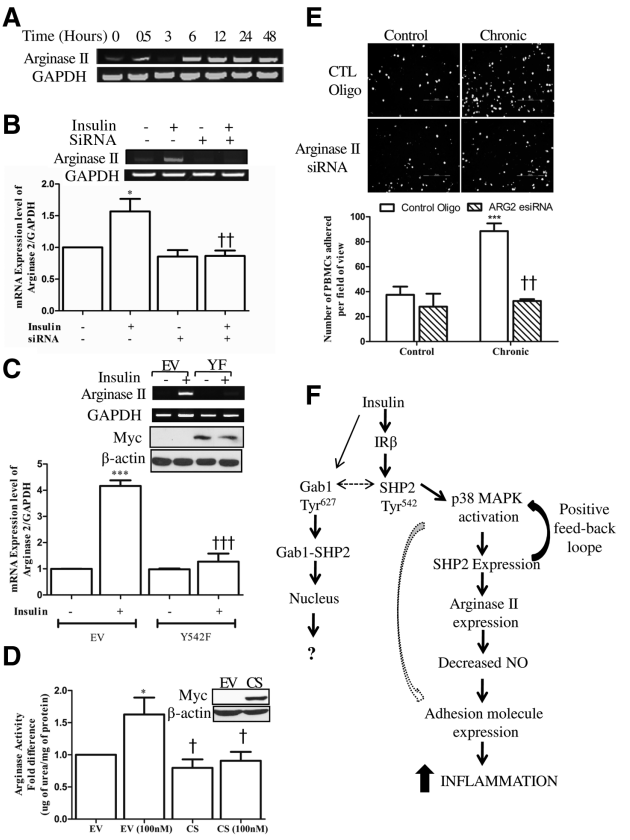

Figure 6. Insulin upregulates arginase II expression via src-homology domain-2–containing protein tyrosine phosphatase 2 (SHP2). **A**, Reverse-transcriptase polymerase chain reaction for time-dependent expression of arginase II in response to 100 nmol/L insulin. **B**, Effect of SHP2 small interfering 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 (*$P \leq 0.05$ and ***$P \leq 0.001$ vs control, and †$P \leq 0.05$ and ††$P \leq 0.01$ vs corresponding insulin treatment).

Tyr^{580} induces Grb2 interaction and subsequent recruitment of the Grb2/Sos complex to the plasma membrane for Ras-MAPK signaling.[11,12] Between the 2, Tyr^{542} appears to be the major Grb2 binding site and is relatively more resistant to autodephosphorylation.[35] However, others have observed a temporal correlation between tyrosine phosphorylation and increased catalytic activity of SHP2.[36] These observations support the enzyme activation model, wherein phosphorylation of Tyr^{542} promotes its intramolecular interaction with N-terminal SH2 domain and subsequent phosphorylation of Tyr^{580} promotes its interaction with C-terminal SH2 domain, thereby stimulating the enzyme activity. This model was confirmed via incorporation of phosphonate moieties at Tyr^{542} and Tyr^{580} residues.[35,37] Interestingly, extracellular signal–regulated protein kinase activation in response to fibroblast growth factor and platelet-derived growth factor is attenuated in fibroblasts overexpressing Y542F mutant of SHP2.[17] Because the YF mutant of SHP2 blocked insulin-induced p38 MAPK activation and arginase II gene expression, we believe this phosphorylation plays a critical role in triggering insulin-induced endothelial inflammation. Whether Ty^{542} 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.[30,38–40] It should also be noted that p38 MAPK and cAMP[24] 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 Tyr^{542} 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 Tyr^{542} in response to insulin in endothelial cells.

Upregulated protein tyrosine phosphatase activities are reported in rodent models of diabetes mellitus[41–44] and in skeletal muscles of insulin-resistant Pima Indians.[45] 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.[46] Increased expression of SHP2 is also observed in *Condyloma accuminatum* and cervical cancer patients after human papillomavirus infections.[46] Given that activating mutations of SHP2 enhance cytokine sensitivity,[47] 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 achieving in current settings presently remains undetermined. Nuclear SHP2 is reported to prevent nuclear export of telomerase reverse transcriptase[48] in endothelial cells. Hence, it is likely that nuclear SHP2 is required for insulin-induced DNA replication. Alternatively, given that nuclear SHP2-signal transducer and activator of transcription-5α complex promotes β-casein gene expression,[49] it is likely that nuclear SHP2 regulates expression of endothelial genes in response to insulin.

At first, the observation of chronic insulin treatment decreasing availability of NO may seem rather odd given that insulin is reported to activate eNOS.[50] However, it should be noted that physiological concentrations of insulin for shorter durations activate eNOS[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.[10] 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.[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. Hence, it seems that SHP2 regulates cellular NO index in multiple ways. On one hand, it appears to stabilize eNOS dimer and mediate Ser^{1177} eNOS phosphorylation[10] to increase cellular NO, whereas on the other through increased expression of arginase II it decreases the availability of L-arginine, the major substrate of eNOS. In physiology, which axis becomes dominant would be dictated by the prevailing conditions at that point of time. It should be noted that use of protein tyrosine phosphatase inhibitors improve diabetes mellitus–induced endothelial dysfunction.[51] Our observation of SHP2 inhibition reversing NO production despite presence of high insulin also supports a negative role of tyrosine phosphatases in endothelial dysfunction. One may argue that decreased NO index in response to chronic insulin treatment can also be attributable to increase in activity and expression of other protein tyrosine phosphatase such as PTP1B. Because we observed abrogation of leukocyte adhesion upon downregulation of endogenous SHP2 with siRNA interference, we believe that although there is every possibility of other protein tyrosine phosphatase being involved, the inflammatory effects of insulin seem to be predominantly mediated via SHP2. In conclusion, we demonstrate that treatment of endothelial cells to insulin elicits an inflammatory response via increased Tyr^{542} phosphorylation of SHP2. This in turn activates p38 MAPK to regulate expression of SHP2 and arginase II. Increased arginase activity consequently leads to decreased cellular NO to promote leukocyte adhesion.

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Disclosures

None.

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