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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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 NH₂ terminus, one near the COOH terminus, and one in the middle. The two near the NH₂ 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 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...
Insulin suppression of MCP-1 and RANTES

Table 1—Demographic data for patients at baseline

| Variable                  | Insulin | Glucose | Saline |
|---------------------------|---------|---------|--------|
| Age (years)               | 47.9 ± 8.9 | 45.8 ± 7.6 | 41.5 ± 8.2 |
| BMI (kg/m²)               | 30.2 ± 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)
- Metformin (all), sulfonylureas, atenolol, atorvastatin, simvastatin, diltiazem, losinopril, levotyroxine, gemfibrozil, enalapril, metoprolol

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 Hank’s 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...
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 receptor (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 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...
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
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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References
1. Dandona P, Aljada A, Mohanty P, Ghanim H, Hamouda W, Assian E, Ahmad S. Insulin inhibits intranuclear nuclear factor κB and stimulates κB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect? J Clin Endocrinol Metab 2001;86:3257–3265
2. Aljada A, Ghanim H, Mohanty P, Kapur N, Dandona P. Insulin inhibits the pro-inflammatory transcription factor early growth response gene-1 (Egr)-1 expression in mononuclear cells (MNC) and reduces plasma tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) concentrations. J Clin Endocrinol Metab 2002;87:1419–1422
3. Dandona P, Aljada A, Mohanty P, Ghanim H, Bandopadhyay A, Chaudhuri A. Insulin suppresses plasma concentration of vascular endothelial growth factor and matrix metalloprotease-9. Diabetes Care 2003;26:3310–3314
4. Ghanim H, Mohanty P, Decopurkar R, Sia CL, Korzeniewski K, Abuaysheh S, Chaudhuri A, Dandona P. Acute modulation of toll-like receptors by insulin. Diabetes Care 2008;31:1827–1831
5. Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat Rev Immunol 2008;8:802–815
6. Mantovani A, Garlanda C, Locati M. Macrophage diversity and polarization in atherosclerosis: a question of balance. Arterioscler Thromb Vasc Biol 2009;29:1419–1423
7. Shantsila E, Lip GY. Monocytes in acute coronary syndromes. Arterioscler Thromb Vasc Biol 2009;29:1433–1438
8. Charo IF, Peters W. Chemokine receptor 2 (CCR2) in atherosclerosis, infectious diseases, and regulation of T-cell polarization. Microcirculation 2003;10:259–264
9. Gautier EL, Jakubzick C, Randolpgh GJ. Regulation of the migration and survival of monocyte subsets by chemokine receptors and its relevance to atherosclerosis. Arterioscler Thromb Vasc Biol 2009;29:1412–1418
10. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Itazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest 2006;116:1494–1505
11. Yu R, Kim CS, Kwon BS, Kawada T. Mesenteric adipose tissue-derived monocyte chemoattractant protein-1 plays a crucial role in adipose tissue macrophage migration and activation in obese mice. Obesity (Silver Spring) 2006;14:1353–1362
12. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in the pathogenesis of vascular disease. Circulation 2004;109:1419–1423
13. Jose PJ, Griffiths-Johnson DA, Collins PD, Walsh DT, Moqbel R, Totty NF, Truong O, Hsuan JJ, Williams TJ. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. J Exp Med 1994;179:881–887
14. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Luuo P. Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8+ T cells. Science 1995;270:1811–1815
15. Aljada A, Ghanim H, Saadeh R, Dandona P. Insulin inhibits NFκB and MCP-1 expression in human aortic endothelial cells. J Clin Endocrinol Metab 2001;86:450–453
16. Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. Circ Res 2004;95:858–866
17. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol Cell 1998;2:275–281
18. Nathan DM, Lachin J, Cleary P, Orchard T, Brillon DJ, Backlund JY, O’Leary DH, Genuith S, Diabetes Control and Complications Trial, Epidemiology of Diabetes Interventions and Complications Research Group. Intensive diabetes therapy and carotid intima-media thickness in type 1 diabetes mellitus. N Engl J Med 2003;348:2294–2303
19. Shamir R, Shehadeh N, Rosenblat M, Eshach-Adir O, Coleman R, Kaplan M, Hamoudi S, Lischinsky S, Hayek T. Oral insulin supplementation attenuates atherosclerosis progression in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2003;23:104–110
20. Teuscher C, Buttersfield RJ, Ma RZ, Zachary JF, Doerge RW, Blankenhorn EP. Sequence polymorphisms in the chemokines Scy1 (TCA-3), Scy2 (monocyte chemoattractant protein (MCP)-1), and Scy12 (MCP-3) are candidates for eae7, a locus controlling susceptibility to monophasic remitting/nonrelapsing experimental allergic encephalomyelitis. J Immunol 1999;163:2262–2266
21. Binder NB, Niederreiter B, Hoffmann O, Stange R, Pap T, Stulng TM, Mack M, Erben RG, Smolen JS, Redlich K. Estrogen-dependent and C-C chemokine receptor-2-dependent pathways determine osteoclast behavior in osteoporosis. Nat Med 2009;15:417–424
22. Bonds DE, Larson JC, Schwartz AV, Strother ES, Robbins J, Rodriguez BL, Johnson KC, Margolis KL. Risk of fracture in women with type 2 diabetes: the Women’s Health Initiative Observational Study. J Clin Endocrinol Metab 2006;91:3404–3410
23. Veillard NR, Kwak B, Pelli G, Mulhaupt F, James RW, Proudfoot AE, Mach F. Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. Circ Res 2004;94:253–261