Suppressive Effect of Insulin Infusion on Chemokines and Chemokine Receptors

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OBJECTIVE — In view of the previously described anti-inflammatory effects of insulin, we investigated the potential suppressive effect of insulin on plasma concentrations and expression of the chemokines, monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normal T-cell expressed and secreted (RANTES) and their receptors, chemokine receptor (CCR)-2 and CCR-5, in mononuclear cells (MNCs). We also investigated the effect of insulin on other chemokines.

RESEARCH DESIGN AND METHODS — Ten obese type 2 diabetic patients were infused with insulin (2 units/h with 100 ml of 5% dextrose/h) for 4 h. Another 8 and 6 type 2 diabetic patients were infused with 100 ml of 5% dextrose/h or saline for 4 h, respectively, and served as control subjects. Blood samples were obtained at 0, 2, 4, and 6 h.

RESULTS — Insulin infusion significantly suppressed the plasma concentrations of MCP-1, eotaxin, and RANTES and the expression of RANTES, macrophage inflammatory protein (MIP)-1β, CCR-2, and CCR-5 in MNCs at 2 and 4 h. Dextrose and saline infusions did not alter these indexes.

CONCLUSIONS — A low-dose infusion of insulin suppresses the plasma concentration of key chemokines, MCP-1, and RANTES, and the expression of their respective receptors, CCR-2 and CCR-5, in MNCs. Insulin also suppresses the expression of RANTES and MIP-1β in MNCs. These actions probably contribute to the comprehensive anti-inflammatory effect of insulin.

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Our work over the past few years has shown that insulin exerts a comprehensive and rapid anti-inflammatory effect as reflected in the suppression of intranuclear nuclear factor-κB and Egr-1 binding in peripheral blood mononuclear cells (MNCs) and the plasma concentrations of adhesion molecules and cytokines, matrix metalloproteinases, tissue factor, plasminogen activator inhibitor-1, and vascular endothelial growth factor (1–3). More recently, a low-dose insulin infusion has also been shown to suppress the expression of several Toll-like receptors including TLR1, 2, 4, 7, and 9 and PU.1, the major transcription factor regulating their synthesis (4). Thus, the anti-inflammatory effect of insulin may be seen at several levels.

Recent work has shown that chemokines are cardinal in the pathogenesis of all inflammation because they mediate the arrival of inflammatory cells to the site of both acute and chronic inflammation. Thus, the injection of endotoxin leads to an increase in interleukin (IL)-8 for polymorphs and monocyte chemoattractant protein-1 (MCP-1) for monocytes. Chronic inflammation in atherosclerosis, as observed in the arterial wall, is dependent on the release of chemokines from inflammatory cells in the atherosclerotic plaque (5). MCP-1 is a major chemokine that attracts more monocytes to the plaque to enhance the inflammation and thus facilitates the formation of foam cells (6,7). MCP-1 is abundantly expressed in atherosclerotic arterial lesions. The chemotactic response of the MNC is dependent on the presence of the chemokine receptor-2 (CCR-2) on its surface (8). Recent work has demonstrated that two other cytokines, regulated on activation normal T-cell expressed and secreted (RANTES) (CCL-5) and fractalkine (CX3CL-1), also play an important role in atherogenesis (9). The deletion of either the cytokines or their respective receptors leads to a marked reduction in atherogenesis in animal models (9). The deletion of two of these three cytokines or their receptors leads to an almost total elimination of atherosclerosis (9). Clearly, their role in atherogenesis is of extreme importance.

It has been shown that MCP-1, secreted by the adipose tissue macrophages, mediates the movement of the circulating monocytes to the adipose tissue to further enhance the inflammatory potential of the adipose tissue (10,11). The chemotactic response of the MNC is dependent on the presence of CCR-2 on its surface (8). MCP-1 is the major natural ligand for this receptor (12). Most chemokines have at least four cysteine residues, two near the NH_2 terminus, one near the COOH terminus, and one in the middle. The two near the NH_2 terminus may either be next to each other (CC motif) or be separated by an amino acid (CXC) motif. MCP-1, RANTES, and eotaxin belong to the CC class, whereas fractalkine, IL-8, and stromal-derived factor-1 (SDF-1) possess the CXC motif (12). CCR-2 is a seven-transmembrane domain G protein–coupled receptor whose activation leads to the rearrangement and intracellular movement of actin, resulting in a change in the shape of the cell and cellular movement (12). CCR-5, a similar receptor, is found on monocytes and T-cells and has RANTES as its ligand. Eotaxin and MIP-1β are other chemokines that bind to CCR-5 and are involved in allergic reactions and in responses to HIV-1 infections, respectively (13,14).

Our previous work has shown that insulin suppresses MCP-1 concentrations in plasma in obese individuals in vivo (1) and its expression by human aortic endothelial cells in vitro (15). Thus, chemokine-based inflammatory processes may be suppressed by insulin. We have now hypothesized that insulin suppresses

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Table 1—*Demographic data for patients at baseline*

	Insulin	Glucose	Saline
Age (years)	47.9 ± 8.9	45.8 ± 7.6	41.5 ± 8.2
BMI (kg/m^2)	39.2 ± 6.5	38.6 ± 7.2	36.9 ± 6.7
A1C (%)	7.00 ± 0.8	7.30 ± 0.9	7.5 ± 1.1
Diabetes diagnosis (years)	4.9 ± 3.5	4.2 ± 3.1	4.2 ± 3.1
Fasting glucose (mg/dl)	123 ± 10	133 ± 14	135 ± 13
Fasting insulin (μU/ml)	20.9 ± 10.9	27.6 ± 5.6	20.6 ± 5.5
Blood pressure (systolic/diastolic)	137 ± 4/102 ± 3	131 ± 4/94 ± 3	133 ± 5/95 ± 4
Other medical conditions	Hypertension (6), hypothyroidism (2), dyslipidemia (8), retinopathy (1)	Hypertension (5), hypothyroidism (1), dyslipidemia (6)	Hypertension (4), hypothyroidism (1), dyslipidemia (4), retinopathy (1)
Medications	Metformin (all), sulfonylureas, atenolol, atorvastatin, simvastatin diltiazem, fosinopril, levothyroxine, gemfibrozil, enalapril, metoprolol	Metformin (all), sulfonylureas, atenolol, atorvastatin, simvastatin diltiazem, fosinopril, levothyroxine, gemfibrozil, enalapril	Metformin (all), sulfonylureas, atorvastatin, diltiazem, fosinopril, levothyroxine, gemfibrozil, Toprol, enalapril, valsartan

Data are means ± SEM.

the plasma concentrations of the chemokines MCP-1, RANTES, eotaxin, MIP-1β, fractalkine, IL-8, and SDF-1 in patients with type 2 diabetes. We also hypothesized that it suppresses the expression of the chemokine receptors CCR-2 (MCP-1 receptor), CCR-5 (RANTES, eotaxin, and MIP-1β receptor), CX3CR-1 (fractalkine receptor), CXCR-1 (IL-8 receptor), and CXCR-4 (receptor for SDF-1) in MNCs.

RESEARCH DESIGN AND METHODS

— Twenty-four obese patients with type 2 diabetes participated in this study. They were taking stable doses of oral antidiabetes medications. All patients were taking metformin (1–2 g/day), and 14 patients were taking sulfonylureas (5–10 mg/day glyburide or glipizide). None of the subjects was receiving insulin or thiazolidinedione therapy or taking any antioxidant or nonsteroidal anti-inflammatory drugs. Demographic data for the patients are summarized in Table 1. After an overnight fast, 10 subjects (5 women) were infused with insulin (2 units/h) with 5% glucose and 20 mEq of potassium chloride for 4 h followed by 2 h of observation and washout. The blood glucose level was maintained at a target level of 80–130 mg/dl and was measured every 15 min. Another 8 (4 women) and 6 (4 women) subjects were infused with either 5% glucose or normal saline alone, respectively, at a rate of 100 ml/h for 4 h and served as control subjects. None of the patients had any hypoglycemic symptoms. Blood samples were collected at baseline and at 2, 4, and 6 h after the start of the infusion. The protocol was approved by the Human Research Committee of the State University of New York at Buffalo. An informed consent form was signed by all subjects.

MNC isolation

Blood samples were collected in Na-EDTA and carefully layered on Lympholyte medium (Cedarlane Laboratories, Hornby, Ontario, Canada). Samples were centrifuged, and two bands separated out at the top of the red blood cell pellet. The MNC band was harvested and washed twice with Hanks' balanced salt solution. This method yields >95% MNC preparation.

Quantification of chemokines and chemokine receptor expression

The mRNA expression of the chemokines MCP-1, MIP-1β, RANTES, eotaxin (CCLs 2, 4, 5, and 11, respectively), fractalkine, IL-8, and SDF-1 and the chemokine receptors CCR-2, CCR-5, CX3CR-1, CXCR1, and CXCR-4 was measured in MNCs by RT-PCR: Total RNA was isolated using the commercially available RNAqueous-4PCR kit (Ambion, Austin, TX). Real-time RT-PCR was performed using an Mx3000P QPCR system (Strategene, La Jolla, CA), SYBR Green MasterMix (Qiagen, Valencia, CA), and gene-specific primers for CCRs and CCLs (Life Technologies, Gaithersburg, MD). All values were normalized to the expression of a group of housekeeping genes including actin, ubiquitin C, and cyclophilin A.

Western blotting

MNC total cell lysates were prepared, and electrophoresis and immunoblotting were performed as described before (1). Monoclonal antibodies against CCR2 and CCR5 (Abcam, Cambridge, MA) and actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used, and all values were corrected for loading to actin.

Plasma measurements

Glucose concentrations were measured in plasma by a YSI 2300 STAT Plus glucose analyzer (YSI, Yellow Springs, OH). ELISA was used to measure plasma concentrations of insulin (Diagnostic Systems Laboratories, Webster, TX), CCL-2/MCP-1, CCL11/eotaxin, CCL5/RANTES, and CCL4/MIP-1β, IL-8, SDF-1, and fractalkine (R&D Systems, Minneapolis, MN).

Statistical analysis

Statistical analysis was conducted using SigmaStat software (SPSS, Chicago, IL). All data are presented as means ± SEM. Changes from baseline were calculated, and statistical analysis was performed using one-way repeated-measures analysis of variance (RMANOVA) with a Holm-Sidak post hoc test. Two-factor RMANOVA

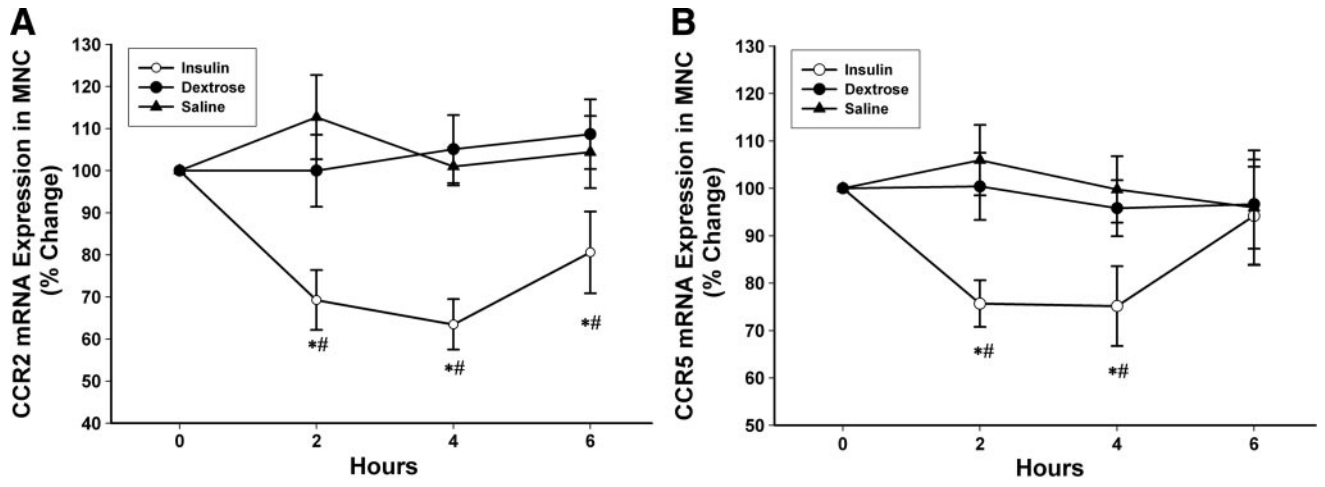

Figure 1—*Change in the expression of CCR2 (A) and CCR5 (B) in MNCs after 2 units/h insulin/dextrose infusion (Insulin), dextrose alone (Dextrose), or saline alone (Saline) in obese type 2 diabetic patients for 4 h. Data are means ± SEM. *P < 0.05 by one-way RMANOVA (compared with baseline). #P < 0.05 by two-way RMANOVA compared with control groups.*

followed by the Dunnett post hoc test was used for multiple comparisons between different treatments.

RESULTS

Insulin and glucose concentrations after insulin infusion

Plasma insulin concentration increased from 20.9 ± 10.9 to 50.5 ± 22.4 μU/ml ($P < 0.001$) during the insulin infusion, whereas it fell slightly in the dextrose groups from 27.6 ± 5.6 to 22.9 ± 6.5 μU/ml at 4 h (NS) and in the normal saline group from 20.6 ± 5.5 to 17.9 ± 4.7 μU/ml at 4 h (NS). The mean blood glucose concentrations changed from 122 ± 15 mg/dl at baseline to 111 ± 10 mg/dl at 4 h (NS) after insulin infusion and from 133 ± 14 mg/dl at baseline to 125 ± 12 mg/dl at 4 h (NS) after dextrose infusion. The blood glucose concentration did not change in the saline group. Blood glucose concentrations at baseline and at 4 h were not significantly different among the three groups.

Effect of insulin infusion on chemokine receptors (CCR-2, CCR-5, CX3CR-1, CXCR-1, and CXCR-4) expression in MNCs

The mRNA expression of CCR-2 and CCR-5 fell significantly by 43 ± 4 and 24 ± 5%, respectively, at 4 h after insulin infusion ($P < 0.05$) (Fig. 1), whereas there was no change in CX3CR-1, CXCR1, and CXCR4 expression. There was also a concomitant fall in CCR2 protein levels by 22 ± 5% at 4 h after in insulin infusion ($P < 0.05$), whereas CCR5 protein levels only showed a trend toward a fall but did not reach statistical significance (Fig. 2). There was no significant change in the expression of these receptors after glucose alone or saline alone infusions.

Effect of insulin infusion on chemokine concentrations (MCP-1, eotaxin, MIP-1β, RANTES, SDF-1, and fractalkine)

After insulin infusion, there was a significant decrease in plasma concentrations of

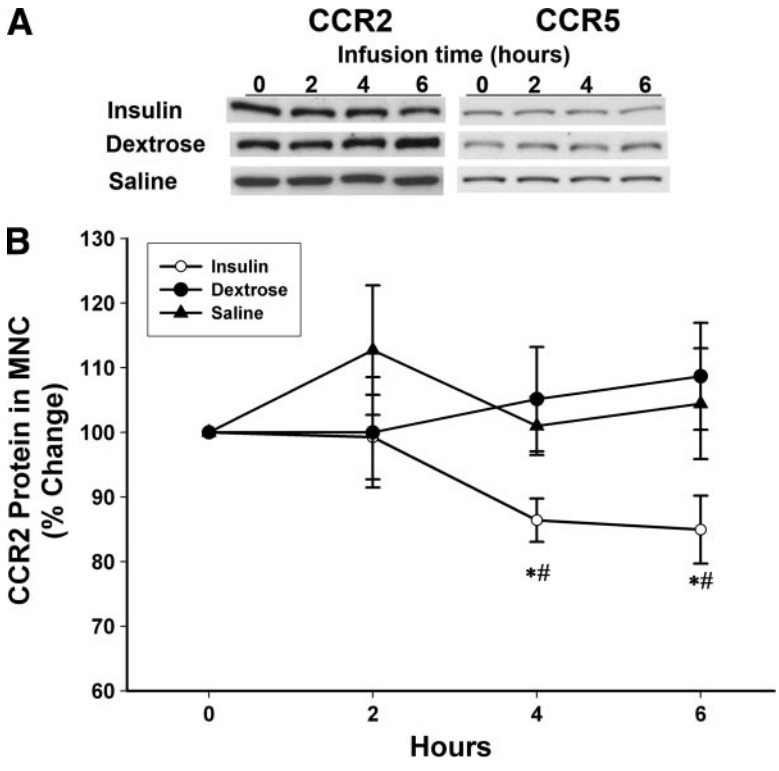

Figure 2—*Representative Western blots of changes in CCR2 and CCR5 protein (A) and percent change in CCR2 (B) protein levels in MNCs after 2 units/h insulin/dextrose infusion (Insulin), dextrose alone (Dextrose), or saline alone (Saline) in obese type 2 diabetic patients for 4 h. Data are means ± SEM. *P < 0.05 by one-way RMANOVA (compared with baseline). #P < 0.05 by two-way RMANOVA compared with control groups.*

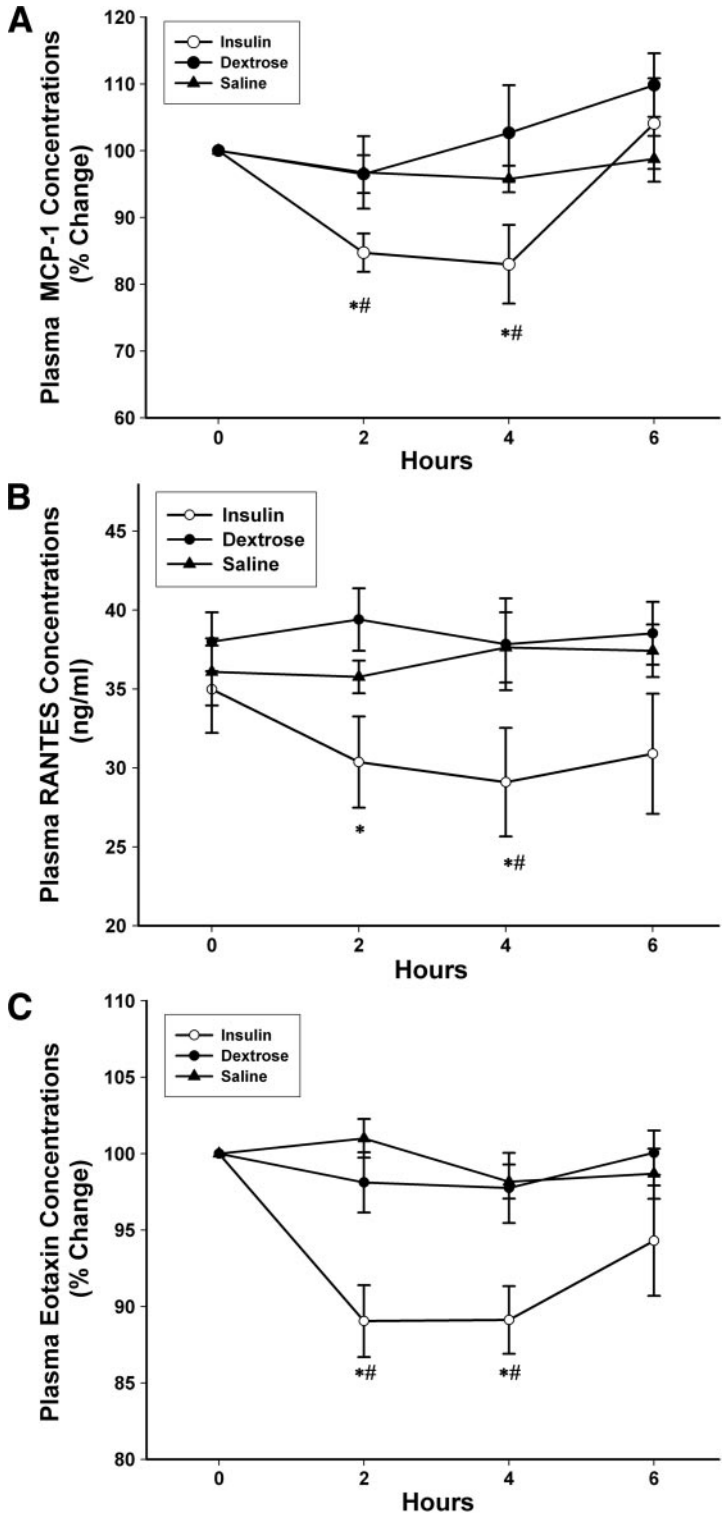

Figure 3—*Change in plasma concentrations of MCP-1 (*A*), RANTES (*B*), and eotaxin (*C*) after 2 units/h insulin/dextrose infusion (Insulin), dextrose alone (Dextrose), or saline alone (Saline) in obese type 2 diabetic patients for 4 h. Data are means ± SEM. *P < 0.05 by one-way RMANOVA (compared with baseline). #P < 0.05 by two-way RMANOVA compared with control groups.*

MCP-1 (by 15 ± 4%), RANTES (by 19 ± 5%), and eotaxin (by 10 ± 3%) at 4 h ($P < 0.05$) (Fig. 3) but not in MIP-1β, fractalkine, or SDF-1 concentrations. There was no significant change in plasma levels of these chemokines after dextrose or saline infusions.

Effect of insulin infusion on chemokine expression in MNCs

We further examined the effect of insulin infusion on chemokine expression in MNCs. The mRNA expression of CCL4 (MIP-1β) and CCL5 (RANTES) fell significantly by 26 ± 10 and 22 ± 8% below the baseline, respectively ($P < 0.05$) (Fig. 4) after insulin infusion, but not after glucose or saline alone, whereas that of MCP-1, fractalkine, and IL-8 did not change significantly.

CONCLUSIONS — Our data show for the first time that a low-dose insulin infusion in type 2 diabetic patients suppresses plasma concentrations of MCP-1, RANTES, and eotaxin. This occurs in parallel with a significant reduction in the expression of CCR-2 and CCR-5 in MNCs within 2 h of the insulin infusion, is maintained for the duration of the infusion, and reverts to the baseline within 2 h of the cessation of the infusion. In addition, insulin also suppressed the expression of RANTES and MIP-1β in MNCs. Thus, insulin exerts an inhibitory effect on chemokine mechanisms at three levels: the expression and plasma concentration of chemokines and the expression of their receptors. These observations are consistent with the previously demonstrated suppression by insulin of MCP-1 in obese individuals in vivo (1) and in endothelial cells in vitro (15). In contrast to the effects described above, insulin exerted no effects on the expression or plasma concentration of fractalkine, IL-8, SDF-1, or their receptors, CX3CR-1 CXCR-1, and CXCR-4, respectively. It is of interest that the cytokines and the receptors that were suppressed by insulin have a CC configuration, whereas those that were not suppressed, fractalkine and IL-8, and their receptors have a CXC configuration. The biological and clinical significance of this difference is not clear at this time.

Insulin suppressed the plasma concentrations of MCP-1 but did not alter the expression of this chemokine in MNCs. It is possible that it is secreted largely at other sites. Indeed, the endothelium is known to be its major source, and, as stated above, its expression in human aortic endothelial cells is suppressed by insulin in vitro (15). CCR-2 is expressed on monocytes, dendritic cells, and memory T-cells and thus determines their movements under the influence of the various chemokines that bind to this receptor. The clinical conditions likely to be affected through the suppression of CCR-2

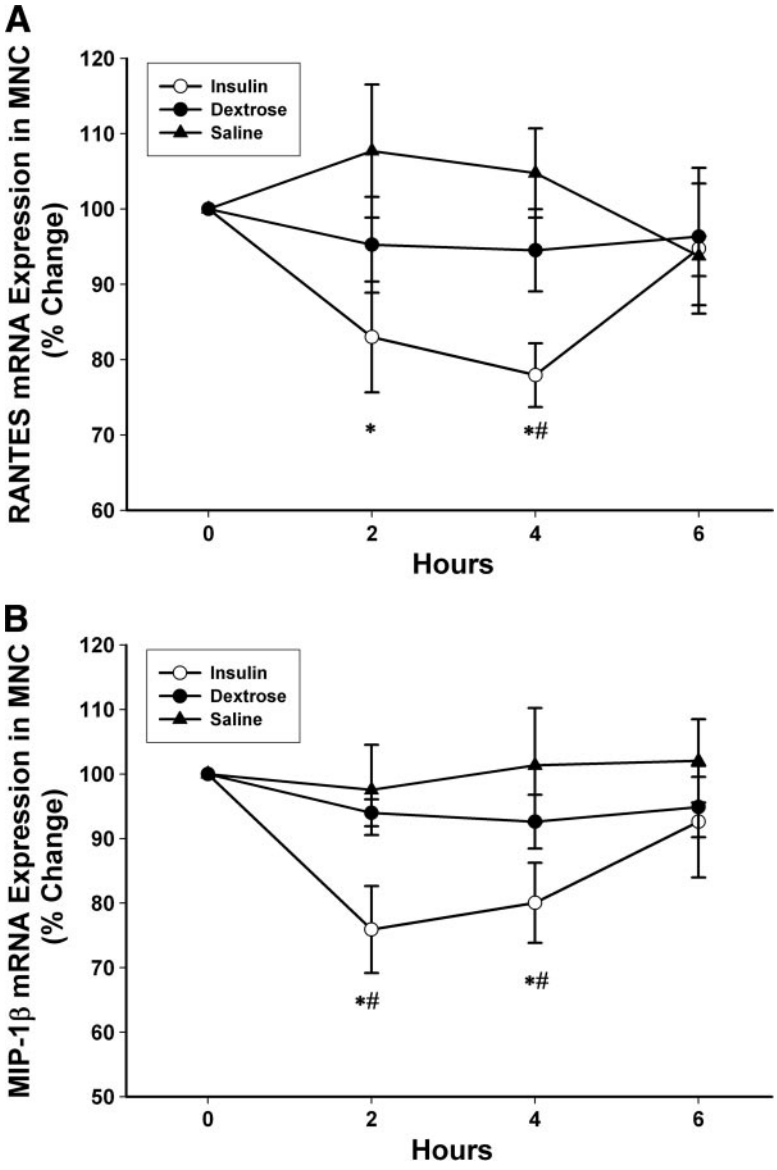

Figure 4—*Change in mRNA expression of CCL5 (RANTES) (*A*) and CCL4 (MIP-1β) (*B*) in MNCs after 2 units/h insulin/dextrose infusion (Insulin), dextrose alone (Dextrose), or saline alone (Saline) in obese type 2 diabetic patients for 4 h. Data are means ± SEM. *P < 0.05 by one-way RMANOVA (compared with baseline). #P < 0.05 by two-way RMANOVA compared with control groups.*

are atherosclerosis, rheumatoid arthritis, and multiple sclerosis (8). MCP-1 is considered to have a nonredundant role in the chemoattraction of monocytes to the endothelium and in the transendothelial transfer of the monocytes (16). Because atherogenesis is dependent on the transendothelial transfer of monocytes into the intima, it is not surprising that deletion of the MCP-1 gene leads to a reduction in atherogenesis in experimental models of atherosclerosis (17). Indeed, data from the Diabetes Control and Complications Trial-Epidemiology of Diabetes Interventions and Complications (DCCT-EDIC) show that intensive therapy with insulin was associated with a reduction in the carotid intimal-medial thickness and the incidence of cardiovascular events (18). In apolipoprotein E–deleted atherogenic mice, the administration of insulin was associated with a reduction in atherogenesis and oxidative stress (19). Furthermore, in genome-wide scans, MCP-1 has been shown to be one of the three genes to be strongly associated with multiple sclerosis (20).

It is of interest that CCR-2 has recently been shown to have an important role in the pathogenesis of osteoporosis (21). The deletion of CCR-2 results in the protection of female mice from bone loss after oophorectomy (21). It is therefore possible that the MCP-1/CCR-2 combination may play an important role in the pathogenesis of postmenopausal osteoporosis. The suppressive action of insulin on MCP-1/CCR-2 implies that insulin may have a potential anti-osteoporotic protective role in the bone. This finding is relevant to the recently demonstrated increase in fracture rates in patients with type 2 diabetes independent of a reduction in bone mineral density (22).

On the other hand, CCR-5 is expressed on T-cells and monocytes, which would respond to the chemokines listed above that bind to CCR-5. CCR-5 is important in mediating transplant rejection (12). Because RANTES is considered important in atherogenesis (23), the suppression of both RANTES and its receptor should have an inhibitory impact on atherogenesis. Interestingly, CCR-5 also serves as a coreceptor for HIV-1 for those strains that are T-cell tropic. It would be of interest to examine whether insulin reduces the entry of appropriate strains of HIV-1 into T-cells.

The suppression of at least two (MCP-1 and RANTES) of the three (MCP-1, RANTES, and fractalkine) most important chemokines involved in the pathogenesis of atherosclerosis in patients with type 2 diabetes is relevant because two-thirds of mortality in this condition is attributable to atherosclerotic complications of coronary heart disease, cerebrovascular disease, and peripheral arterial disease. The occurrence of insulin resistance in this condition may result in an increase in these chemokines and their receptors and thus potentially promote atherogenesis. Indeed, MCP-1 concentrations are known to be increased in obese individuals and in type 2 diabetic patients.

In summary, a low-dose infusion of insulin suppresses plasma concentrations of MCP-1, eotaxin, and RANTES and the expression of their respective receptors, CCR-2 and CCR-5, in patients with type 2 diabetes. In addition, it suppresses the expression of RANTES and MIP-1β in MNCs. On the other hand, fractalkine, IL-8, and SDF-1 and their respective receptors are not affected by insulin, at least during 4 h of insulin infusion. The overall anti-inflammatory effect of insulin thus includes its suppressive effect on chemokines and chemokine receptors with the CC motif but not the CXC motif. These

effects are potentially important because they may contribute significantly to the treatment of a wide range of pathological inflammatory processes, especially atherosclerosis.

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No potential conflicts of interest relevant to this article were reported.

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