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Insulin Modulates Protease-Activated Receptor 2 Signaling: Implications for the Innate Immune Response

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Given the anti-inflammatory effects of insulin in human and animal studies done in vivo and given the signaling pathways in common between insulin and the protease-activated receptor 2 (PAR2), a G protein-coupled receptor, we hypothesized that insulin would have an impact on the inflammatory actions of PAR2. We found that low doses or concentrations of insulin in the subnanomolar range reduced PAR2-induced inflammation in a murine paw edema model, attenuated PAR2-induced leukocyte trafficking in mouse intestinal venules, and reduced PAR2 calcium signaling in cultured dorsal root ganglion neurons and endothelial cells. This effect of insulin to attenuate PAR2-mediated inflammation was reversed when cells were preincubated with LY294002 (a PI3K inhibitor) and GF 109203X (a pan-protein kinase C inhibitor). The enhanced inflammatory effect of PAR2 observed in vivo in an insulin-deficient murine type 1 diabetes model was attenuated by the local administration of insulin at the inflammatory site. Our data point to an anti-inflammatory action of insulin that targets the acute innate inflammatory response triggered by PAR2. The Journal of Immunology, 2010, 184: 000-000.

Increasing evidence points to an association between diabetes and chronic activation of the innate immune system, with elevated low-grade inflammation. Insulin has been shown to exert anti-inflammatory properties in a number of settings, including several animal models of inflammation (summarized in Ref. 1). To date, the anti-inflammatory action of insulin has been attributed to its effects on mononuclear cells (2), possibly via the suppression of endothoxin-induced proinflammatory transcription factors and their gene targets. In a cardiac ischemia-reperfusion model, the ability of insulin to reduce infarct size has been attributed to its antiapoptotic action, mediated via PI3K, Akt (protein kinase B), Bcl-2–associated death promoter, and reduced NO production via NO synthase phosphorylation (reviewed in Ref. 1). However, a modulatory role for insulin on inflammatory signals induced by mediators signaling through G protein-coupled receptors is still largely unknown. Serine proteases, through the activation of protease-activated receptors (PARs), are now considered as key mediators of the innate immune response (3). Several studies have shown the involvement of this family of G protein-coupled receptors in both acute and chronic inflammatory states (3, 4). PARs belong to a unique four-member G protein-coupled receptor family (PAR1–4), which are proteolytically activated by the unmasking of a cryptic N-terminal sequence that becomes a tethered receptor-activating ligand (5). In isolation, synthetic peptides having sequences in common with the proteolytically revealed tethered ligand (e.g., SLIGRL-NH2 derived from PAR2) can selectively activate PARs in the absence of enzymatic cleavage. By cleaving and activating PAR2, trypsin and other serine proteases, such as tryptase or tissue kallikreins, have been found to trigger a variety of inflammatory responses, including edema, leukocyte infiltration, and pain (6–8). These inflammatory actions of trypsin, due in large part to a neurogenic mechanism (9), are mimicked by the receptor-selective PAR2-activating peptide (PAR2-AP) SLIGRL-NH2. A number of studies have highlighted, particularly for PAR2, a prominent inflammatory role as a mediator of innate immunity, singling out PAR2 as a new molecular target for anti-inflammatory and analgesic treatments (10, 11). Although some studies have identified factors that are able to control the expression of PAR2, no studies have identified so far the endogenous signaling pathways that can modulate its proinflammatory effects.

In the context of an association among diabetes, low insulin levels, and an inflammatory state and in view of the prominent role of proteases in initiating inflammatory processes via PAR2, we hypothesized a potential interaction between insulin and PAR2 agonists to affect inflammation. This possible interaction between insulin and PAR2 signaling might relate pathophysiologically to inflammation in the setting of diabetes.

To test the hypothesis that PAR2 and insulin signaling pathways can interact in the triggering of an acute inflammatory response, we used: 1) a PAR2-selective agonist, SLIGRL-NH2, to activate PAR2 without affecting other PARs, like trypsin would do, and 2) appropriately low doses or concentrations of insulin (1 nM in vitro; 150 pmol, administered locally in vivo) with or without activation of PAR2 by SLIGRL-NH2. The PAR2-selective agonist and insulin were used to evaluate several inflammation-related targets: 1) an in vivo paw edema inflammation model for which we have previously documented the inflammatory action of trypsin and PAR2 (6), 2) an in vivo leukocyte-endothelium interaction model, documented with intravital microscopy (7), and 3) a PAR2-stimulated calcium signaling model, using the two cell types that have been...
showed an important role in PAR-2-induced inflammation, isolated dorsal root ganglion (DRG) neurons (12) and cultured microvascular endothelial cells (13). Further, we evaluated the inflammatory action of SLIGRL-NH$_2$ in mice rendered diabetic via pancreatic β cell elimination.

**Materials and Methods**

**Mice**

Six- to eight-week-old C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) or Janvier (St. Quentin Fallavier, France). RIP-HA mice expressing influenza hemagglutinin (HA) on pancreatic islet β cells under the control of a rat insulin promoter (14) were bred at the INSERM U563 Animal Care Facility. CL-4T CR transgenic mice express on most CD8 T cells a TCR specific for the influenza virus HA$_{152-159}$ peptide-K$^d$ complex (15). RIP-HA and CL-4T CR transgenic mice were backcrossed at least 10 times onto the BALB/c background. Animals were kept under pathogen-free conditions and given free access to food and water. All of the experimental protocols were approved by local Animal Care and Ethics Committees and followed the guidelines of the Canadian and French Councils on Animal Care.

**Chemicals**

SLIGRL-NH$_2$ (a receptor-selective PAR-2-AP) and a control peptide LRGILS-NH$_2$ (the reverse sequence of PAR2-AP that cannot activate PARs) were obtained from the protein synthesis facility at the University of Calgary (Calgary, Alberta, Canada). HPLC and mass spectrometry were used to assess the purity of the peptides. Peptides that were >95% pure (HPLC and mass spectrometry criteria) were dissolved for paw edema studies in isotonic 0.9% NaCl (saline) or for intravital studies in an 80:10:10 (v/v/v) saline solution supplemented with Tween 80 (80% saline, 10% ethanol, and 10% Tween 80). This vehicle is necessary to enable the peptide to gain access to the tissue. Carrageenan and a recombiant yeast-produced human insulin (2643, 27.5 U/mg) were from Sigma-Aldrich (St. Louis, MO). The PI3K inhibitor LY294002 was from Cayman Chemical (Ann Arbor, MI), and the protein kinase C (PKC) inhibitor GF 109203X was from Tocris Bioscience (Ellisville, MO).

**Paw edema**

Paw edema was monitored with an electronic caliper (resolution 0.01; Mitutoyo, Aurora, IL), using C57BL/6 mice, in keeping with previous studies with rats (6). Basal paw thickness (at time 0) was measured, and immediately thereafter 10 μl solution containing various test compounds was administered via intraplantar injection. The test substances included vehicle (isotonic saline), SLIGRL-NH$_2$ (50 μg), LRGILS-NH$_2$ (50 μg), insulin (150 pmol), and carrageenan (200 μg). The inflammatory agonists (peptides or carrageenan) were administered either alone, or in combination with insulin (e.g., SLIGRL-NH$_2$ plus insulin [50 μg and 150 pmol, respectively] and carrageenan plus insulin [200 μg and 150 pmol, respectively]). Isotonic saline served as a vehicle control for all of the experiments using insulin. The change in paw thickness was measured every hour for 6 h and recorded as the differences between the measurement at each time point and the basal measurement done immediately prior to the intraplantar injections. After the last paw edema measurement, the animals were sacrificed by cervical dislocation, and paws were removed for the extraction and measurement of myeloperoxidase (MPO) activity as an index of granulocyte infiltration. Similar procedures were followed for in vivo experiments with kinase inhibitors, but 15 min prior to the intraplantar injections with SLIGRL-NH$_2$ or SLIGRL-NH$_2$ plus insulin, mice received an intraplantar injection of 2 μl distilled water to cause osmotic shock, immediately followed by a 5 μl intraplantar injection of LY294002 (1 μg) or GF 109203X (1 μg).

**MPO activity**

Tissues collected for MPO assay were processed as outlined previously (16). In brief, dissected paw samples were homogenized in 0.5% hexadecyltrimethylammonium bromide solution (dissolved in phosphate buffer solution). The homogenized tissues were centrifuged at 15,000 × g for 4 min. Supernatants were placed into a 96-well plate, and 1% hydrogen peroxide/O-dianisidine dihydrochloride-containing buffer was added. OD readings were at a wavelength of 450 nm for 1 min at 30 s intervals.

**Intravital microscopy**

Mice were anesthetized by a mixture of xylazine (10 mg/kg; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and ketamine (200 mg/kg; Rogar/STB, London, Ontario, Canada) by i.p. injection. Mice were given 100 μl rhodamine 6G (Sigma-Aldrich) in NaCl 0.9% (0.3 mg/kg, administered i.v. in 0.1 ml). The dosage of rhodamine 6G used in this study has been shown to have no effect on leukocyte kinetics while effectively labeling leukocytes and platelets (17). Microcirculation was observed using an inverted fluorescent microscope (Nikon, Melville, NY) with a 20× objective lens by epi-illumination at 510-560 nm, using a 590 nm emission filter. After anesthesia, a midline abdominal incision was made, and a segment from the small intestine (jejunum) was exteriorized carefully. The exposed pedicle was placed on top of a viewing pedestal and superfused with bicarbonate-buffered saline (pH 7.4). After 10 min of equilibration, single venules (20–40 μm in diameter) were selected and visualized, with images recorded for 5 min. The end of the first 5 min of recording was designated as time 0. Immediately thereafter at the jejunal site of visualization, agonist or control peptides were administered by an intraluminal injection: SLIGRL-NH$_2$ (100 μg insulin-stimulated injection at 80× 95% pure, or SLIGRL-NH$_2$ plus insulin (150 pmol). The polypeptide was administered in 0.1 ml physiological isotonic saline (pH 7.4), supplemented with 10% (v/v) absolute ethanol and 10% (v/v) Tween 80. The control mice were treated with vehicle alone. Additional images of the selected venules then were recorded for 5 min intervals at times 20, 35, 50, 65, and 80 min. Upon video playback, leukocyte rolling, adherence, and vessel diameter were measured. Leukocyte flux was defined as the number of leukocytes per minute moving at a velocity less than that of the erythrocytes that passed a reference point in the venule. A leukocyte was considered adherent to the vessel wall if it remained stationary for 30 s or longer. The change in vessel diameter and leukocyte flux was evaluated as the differences between the values at each interval and the basal value observed at time 0. The parameters measured were plotted over the 80 min time interval of observation, and the area under the curve was calculated.

**Endothelial and primary afferent neuronal DRG cell cultures**

Human microvascular endothelial cells (HMEC-1) (Centers for Disease Control and Prevention, Atlanta, GA) were grown in a cell culture flask containing growth medium MCDB 131 (Invitrogen, Burlington, Ontario, Canada) supplemented with epidermal growth factor (10 ng/ml), hydrocortisone (1 μg), 10% FBS, and 1% penicillin/streptomycin.

DRG neurons were isolated from C57BL/6 mice as described previously (12, 18). DRGs were dissociated in HBSS and triturated in a 0.25% trypsin solution containing 27 μg/ml papain (Sigma-Aldrich) for 10 min at 37°C. After being washed first with L-15 buffer (Leibovitz’s L-15 medium [Invitrogen], supplemented with 2 mM glutamine [Sigma-Aldrich] and 10% FBS [Invitrogen]) and then with HBSS, DRGs were incubated in HBSS containing 1 mg/ml collagenase type I (Sigma-Aldrich) and 4 mg/ml dispase II (Sigma-Aldrich). L-15 wash buffer then was added to neutralize enzymatic activities, and the suspension was centrifuged at 600 × g for 5 min. Neurons in the pellet were suspended in DMEM (Invitrogen) containing 2.5% FBS, 2 mM glutamine, 1% penicillin/streptomycin, and 10 μM each of cytosine arabinoside, flurodeoxyuridin, and uridine (all from Sigma-Aldrich). Cells were plated on CC2 LabTek II culture slides (Nunc, Domnicutsch, Braumath, France) for measurements of calcium signaling assay Ca$_2^{+}$ (see below). Neuronal cultures were allowed to be grown for 24 h or more to enable a regeneration of cell surface receptors, such as the PARs, that may have been affected by papain treatment.

**Imaging to measure calcium transients in HMECs and DRGs**

Mobilization of intracellular Ca$_2^{+}$ in HMECs was measured using cell monolayers in accord with methods described previously (20). In brief, HMECs were seeded in glass-bottom petri dishes (MatTek, Ashland, MA). HMECs grown to 90% confluence were incubated for 30 min at 37°C with serum-free MCDB 131 containing calcium-containing medium containing 10 μM fluo-3-acetoxymethyl ester (2.5 μM) (Molecular Probes, Eugene, OR). Cell monolayers then were washed briefly with dye-free calcium assay buffer (pH 7.4) containing NaCl (150 mM), KCl (3 mM), CaCl$_2$ (1.5 mM), glucose (10 mM), HEPES (20 mM), and sulfinpyrazone (25 mM) and then covered with 500 μl assay buffer. Test agonists diluted in this assay buffer were added to the monolayers, and the elevation of the intracellular calcium concentration was monitored. Increased levels of intracellular calcium concentration were monitored. Increased levels of intracellular calcium concentration, an index of increased intracellular calcium concentration, was measured using an inverted microscope with a ×20 objective. The fluorescence signal was analyzed by ImageMaster system software (Phon Technology International, London, Ontario, Canada) with excitation at 480 nm and emission recorded at 530 nm through a dichroic filter cube with the appropriate filter and an intensified charge-coupled device video camera (Phon Technology International). The magnitude of the calcium signal observed was expressed as a percentage (% A23187) of the signal caused in the same cell monolayers by the calcium ionophore A23187 (4 μM; Sigma-Aldrich).
The uptake of calcium indicator dye by DRG neurons was achieved over a 30 min incubation at 37°C in HBSS (pH 7.45) (Invitrogen) containing 0.1% BSA (Sigma-Aldrich) and 2.5 mM probenecid (Sigma-Aldrich), supplemented with 10 μM fluo-3-acetoxymethyl (Invitrogen), and 20% pluronic F-127 (Sigma-Aldrich). After the incubation period, the plates were washed twice with the dye-free solution and placed into a 37°C incubator in the dark for 30 min. The increase in cellular fluorescence (excitation at 460–490 nm; emission at 515 nm) was recorded using an inverted microscope (Zeiss, Oberkochen, Germany) and a (×10, 0.5 numerical aperture objective). Images were acquired using a charge-coupled device camera (Zeiss) and Metamorph software (Molecular Devices, Sunnyvale, CA). Acquisition parameters were kept constant within each experiment. A kinetic of 200 recordings (one per second) was performed. From 10 to 65 s, neurons were exposed to agonists, then from 65 to 155 s, neurons were exposed to HBSS-BSA-probenecid buffer containing 50 μM thapsigargin (Calbiochem, San Diego, CA), 5 μM ionomycin (Calbiochem), and 10 μM EGTA (Sigma-Aldrich). Finally, from 155 to 200 s, neurons were exposed to HBSS-BSA-probenecid buffer containing 120 mM CaCl2. Regions of interest were fitted around the perimeter of one neuron, and intensity variations for each region of interest were measured using Metamorph software. Intracellular calcium concentrations \([Ca^{2+}]_i\), in nanomolar, were calculated using the equation 

\[
[Ca^{2+}]_i = K_0 \left( F - F_{min} \right) / \left( F_{max} - F \right),
\]

where \(K_0\) is the dissociation constant of the Ca\(^{2+}\)-fluor-3-acetoxymethyl ester complex (390 nM) and \(F\) represents the fluorescence intensity of the cells expressed as the ratio between the highest fluorescence measurement between 10 and 65 s and the baseline. \(F_{min}\) corresponds to the minimum fluorescence between 65 and 155 s, and \(F_{max}\) represents the maximum fluorescence between 155 and 200 s. The data were expressed as the increase in fluorescence with respect to the baseline fluorescence intensity measured during the 10 s time period before the addition of agonists. Cells were treated with various compounds including SLIGRL-NH₂ (100 μM) with or without added native or boiled insulin (1 nM). The impact of insulin on signaling was monitored in the absence or presence of the PI3K inhibitor LY294002 (10 μM), the PKC inhibitor GF 109203X (10 μM), or both. When assessed for its effects in the calcium signaling assays, insulin was added to the monolayers for 5 min before the addition of an agonist. The effects of the PI3K and PKC inhibitors on the effects of insulin were assessed for monolayers treated for at least 30 min with the inhibitors before the addition of insulin or any other test compounds.

**Induction of diabetes in mice**

To induce diabetes, influenza HA-specific Tc1 cells were generated. A total of 10\(^7\) spleen and lymph node cells from CL4-TCR transgenic mice were collected by Ficoll density separation and used in adoptive transfer experiments. At day 6, viable cells were stimulated with 1 μg/ml HA, 20% FCS, and 10% BSA (Sigma-Aldrich) and 2.5 mM probenecid (Sigma-Aldrich). Finally, from 155 to 200 s, neurons were exposed to HBSS-BSA-probenecid buffer containing 120 mM CaCl₂. Regions of interest were fitted around the perimeter of one neuron, and intensity variations for each region of interest were measured using Metamorph software. Intracellular calcium concentrations \([Ca^{2+}]_i\), in nanomolar, were calculated using the equation 

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**Statistical analysis**

One-way ANOVA followed by the Student-Newman-Keuls test was used to assess statistical difference among experimental groups. All of the results are expressed as mean ± SEM. Statistical significance was assessed according to a p value <0.05.

**Results**

**PAR₂-induced inflammation is modulated by insulin treatment: reduced paw edema**

Intraplantar injection of PPAR₂-AP (SLIGRL-NH₂) induced inflammation that was characterized by edema and granulocyte infiltration, as indicated by increases in paw thickness and MPO activity, respectively (Fig. 1A, 1B). Mice that received a coinjection of insulin along with PPAR₂-AP also showed a significant increase in paw thickness as compared with saline-treated mice, but the edema observed in the presence of insulin was significantly lower at the 2, 3, 5, and 6 h time points, as compared with the mice treated with PPAR₂-AP alone (Fig. 1A, open circles). The increase in MPO activity induced by PPAR₂-AP injection (Fig. 1B, gray bar) was abolished when insulin was coadministered along with PPAR₂-AP (Fig. 1B, black bar). These anti-inflammatory effects were not caused by insulin that had been treated by boiling before its coadministration with SLIGRL-NH₂ (Fig. 1C, 1D for edema and MPO activity, respectively). The anti-inflammatory effect of insulin in the paw edema assay seen with PPAR₂-AP-induced inflammation was not observed when inflammation was caused by the intraplantar injection of carrageenan (Fig. 1E, 1F).

**Insulin treatment reduces PAR₂-triggered leukocyte recruitment**

Because the inflammatory action of PPAR₂ in the paw edema model is known to involve the endothelium-dependent recruitment of inflammatory leukocytes (7, 11), we evaluated the effects of insulin on PPAR₂-induced leukocyte rolling and adhesion onto the endothelial wall in vivo. Leukocyte trafficking in the microcirculation of intestinal venules was monitored both before and at different times after the intraluminal administration of PPAR₂-AP alone or in combination with insulin. Intraluminal administration of PPAR₂-AP, SLIGRL-NH₂, but not the reverse PPAR₂-inactive peptide, induced a significant increase in the area under the curve for the flux of rolling leukocytes and their adherence to the vessel wall (Fig. 2A, 2B).

**FIGURE 1.** Effects of insulin on paw inflammation. Changes in paw thickness (A, C, E) were measured every hour for 6 h using an electronic caliper and reported as a difference between the measurement at each time point and the basal measurement. MPO activity (B, D, F), a marker of granulocyte infiltration, was measured from the paws isolated 6 h after the injection of indicated solutions. A–F, C57BL/6J mice received intraplantar injections of saline, PPAR₂-AP (SLIGRL-NH₂, 5 μg per paw), insulin (150 pmol), PPAR₂-AP (50 μg per paw) plus insulin (150 pmol) (native or boiled), carrageenan (200 μg), or carrageenan (200 μg) plus insulin (150 pmol). *p < 0.05, significantly different from mice injected with saline; *p < 0.05, significantly different from mice injected with PPAR₂-AP alone. Values are shown as mean ± SEM (n = 8).
PAR2-AP observed after PAR2 activation was attenuated significantly by preincubation of the cells with insulin for either 1 or 5 min (Fig. 3A). The inhibitory effects of insulin on the PAR2 agonist-induced calcium signal in endothelial cells appeared to be receptor-specific because the elevation of HMEC intracellular calcium concentration caused by activation of PAR1 with the PAR1-activating peptide (PAR1-AP) TFLLR-NH₂ was not reduced by pretreatment with insulin (0.1 and 1 nM) but, in contrast, was increased slightly at the highest concentration of insulin used (10 nM) (Fig. 3B). Similarly, in vivo, PAR1-AP-induced edema was not reduced by coinjection with insulin at all of the time points after intraplantar injection, except at the first hour, where insulin treatment slightly increased PAR1 agonist-induced edema (Fig. 3D).

**Insulin reduces endothelial cell Ca²⁺ signaling in response to PAR2 activation**

Because leukocyte trafficking along intestinal venules depends on endothelial function and the expression by both leukocytes and the endothelium of adhesion molecules and because PAR2 has been shown to play a role in the upregulation of ICAMs in endothelial cells (22), we hypothesized that insulin would be able to affect PAR2-AP signaling in HMECs (23). To test this hypothesis, we evaluated the ability of insulin to modulate PAR2-activated calcium signaling in these cells. Treatment of HMECs with PAR2-AP elicited an elevation of intracellular calcium concentration in the HMECs (Fig. 3A, 3C). The increase in HMEC intracellular calcium concentration caused by PAR2-AP observed after PAR2 activation was attenuated significantly by preincubation of the cells with insulin for either 1 or 5 min (0.1, 1, and 10 nM; Fig. 3A). The inhibitory effects of insulin on the PAR2 agonist-induced calcium signal in endothelial cells appeared to be receptor-specific because the elevation of HMEC intracellular calcium concentration caused by activation of PAR1 with the PAR1-activating peptide (PAR1-AP) TFLLR-NH₂ was not reduced by pretreatment with insulin (0.1 and 1 nM) but, in contrast, was increased slightly at the highest concentration of insulin used (10 nM) (Fig. 3B). Similarly, in vivo, PAR1-AP-induced edema was not reduced by coinjection with insulin at all of the time points after intraplantar injection, except at the first hour, where insulin treatment slightly increased PAR1 agonist-induced edema (Fig. 3D).

**Insulin reduces PAR2-stimulated Ca²⁺ signaling in primary cultures of DRG neurons**

Our previous studies have shown that PAR2 agonist-induced paw edema involves a neurogenic mechanism, with activation of the receptor on sensory neurons that in turn releases the vasoactive neurokinin substance P and calcitonin gene-related peptide through a calcium-dependent mechanism (9). Given the ability of insulin to reduce the edema response (Fig. 1), we wondered whether insulin might modulate the action of PAR2 in sensory neurons. To explore this possibility, we evaluated the effect of insulin on PAR2-mediated calcium signaling in isolated sensory DRG cells. As described previously (12, 18, 19), activation of PAR2 in sensory neurons...
isolated from mouse DRGs led to rapid mobilization of intracellular calcium (Fig. 4). Cultured DRGs preincubated with insulin (1 nM) for either 1 (data not shown) or 5 min showed a substantial reduction in the elevation of intracellular calcium concentration caused by PAR2-AP (Fig. 4A). The proportion of cultured neurons that responded to PAR2-AP with an increase in calcium signaling also was reduced significantly when the cultures were treated with insulin (1 nM) (Fig. 4B). In contrast, when the cultured DRG neuronal cells were preincubated with boiled insulin (1 nM), the PAR2-AP–induced elevation of intracellular calcium concentration was not significantly different from that of cells treated with PAR2-AP alone (data not shown).

**Intracellular signaling pathways associated with insulin-induced reduction of PAR2 signals**

To assess the mechanism(s) by which insulin was able to diminish PAR2 calcium signaling at a cellular level, either HMECs or primary afferent neurons were preincubated with either LY294002 (an inhibitor of PI3K) or GF 109203X (a nonselective inhibitor of PKC) prior to monitoring the effects of insulin on PAR2-induced calcium mobilization in those cell types. The PI3K and PKC inhibitors on their own had no effect on the ability of PAR2-AP to elevate intracellular calcium concentration in either HMECs (Fig. 3C) or DRGs (Fig. 4A). However, in the presence of either inhibitor, the ability of insulin to reverse the PAR2-triggered increase in intracellular calcium concentration was blocked in both the HMECs (Fig. 3C) and the cultured DRGs (Fig. 4A). This reversal of the inhibitory action of insulin also was reflected in terms of the proportion of responding cells in the DRG cultures (Fig. 4B). Thus, in both the HMECs and the cultured primary afferent neurons, the ability of insulin to diminish the cell response to PAR2-AP (elevated intracellular calcium concentration) appeared to depend on the PI3K and PKC signaling pathways.

We have investigated in vivo whether PI3K or PKC inhibitors, when injected into the mouse paw before the injection of PAR2 agonist, or the coinjection of PAR2 agonist and insulin can modify the inflammatory parameters of edema and granulocyte infiltration (MPO activity). Pretreatment with the PI3K or PKC inhibitors showed no significant effect on PAR2-AP–induced paw edema and increased MPO activity (Fig. 5A, 5B). However, in mice that received a pretreatment with those inhibitors into the paw, the ability of insulin to significantly reduce PAR2-AP–induced paw edema, and increased MPO activity was blocked (Fig. 5C, 5D). This result suggests that in vivo as well the ability of insulin to reduce PAR2 agonist-induced inflammation depends on the PI3K and PKC signaling pathways.

**Inflammatory response to PAR2-AP in the paw edema model is increased in mice with type 1 diabetes**

Because our data showed that insulin could diminish PAR2-induced inflammation, ostensibly via an effect on the endothelium and sensory nerve components, we hypothesized that the inflammatory effect of PAR2 activation might be affected in the setting of insulin-deficient diabetes. To test this hypothesis, we evaluated PAR2-triggered paw inflammation in mice rendered diabetic via the immune destruction of pancreatic β cells. In keeping with the data in Fig. 1, nondiabetic RIP-HA transgenic mice that received an intraplantar injection of the PAR2 agonist SLIGRL-NH2 developed an inflammatory response characterized by an increase in paw diameter (Fig. 6A, open squares) and an influx of inflammatory cells (increased MPO activity; Fig. 6B, white bars). In support of our working hypothesis, the inflammatory response of the diabetic RIP-HA mice to the intraplantar administration of SLIGRL-NH2 was enhanced, in terms of both edema (Fig. 6A, solid squares) and increased neutrophil infiltration (enhanced MPO activity; Fig. 6B, solid histograms). Thus, the insulin-deficient diabetic mice appeared to be more prone to a PAR2-mediated inflammatory response. Acute administration of insulin together with the PAR2 agonist in diabetic mice was able to inhibit PAR2 agonist-induced edema significantly, to the same extent as in control mice (Fig. 6C). This result suggests further that the increased inflammatory response associated with PAR2 activation is due to the lack of modulatory effects of insulin in the diabetic mice.

**Discussion**

The main finding of our study was that insulin, at low doses, can attenuate PAR2-mediated inflammation. The concentrations of insulin used in vitro were in the physiological range (1 nM). It is difficult to predict the absolute concentrations of insulin reached in the experiments done in vivo, even though comparatively low doses were administered. This action of insulin to mitigate the acute inflammatory effects of PAR2 activation was reflected not only by diminished edema and neutrophil infiltration (MPO activity) caused by the intraplantar administration of a PAR2 agonist but also in terms of markedly attenuated PAR2-induced leukocyte trafficking and a reduction in intracellular calcium signaling triggered by PAR2 in sensory neuronal cells (DRGs) and endothelial cells. Thus, insulin action can be seen to diminish the inflammatory effects of PAR2 in an intact tissue by targeting a number of key sites: the endothelium (intracellular signaling and leukocyte adherence), the sensory nerve that can release vasoactive neuropeptides (calcium signaling), and vascular permeability (edema in vivo). All of these sites have been documented to play roles in PAR2-triggered neurogenic inflammation (9, 24). The time frame of the PAR2-triggered effects on leukocyte trafficking (seen within 15–20 min) suggests a rapid P-selectin–initiated process followed by interactions with constitutively expressed endothelial VLA4–VCAM1/LFA1–ICAM1 (25). This mechanism, reversed by insulin, would merit further study.

Our data provide a new link between the action of insulin and the innate immune response system, a topic that has attracted considerable attention over the past decade (1, 26). To date, in the

**FIGURE 4.** Effects of insulin on the increase in intracellular calcium concentration (nanomolar) (A) and number of responding neurons (B) in primary afferent DRG cultures stimulated by PAR2-AP (SLIGRL-NH2, 100 μM) in the presence or absence of insulin (1 nM, with 5 min preincubation), and in the presence or absence of the PI3K inhibitor (LY294002, 50 μM) or the PKC inhibitor (GF 109203X, 100 μM) (C). **p < 0.01, significantly different from PAR2-AP–treated neurons. Values, representing the increase in calcium concentration (nanomolar), are shown as mean ± SEM. Observations were made for a minimum of 92 cells for each condition.
setting of diabetes, the role of the innate immune system has been viewed as diminishing insulin responses, due to inflammatory cytokine action (24). In contrast, our data now reveal that reciprocally insulin action can diminish a number of inflammatory parameters triggered by an acute stimulation of the innate immune system itself, as typified by PAR2 activation. Because the PAR2 system appears to play a prominent role in inflammatory diseases, such as arthritis (27) and intestinal inflammatory disease (3, 4, 11, 28), one can suggest that PAR2 activation also may enhance inflammatory responses in type 1 and type 2 diabetes. It is thus of much interest that insulin itself can be a negative regulator of the acute inflammatory action of PAR2. This action of insulin is in keeping with its anti-inflammatory and cardioprotective effects observed in several animal models in vivo (1) and with the clinical use of rigorous insulin therapy in humans in an intensive care setting (29). However, it can be noted that rigorous treatment of patients with type 2 diabetes with insulin or metformin to optimize blood glucose levels failed to reduce chronic inflammatory biomarkers compared with those of control patients [e.g., high-sensitivity C-reactive protein (30)]. Thus, the data that we report may apply only to an acute and not a chronic inflammatory process in diabetic humans. This issue merits further study.

The increased paw inflammation caused by SLIGRL-NH_{2} in insulin-deficient diabetic mice was in complete accord with our other data, because in these animals the anti-inflammatory action of insulin would be diminished due to the reduced insulin levels. Indeed, local treatment of the diabetic mice with low doses of insulin, administered at the inflammatory site so as not to affect systemic insulin levels, attenuated PAR2-induced inflammation. Notwithstanding, the enhanced inflammation triggered by PAR2 in the diabetic animals also might have been influenced by hyperglycemia, which also is recognized to have proinflammatory effects (1). Thus, in a type 1 diabetes setting mimicked by the murine model that we used, one can suggest that increased inflammation resulting from an acute activation of the innate immune response would be due to both hyperglycemia and a relative...
reduction in the anti-inflammatory action of insulin. This increase in inflammation, due to protease activation of the innate immune response, also may occur in a type 2 diabetes setting, where hyperglycemia is accompanied by a reduced sensitivity to the action of insulin. Thus, an interaction between the actions of insulin and the inflammatory effects of endogenous activation of PAR2 may be of importance in both type 1 and type 2 diabetes.

The mechanism(s) whereby insulin was able to mitigate the inflammatory response to the PAR2 agonist appears complex, involving both the P3K and PKC signaling pathways. Reciprocal interactions between signaling by insulin and G protein-coupled receptors have been known for over 30 y, best typified by the inhibitory effect of insulin on norepinephrine, adrenocorticotropin hormone, and glucagon-stimulated lipolysis in isolated adipocytes (31).

More recently, it has been demonstrated that for certain G protein-coupled receptor-mediated increases in intracellular calcium concentration (vasopressin, bradykinin, angiotensin II, neurotensin, and bombesin) insulin can enhance rather than inhibit signaling via a mammalian target of rapamycin-dependent pathway (32, 33). However, our data demonstrating an insulin-mediated decrease rather than an increase in PAR2-triggered calcium signaling are in keeping with the ability of insulin-like growth factor I and insulin to induce phosphorylation and desensitization of the G protein-coupled α1B and α1D adrenergic receptors (34, 35).

In those studies, as in ours, inhibitors of PI3K and PKC blocked receptor phosphorylation (on serines in the α1D adrenergic receptor) caused by the growth factors. It can be noted that the concentrations of insulin used in other studies done in vitro (100 nM or higher) all have been well above the near-physiological concentrations that we employed for our cell culture work (~1 nM). Significantly, our data showed that insulin treatment desensitized PAR2-mediated HMEC calcium signaling but did not decrease or even slightly increased calcium signaling via PAR1 (Fig. 3). Thus, in summary, activation of the insulin receptor may differentially affect signaling not only by the PARs but also by other G protein-coupled receptors. This ability of insulin to affect G protein-coupled receptor signaling in a differential manner may explain why inflammation triggered by carrageenan was not affected by insulin treatment, whereas PAR2-induced inflammation was reduced.

To conclude, our data reveal an unexpected inhibitory interaction between insulin and the acute inflammatory arm of the innate immune response triggered by PAR2. The data bring into focus a potential pathophysiological role for serine proteases, such as those of the coagulation pathway. In principle, those enzymes, via a potential pathophysiological role for serine proteases, such as between insulin and the acute inflammatory arm of the innate immune response. In type 2 diabetes, the diminished impact of insulin signaling may be responsible for the decrease in the acute inflammatory symptoms.

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Disclosures
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References
patients and counteracts the adverse effect of low mannose-binding lectin levels. 