Effect of Granulocyte-Colony Stimulating Factor on Generation of Oxygen-Derived Free Radicals and Myeloperoxidase Activity in Neutrophils From Poorly Controlled NIDDM Patients

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To evaluate whether granulocyte-colony stimulating factor (G-CSF) improves an impaired production of oxygen-derived free radicals by neutrophils from poorly controlled NIDDM patients, we studied the effect of G-CSF on myeloperoxidase (MPO) activity and chemiluminescence amplified by a *Cypridina* luciferin analog (CLA-DCL), which is dependent on O_2^- generation, and luminol (L-DCL), which is dependent on OCl^- generation, in response to formyl-methonyl-leucyl-phenylalanine. Both CLA-DCL and L-DCL by neutrophils from the diabetic group (n = 15, HbA_{1c} >10%) were significantly decreased (26 and 37%, respectively: $P < 0.01$) compared with the age-matched normal control group (n = 15), and L-DCL was more sensitive to this inhibition than CLA-DCL ($P < 0.05$). In both control and diabetic neutrophils, G-CSF significantly enhanced both CLA-DCL (175% in control and 156% in diabetic) and L-DCL (283% in control and 346% in diabetic). In diabetic neutrophils, the enhancing effect of G-CSF on L-DCL was more sensitive than on CLA-DCL ($P < 0.001$). There was a positive correlation between HbA_{1c} and the enhancing effect of G-CSF on L-DCL in diabetic patients ($P < 0.05$), but not on CLA-DCL. MPO activity was also decreased in the diabetic group (63%, $P < 0.05$), and G-CSF improved this impaired MPO activity (184%, $P < 0.01$). Furthermore, there was a positive correlation between HbA_{1c} and the improving effect of G-CSF on MPO activity ($P < 0.05$). Because bacterial infection still accounts for an important cause of morbidity and mortality in diabetic patients, these data suggest that G-CSF may be useful as a drug to prevent the aggravation of bacterial infection by improving neutrophil function, especially through H_2O_2-MPO-OCl^- mechanism, in poorly controlled diabetic patients. *Diabetes* 46:133–137, 1997

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CLA-DCL, *Cypridina* luciferin analog–dependent chemiluminescence; fMLP, formyl-methonyl-leucyl-phenylalanine; G-CSF, granulocyte-colony stimulating factor; HBSS, Hanks' balanced salt solution; H_2O_2; hydrogen peroxide; L-DCL, luminol-dependent chemiluminescence; MPO, myeloperoxidase; OCl^-, hypochlorite; O_2^-, superoxide anion; STZ, streptozotocin.

Neutrophils play a critical role in the host defense mechanism against various bacterial infections, and it is suggested that the impaired neutrophil functions (e.g., chemotaxis, phagocytosis, and bactericidal functions) are factors that cause the susceptibility to infections in diabetic patients (1,2).

The bactericidal mechanism of neutrophil consists of oxygen-dependent and nondependent processes. In the oxygen-dependent bactericidal process, the importance of several oxygen-derived free radicals (e.g., superoxide anion [O_2^-], hydrogen peroxide [H_2O_2], and hypochlorite [OCl^-]) has been recognized (3). These oxygen-derived free radicals are produced during a burst of oxidative metabolism (Fig. 1). Most of the increased oxygen uptake is used to form O_2^-, which is weakly antimicrobial by itself. However, it is a major intermediate in the formation of H_2O_2, which in turn participates in the well-established hydrogen peroxide-myeloperoxidase-hypochlorite (H_2O_2-MPO-OCl^-) system, which has a most powerful bactericidal activity. Recently, we (4–7) and others (8–10) have demonstrated that this oxygen-dependent bactericidal function was impaired in neutrophils from poorly controlled diabetic patients and streptozotocin (STZ)-induced diabetic rats.

When poorly controlled diabetic patients suffer from some infectious disease, it is well known that the vicious circle of infection, diabetic metabolism, and host defense is accelerated; an infection aggravates diabetic metabolism, this aggravated diabetic metabolism causes the host defense to be impaired, this impaired host defense worsens infection, and the worsening infection aggravates diabetic metabolism. To improve this vicious circle, we usually use antibacterial drugs and insulin as a treatment for infection and diabetic metabolism, respectively. However, to our knowledge, no clinical drug trials are being conducted to find agents to improve the impaired host defense.

Recently, granulocyte-colony stimulating factor (G-CSF) appears not only to stimulate the formation of granulocyte colonies from bone marrow–derived precursors, but also to enhance the function of mature neutrophils (e.g., superoxide anion generation) (11). We have recently reported that G-CSF improved an impaired neutrophil bactericidal function from STZ-induced diabetic rats (12). To know the clinical evidence of G-CSF as a useful drug, we have studied the effect of G-CSF on the ability of production of oxygen-

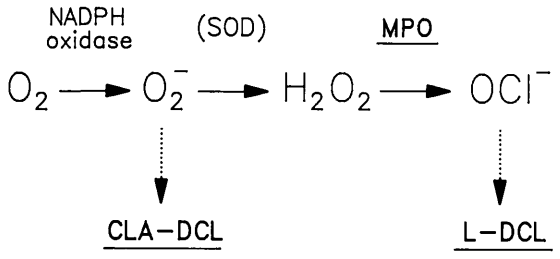

FIG. 1. Generation of oxygen-derived free radicals in neutrophils and measurement of oxygen-derived free radicals. O_2^-, H_2O_2, and OCl^- are oxygen-derived free radicals, which are generated during a burst of oxidative metabolism. CLA-DCL and L-DCL are highly dependent on O_2^- and OCl^- generation, respectively. SOD; superoxide dismutase.

derived free radicals by neutrophils from poorly controlled diabetic patients. In this study, to measure the ability of production of oxygen-derived free radicals, we used a *Cypridina* luciferin analog (CLA; 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]-pyrazin-3-one) and luminol (L; 5-amino-2,3-dihydro-1,4-phthalazine-dione) as agents to amplify the chemiluminescence. *Cypridina* luciferin analog–dependent chemiluminescence (CLA-DCL) and luminol-dependent chemiluminescence (L-DCL) appear to be highly dependent on O_2^- (13) and OCl^- (14) generation, respectively (Fig. 1). Furthermore, we have studied the effect of G-CSF on MPO activity because it is suggested that the reduction of MPO activity in poorly controlled diabetic patients is one important factor of the impaired neutrophil function (5).

RESEARCH DESIGN AND METHODS

Subjects. There were 15 NIDDM outpatients and 15 healthy control subjects studied in this experiment. Primary selection criteria were age 30–60 years and treatment of diabetes with sulfonylureas. All subjects were without any clinical diseases, including infectious disease, and any medications except sulfonylureas. Initially, patients were recruited on the basis of the following: HbA_{1c} level >10%, no diabetic retinopathy, no diabetic nephropathy (defined as urinary albumin excretion level <20 μg/min), no significant symptoms of diabetic neuropathy, and no ketonuria. Diabetic patients and control subjects were matched for age and sex (age [diabetic/control]: 42.6 ± 3.2/43.1 ± 2.6 years; sex: 8 men and 7 women/8 men and 7 women). The average duration after diagnosis of NIDDM was 0.8 ± 0.2 years. In all subjects at experiment day, C-reactive protein level was not detected, and white blood cell count was <8,000 counts/μl.

Measurements of oxygen-derived free radicals generation and MPO activity in neutrophils. Blood samples were drawn after an overnight fast. Blood glucose level was measured by a glucose oxidase technique. HbA_{1c} level was measured in a single laboratory by high-performance liquid chromatography (Hi-auto A_{1c}; Kyoto Daiich Kagaku, Kyoto, Japan). The leukocytes were separated by 6% dextran and hypotonic lysis method, as previously described (12). The obtained cells consisted of 95–98% neutrophils and were suspended in Hanks' balanced salt solution (HBSS), and final suspensions were adjusted to give 1×10^6 neutrophils/ml. The cells were preincubated with or without 30 pmol/l recombinant human G-CSF (Kirin, Tokyo, Japan: the purity is 99.9%) at 37°C for 30 min because *1*) the dose and time were almost the median effective dose (ED_{50}) for oxidative metabolism stimulated by formyl-methonyl-leucyl-phenylalanine (fMLP) in data previously reported (12,15), and *2*) a peak G-CSF concentration in serum as clinical usage is almost 50 pmol/l (16). CLA-DCL and L-DCL were measured with a Luminescence Reader (Model BLP 102, Aloka, Tokyo, Japan), as described previously (12). The standard reaction mixture for chemiluminescence assay contained 5×10^4 cells, 100 nmol/l fMLP (Sigma), and 5 μmol/l *Cypridina* analog (Tokyo Kasei Kogyo, Tokyo, Japan; for CLA-DCL assay) or 50 μmol/l luminol (Aldrich, Milwaukee, WI; for L-DCL assay) in HBSS in a total volume of 2 ml. All components, except for fMLP, were preincubated for 2 min; the reaction was initiated by the addition of fMLP. The CLA-DCL and L-DCL were assessed with the maximally changed value of chemiluminescence as kilocounts (KC) · min^{-1} · 10^{-6} cells. MPO activity was assayed by Klebanoff's method, with guaiacol as the substrate (5,17). The neutrophils preincubated with or without G-CSF were sonicated for 5 s in an ice bath. The standard reaction mixture for MPO assay contained the disrupted solution (1×10^4 cells), 40 mmol/l guaiacol, and 0.02 mol/l cetyltrimethylammonium bromide in 0.2 mmol/l sodium phosphate buffer (pH 7.0) in a total volume of 2 ml. After the addition of 0.5 mmol/l H_2O_2, the absorbance was determined at 470 nm for 1 min in a recording spectrophotometer, and the optical density change per minute is calculated from the initial rate. Intracellular MPO activity was expressed as the ability of tetraguaiacol production (μmol · min^{-1} · 10^{-6} cells).

Statistical analysis. Statistical analysis was made with Student's *t* test or with linear correlation. All values are expressed as means ± SE. Viability of cells measured by Trypan blue staining was >98% at both the beginning and end of the experiments.

RESULTS

Fasting blood glucose and HbA_{1c} levels. The mean fasting blood glucose level from diabetic patients was 10.1 ± 0.4 mmol/l and significantly higher than that from control subjects (4.8 ± 0.2 mmol/l, $P < 0.001$). HbA_{1c} level from diabetic patients was 12.5 ± 0.4% and higher than that from control subjects (4.4 ± 0.1%, $P < 0.001$).

Effect of G-CSF on CLA-DCL and L-DCL. From control neutrophils preincubated without G-CSF, fMLP caused a prompt rise in chemiluminescence (Fig. 2). CLA-DCL and L-DCL were 1,697 ± 118 and 2,274 ± 184, respectively. In the neutrophils from diabetic patients, mean CLA-DCL and L-DCL were significantly lower than those from control subjects (Figs. 2*C* and *D*), and the L-DCL was clearly more inhibited than the CLA-DCL (L-DCL = 62.6% and CLA-DCL = 74.4% of control, $P < 0.05$) (Fig. 3*A*). However, the dynamics of chemiluminescence in neutrophils from diabetic patients were similar to those from control subjects (Figs 2*A* and *B*).

In neutrophils of either the control or diabetic group, the 30-min pretreatment of 30 pmol/l G-CSF apparently enhanced the fMLP-induced rise in both CLA-DCL and L-DCL without changing the dynamics (Fig. 2). In both the control and diabetic groups, G-CSF enhanced more L-DCL than CLA-DCL ($P < 0.001$) (Fig. 3*B*). In the diabetic group, L-DCL was clearly more enhanced by G-CSF than that from control neutrophils; however, CLA-DCL was enhanced by G-CSF as the same level of control neutrophils (Fig. 3*B*).

Correlations between HbA_{1c} and CLA-DCL/L-DCL and between HbA_{1c} and the enhancing capacity of G-CSF on CLA-DCL/L-DCL in diabetic patients. There were significant negative correlations between HbA_{1c} and CLA-DCL/L-DCL in diabetic patients (Figs. 4*B* and *D*). In CLA-DCL, the enhancing capacity of G-CSF was not affected by HbA_{1c} level (Fig. 4*A*). However, G-CSF enhanced the L-DCL more when the HbA_{1c} level was higher, and there was a significant positive correlation between HbA_{1c} and the enhancing capacity of G-CSF (Fig 4*C*).

Correlations between HbA_{1c} and MPO activity and between HbA_{1c} and the enhancing capacity of G-CSF on MPO activity in diabetic patients. MPO activity in diabetic neutrophils was 0.37 ± 0.05 μmol · min^{-1} · 10^{-6} and lower than that in control neutrophils (0.60 ± 0.07 μmol · min^{-1} · 10^{-6}; $P < 0.01$) (Fig. 5*A*). In either control or diabetic neutrophils, G-CSF significantly increased MPO activity ($P < 0.01$). In diabetic neutrophils, there was a negative correlation between HbA_{1c} and MPO activity (Fig. 5*C*). Furthermore, there was a positive correlation between HbA_{1c} and the enhancing capacity of G-CSF on MPO activity (Fig. 5*B*).

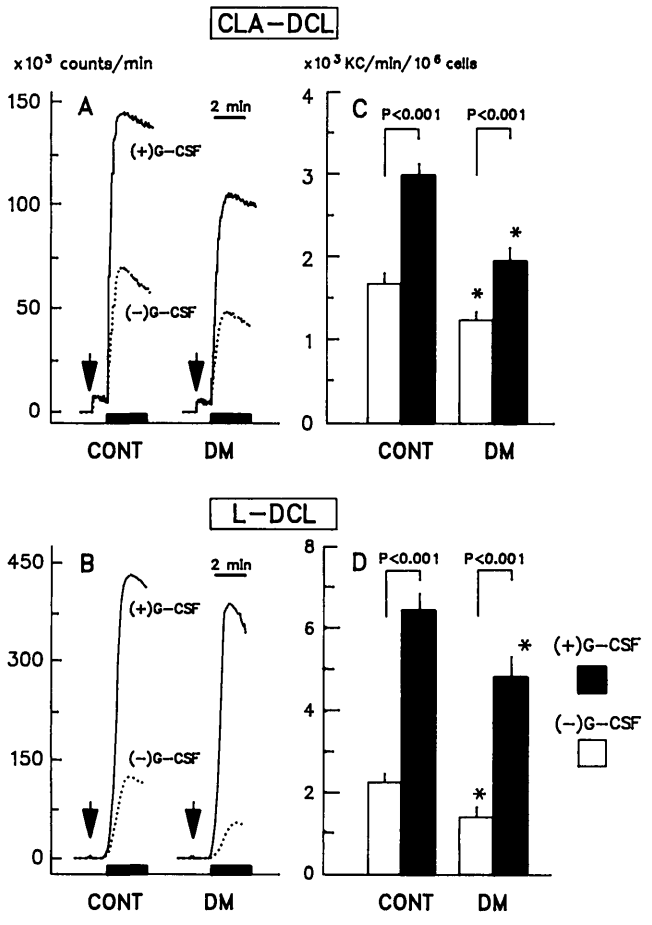

FIG. 2. Effect of G-CSF pretreatment on CLA-DCL and L-DCL in neutrophils from control subjects (CONT) and poorly controlled NIDDM patients (DM). *A* and *B*: representative dynamics of chemiluminescence amplified with a *Cypridina* luciferin analog (*A*) or luminol (*B*) in response to fMLP in neutrophils pretreated with (solid lines) or without (dotted lines) G-CSF. The arrows indicate the addition of *Cypridina* luciferin analog (*A*) or luminol (*B*). The horizontal solid bars indicate the duration of fMLP stimulation (2 min). *C* and *D*: statistical analysis of two groups (CONT: n = 15, and DM: n = 15). $^*P < 0.01$ vs. similarly treated control subjects (Student's t test).

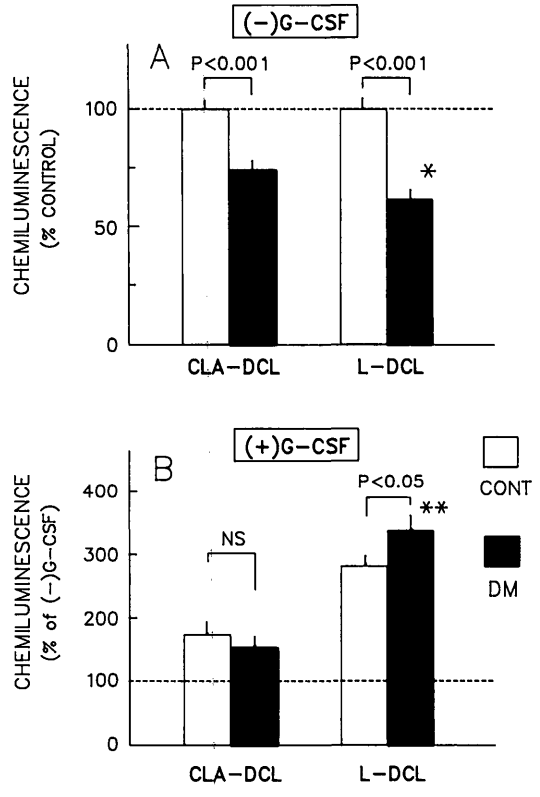

FIG. 3. Comparison of the effect of G-CSF on either CLA-DCL or L-DCL between control subjects (CONT) and poorly controlled NIDDM patients (DM). *A*: effect of diabetic condition on CLA-DCL and L-DCL. Data are expressed as a percent of the responses from normal control neutrophils. Neither diabetic nor control neutrophils were pretreated with G-CSF. $^*P < 0.05$ vs. CLA-DCL in DM (Student's t test). *B*: comparison of the effect of G-CSF on chemiluminescence (CLA-DCL and L-DCL) between control and diabetic neutrophils. Data are expressed as a percent of the responses achieved without G-CSF pretreatment (100% = responses achieved without G-CSF pretreatment). $^{**}P < 0.001$ vs. L-DCL in DM (Student's t test).

DISCUSSION

In this study, both CLA-DCL and L-DCL in response to fMLP were decreased in poorly controlled NIDDM patients. These results were consistent with our previous observation that O_2^- and OCl^- generations were reduced in poorly controlled diabetic patients (5) and STZ-induced diabetic rats (4). Furthermore, the facts in this study that L-DCL was more suppressed than CLA-DCL and that MPO activity was significantly decreased in diabetic neutrophils suggest that (1) OCl^- generation is more impaired than O_2^- generation, and (2) the impaired OCl^- generation is partly caused by the reduced MPO activity. This impaired H_2O_2-MPO-OCl^- system may contribute to greater susceptibility to infection in poorly controlled NIDDM patients because O_2^- by itself is well known to be weakly antimicrobial, and the H_2O_2-MPO-OCl^- system is one of the most powerful antimicrobial systems (3).

Extensive evidence from a number of laboratories indicates that G-CSF acts as enhancing agents on normal neutrophils, enhancing O_2^- generation and H_2O_2-MPO-OCl^- system in response to fMLP (16). However, there are no reports about the effect of G-CSF on oxygen-derived free radicals generation and MPO activity in neutrophils from poorly controlled diabetic patients. Our data firstly demonstrated that G-CSF apparently enhanced both fMLP-induced CLA-DCL and L-DCL and increased MPO activity by the normal and diabetic neutrophils. It may be possible that the enhancing effect of G-CSF on L-DCL is dependent on the induced increase in MPO activity.

To prevent infection in diabetic patients, good control of blood glucose level is most important. Our data support this because there were negative correlations between HbA_{1c} and CLA/L-DCL. However, when poorly controlled diabetic patients suffer from infectious disease, it is well known that the vicious circle of infection, diabetic metabolism, and host defense is accelerated. Despite the great improvement brought by insulin and antimicrobial agents, bacterial infection is still an important cause of morbidity and mortality in diabetic patients (1,2) and no clinical and experimental drug trials are being conducted to find agents to improve the impaired host defense. This study has demonstrated that G-CSF improves the impaired generation of oxygen-derived free radicals by neutrophils from poorly controlled diabetic patients. Since the discovery as potent activator of enhancing the matured neutrophils for oxygen-derived free radi-

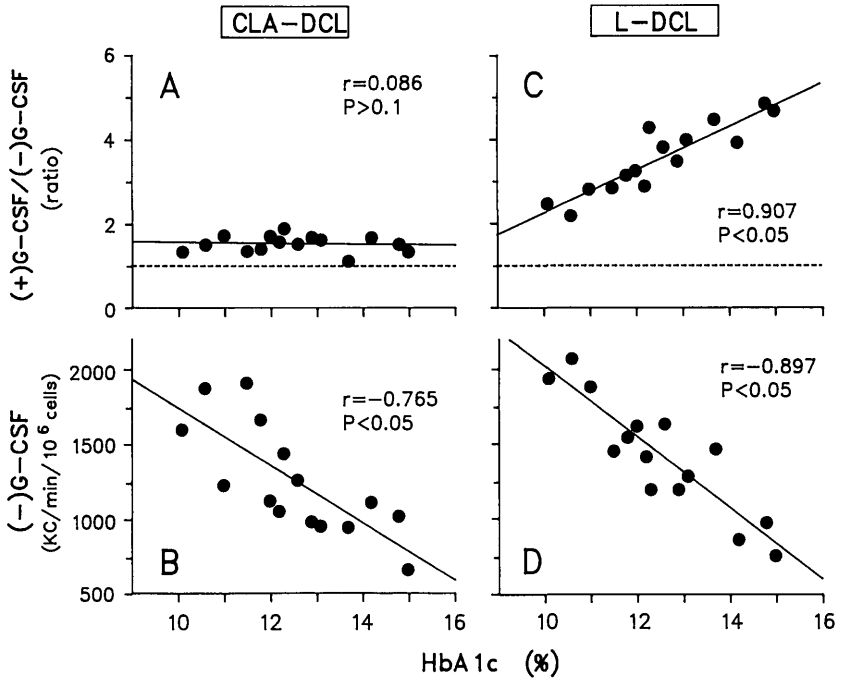

FIG. 4. Correlations between HbA_{1c} and CLA-DCL/L-DCL in diabetic neutrophils without G-CSF (*B* and *D*) and correlations between HbA_{1c} and enhancing capacity of G-CSF on the CLA-DCL/L-DCL (*A* and *C*). *A* and *B* show CLA-DCL data, and *C* and *D* show L-DCL data. There were significant negative correlations between HbA_{1c} and CLA-DCL/L-DCL (*B*; $y = -191x + 3{,}661$, $r = -0.765$, $n = 15$, and $P < 0.05$. D; $y = -237x + 4{,}386$, $r = -0.897$, $n = 15$, and $P < 0.05$). *A* and *C*: data are expressed as a ratio of the responses achieved with/without G-CSF pretreatment. There was a significant positive correlation in L-DCL (*A*, $y = 0.512x - 2.85$, $r = 0.907$, and $P < 0.05$), however, not in CLA-DCL (*C*, $P > 0.1$).

cals, G-CSF has been used in clinical trials to improve infections in patients with acquired immunodeficiency syndrome (18), myelodysplastic syndrome (19), and congenital agranulocytosis (20). Because our present study demonstrates that G-CSF is a useful drug for improving the impaired diabetic neutrophil function, especially an impaired H_2O_2-MPO-OCl^- system, G-CSF may help to prevent morbidity and mortality from bacterial infections in poorly controlled diabetic patients. Obviously, the notion that stimulating neutrophil function is beneficial in diabetes needs to be considered carefully. Oxidative damage has been implicated in the pathogenesis of atherosclerosis, and myeloperoxidase has been reported to be present in human atherosclerotic lesions (21), as are HOCl-damaged proteins (22). Although it is unknown whether G-CSF initiates or aggravates atherosclerotic lesions, it may be of significance to have a prospective randomized trial of the effect of G-CSF on the risk of infection in diabetic subjects.

In this study, the priming ability of G-CSF in diabetic neutrophils was more effective in L-DCL than in CLA-DCL, and G-CSF enhanced MPO activity. G-CSF enhanced the L-DCL and MPO activity more because the HbA_{1c} level was higher. These results suggest that G-CSF is a useful drug to improve the impaired H_2O_2-MPO-OCl^- system in poorly controlled diabetic patients. However, the mechanisms by which G-CSF enhances CLA-DCL/L-DCL and MPO activity were not clarified in this study. We acknowledge that these observations are difficult to explain.

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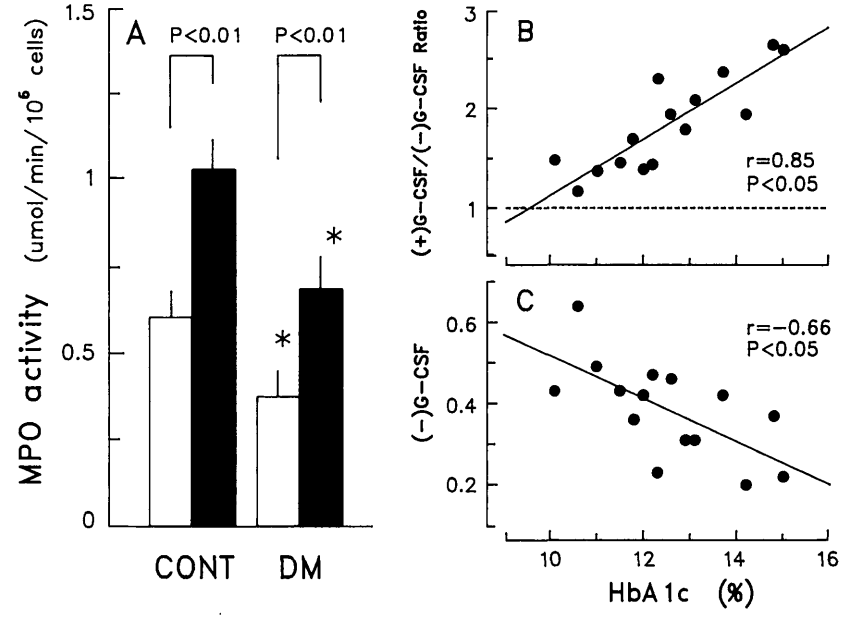

FIG. 5. Effect of G-CSF on MPO activity in control and diabetic neutrophils. *A*: comparison of the effect of G-CSF on MPO activity between control (CONT) and diabetic (DM) neutrophils. $n = 15$ in both groups. *$P < 0.01$ vs. similarly treated control subjects (Student's t test). *B*: correlation between HbA_{1c} and enhancing capacity of G-CSF on MPO activity in diabetic neutrophils ($y = 0.279x - 1.667$, $r = 0.853$, $n = 15$, and $P < 0.05$). Data are expressed as a ratio of the responses achieved with/without G-CSF pretreatment. *C*: correlation between HbA_{1c} and MPO activity without G-CSF pretreatment in diabetic neutrophils ($y = -0.053x + 1.04$, $r = -0.656$, $n = 15$, and $P < 0.05$).

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