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Original Research Communication

High Glucose and Ketosis (Acetoacetate) Increases, and Chromium Niacinate Decreases, IL-6, IL-8, and MCP-1 Secretion and Oxidative Stress in U937 Monocytes

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ABSTRACT

Elevated blood levels of the proinflammatory cytokines interleukin-6 (IL-6), interleukin-8 (IL-8), and MCP-1 (monocyte chemoattractant protein-1) increase insulin resistance and the risk of cardiovascular disease (CVD). There is no previous study that has examined the effect of ketosis and trivalent chromium on IL-6, IL-8, or MCP-1 secretion in any cell type or in human or animal model. The authors examined the hypothesis that ketosis increases and trivalent chromium decreases the levels of cytokines and oxidative stress in diabetes using a U937 monocyte cell culture model. Cells were cultured with control, high glucose (HG), and acetoacetate (AA) in the absence or presence (0.5–10 μM) of $CrCl_3$, chromium picolinate (Cr-P), or chromium niacinate (Cr-N) at 37°C for 24 h. The data show a significant stimulation of IL-6, IL-8, and MCP-1 secretion and an increase in oxidative stress in cells treated with HG or AA. The effect of HG on cytokine secretion was reduced by Cr-N, and to a lesser extent by $CrCl_3$ and Cr-P. The effect of HG on oxidative stress was reduced by Cr-N and $CrCl_3$, but not by Cr-P. Similarly, Cr-N decreased the cytokine secretion in HG+AA-treated cells. Cr-N significantly decreased standard oxidant (H_2O_2) induced cytokine secretion, which suggests that reduction of cytokine secretion by Cr-N is in part mediated by its antioxidative effect. In a cell culture model, Cr-N appears to be the most effective form of chromium in inhibiting oxidative stress and proinflammatory cytokine secretion by monocytes. This study suggests that chromium niacinate supplementation may be useful in reducing vascular inflammation and the risk of CVD in diabetes. *Antioxid. Redox Signal.* 9, 1581–1590.

INTRODUCTION

Interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1) are proinflammatory cytokines produced by macrophages and other cell types in response to various stimuli (11, 21, 58). The levels of these cytokines are elevated in the blood of many diabetic patients (7, 27, 33). An increase in circulating levels of IL-6, IL-8, and MCP-1 can lead to increased insulin resistance, vascular inflammation, and the development of vascular disease (7, 26, 43, 51). Concentrations of chromium in the blood, lenses, and toenails are lower in diabetic patients compared with those of the normal population (22, 37, 41, 48, 49), and several studies have suggested that chromium picolinate (Cr-P) or chromium niacinate (Cr-N) supplementation may be beneficial in individuals with Type 2 and Type 1 diabetes, as evidenced by decreased blood glucose values or decreased insulin requirements (4, 6, 14–16, 31, 35, 38, 39, 42, 50). Results from epidemiological studies suggest an inverse association between chromium levels in toenails and the risk of cardiovascular disease (CVD) in the diabetic and general population (22, 49). The mechanism by which chromium supplementation may increase insulin sensitivity and lower vascular inflammation in diabetes is not known. No previous studies have examined the effect of triva-

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lent chromium supplementation on blood levels of proinflammatory cytokines in diabetic patients or animals.

In addition to hyperglycemia, Type 1 diabetic patients frequently experience ketosis (hyperketonemia) because, in a state of insulin deficiency, body fuel is derived mainly from fat. The blood concentration of ketone bodies [acetoacetate (AA), β-hydroxybutyrate, and acetone] reaches >25 m*M* in diabetics with severe ketosis, compared with concentrations of <0.5 m*M* in normal individuals (13, 36). Recent studies have suggested that hyperketonemia plays a role in the elevated blood levels of IL-6, TNF-α, and ICAM in diabetes (1, 25, 27, 30, 57). However, no studies exist in the literature concerning the effect of ketosis on IL-8 or MCP-1 secretion in monocytes or any other cell type, nor has any study examined the effect of chromium on any of the proinflammatory cytokines in diabetic patients or in experimental models of diabetes. The present study examined the hypothesis that high glucose and ketosis increases and that trivalent chromium lowers proinflammatory cytokines and oxidative stress levels in diabetes. To examine this hypothesis, the effect of hyperglycemia and ketosis (mimicking diabetes) was determined in the absence and presence of trivalent chromium (chromium niacinate, chromium chloride, chromium picolinate) on secretion of IL-6, IL-8, and MCP-1 in a U937 human monocyte cell culture model.

MATERIALS AND METHODS

Human pro-monocytic cell line

The U937 monocyte cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells

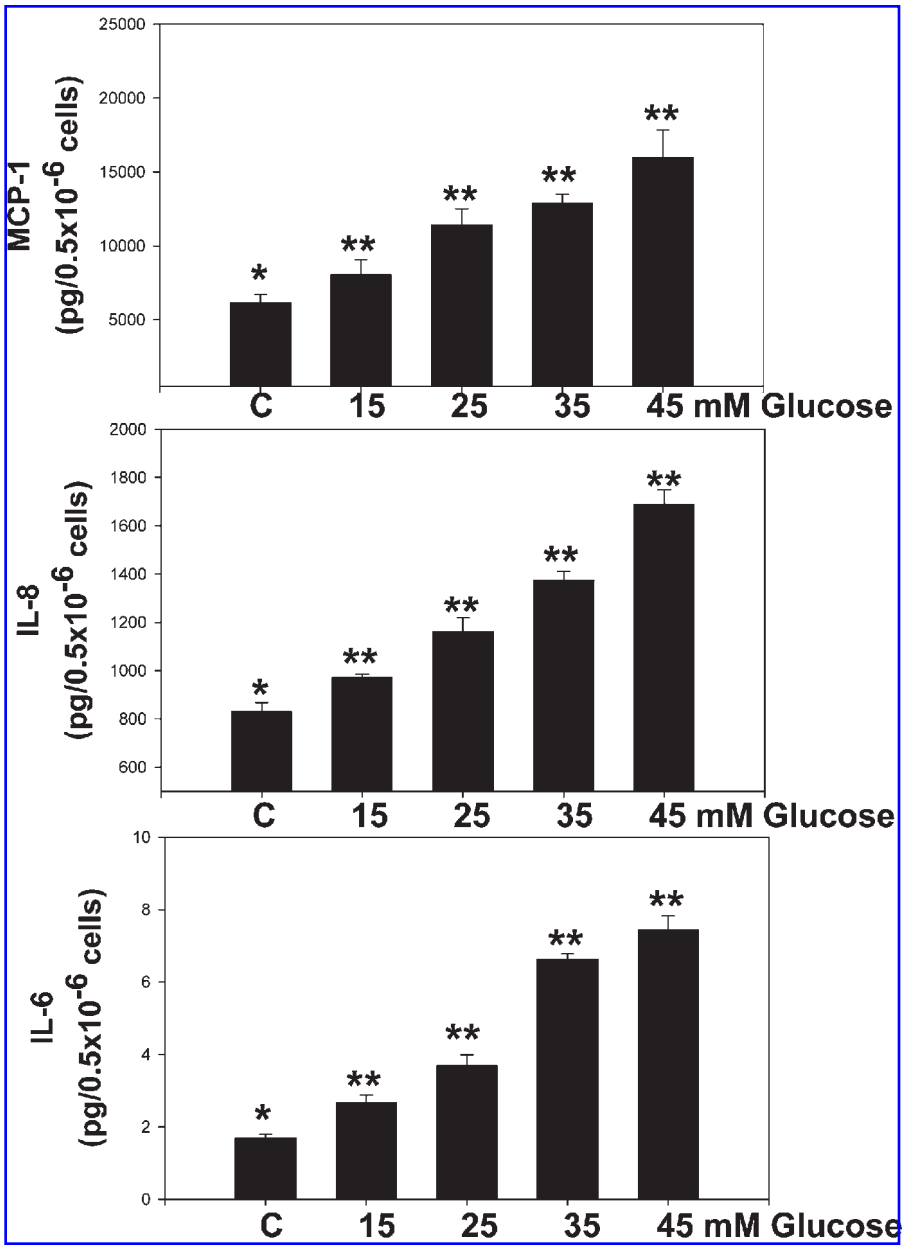

FIG. 1. Effect of increasing glucose concentrations on secretion of IL-6 (*bottom*), IL-8 (*middle*), and MCP-1 (*top*) in activated monocytes. Values obtained with only LPS-activated cells were considered as controls. Values are mean ± SE ($n = 4$). Differences in values marked * *vs.* ** are significant ($p < 0.05$). Note that secretion of cytokines increased with increasing concentrations of glucose.

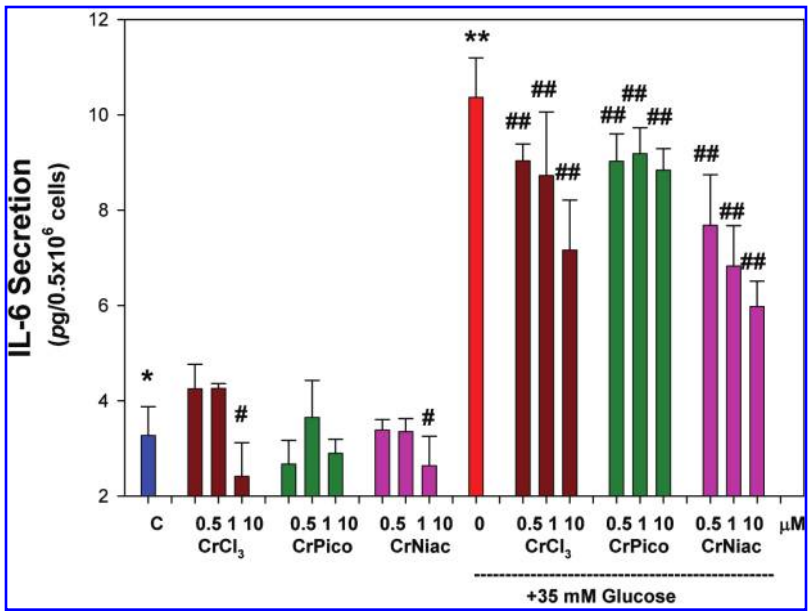

FIG. 2. Effect of different forms of chromium supplementation on IL-6 secretion in high glucose-treated activated monocytes. Values obtained with only LPS-activated cells were considered as controls. Values are mean ± SE ($n = 6$). Differences between * *vs.* # and ** *vs.* ## are significant ($p < 0.05$).

were maintained at 37°C in RPMI 1640 medium containing 7 m*M* glucose, 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 12 m*M* sodium carbonate, 12 m*M* HEPES, and 2 m*M* glutamine in a humidified atmosphere containing 5% (vol/vol) CO_2. For treatments, cells were washed once in plain RPMI 1640 before being suspended in fresh medium (complete) containing serum and other supplements (29).

Treatment with high glucose (HG), acetoacetate (AA), and chromium

U937 (500,000 cells/ml) were treated with normal glucose (7 m*M*), HG (15–35 m*M*), and AA (0–4 m*M*) without and with chromium niacinate, chromium chloride, or chromium picolinate, three different commercially available forms of trivalent chromium. Chromium niacinate (ChromeMate, lot #0410013) was obtained from InterHealth Nutraceutical (Benicia, CA) and chromium picolinate (Chromax, lot #00225720) was obtained from Nutrition 21 (Purchase, NY). Chromium picolinate, chromium niacinate, and chromium chloride were all dissolved in 0.03 *M* NaOH-PBS buffer. Specifically, 10.45 mg of chromium chloride, chromium picolinate, or chromium niacinate was mixed in 50 ml of 0.03 *M* NaOH-PBS buffer solution. The mixture was stirred overnight, which completely dissolved the compounds. This working solution contained 0.5 m*M* each of chromium chloride, chromium picolinate, or chromium niacinate. Four μL of this working solution were added to 2 ml medium-cells suspension for final chromium concentration of 1 μM; and 2 μL was added for 0.5 μM final chromium concentration. Chromium-untreated cells were added buffer volume of 4 μL. There was no change in pH of the medium-cells

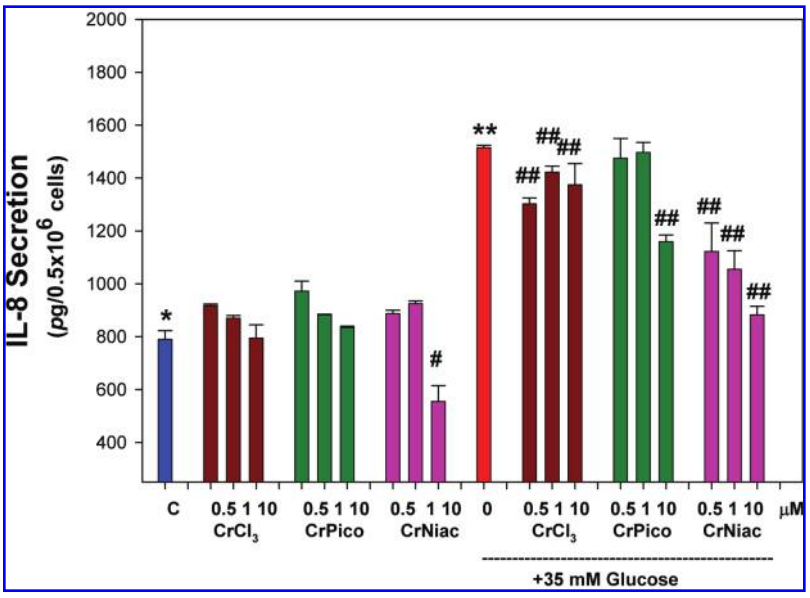

FIG. 3. Effect of different forms of chromium supplementation on IL-8 secretion in high glucose-treated activated monocytes. Values obtained with only LPS-activated cells were considered as controls. Values are mean ± SE ($n = 6$). Differences between * *vs.* # and ** *vs.* ## are significant ($p < 0.05$).

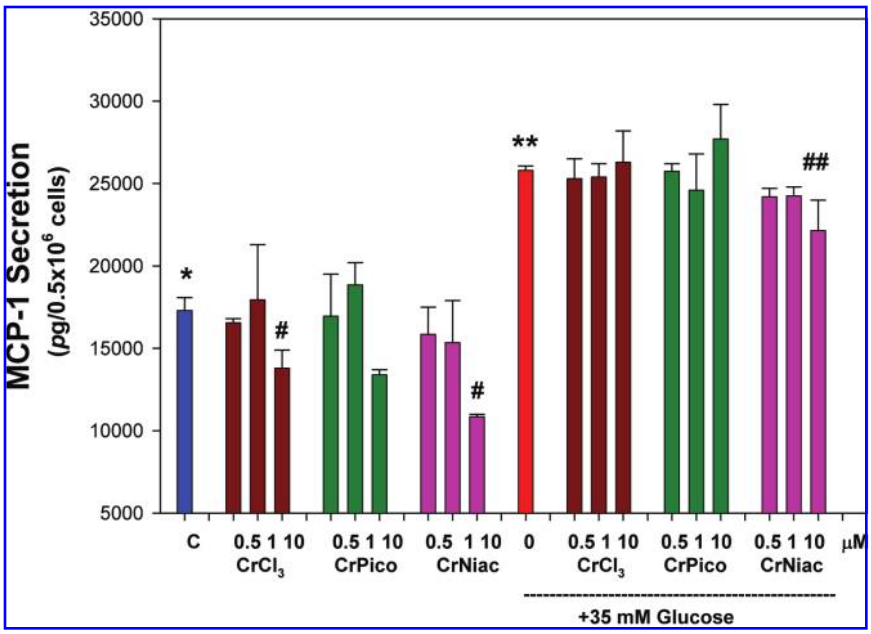

FIG. 4. Effect of different forms of chromium supplementation on MCP-1 secretion in high glucose-treated activated monocytes. Values obtained with only LPS-activated cells were considered as controls. Values are mean ± SE ($n = 6$). Differences between * *vs*. # and ** *vs*. ## are significant ($p < 0.05$).

after the addition of working chromium solution at all of the concentrations used in this study. Mannitol (35 m*M*) was used as an osmolarity control. For cytokine secretion studies, cells were treated with lipopolysaccharide (LPS, 2 μg/ml) at 37°C for 24 h. Values obtained with cells incubated with LPS alone were considered as controls. All experiments were repeated at least four times.

In this study, cells were exposed to a high glucose concentration of 35 m*M* to mimic diabetic conditions. Many previous studies have reported that glucose concentrations as high as 50 m*M* have been found in the blood of uncontrolled diabetic patients (13, 40, 44). It is true that blood glucose levels in patients are not likely to stay as high as 35 m*M* or hyperketonemic for 24 h. However, tissue damage in diabetic patients occurs over many years of countless hyperglycemic and/or ketotic episodes. Thus, the glucose concentration of 35 m*M* used in this cell culture study and by other investigators (47) does not seem unreasonable.

Cytokine secretion, cell viability, and lipid peroxidation

IL-6, IL-8, and MCP-1 levels in the supernatant of treated cells were determined by the sandwich ELISA method using a commercially available kit from Pierce–Endogen (Rockford, IL). All appropriate controls and standards as specified by the manufacturer's kit were used; the data are expressed as pg per ml supernatant. In the cytokine assay, control sam-

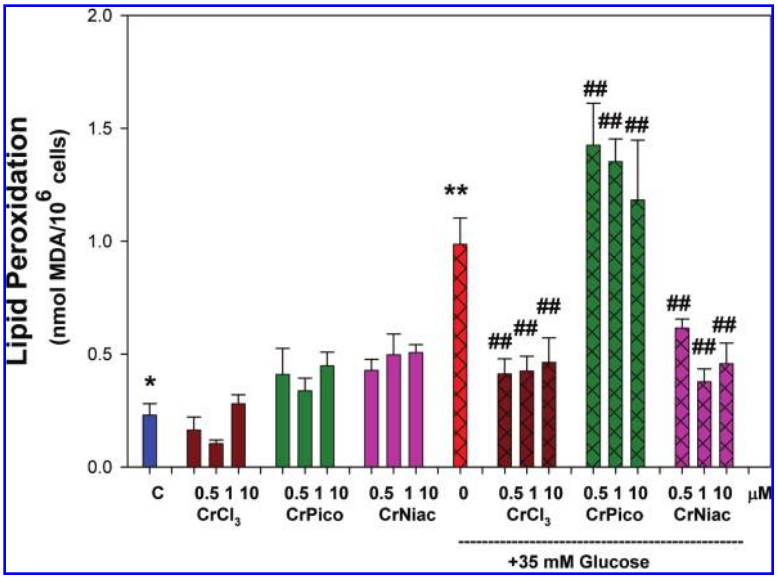

FIG. 5. Effect of different forms of chromium supplementation on lipid peroxidation in high glucose-treated activated monocytes. Values are mean ± SE ($n = 6$). Differences between * *vs*. # and ** *vs*. ## are significant ($p < 0.05$).

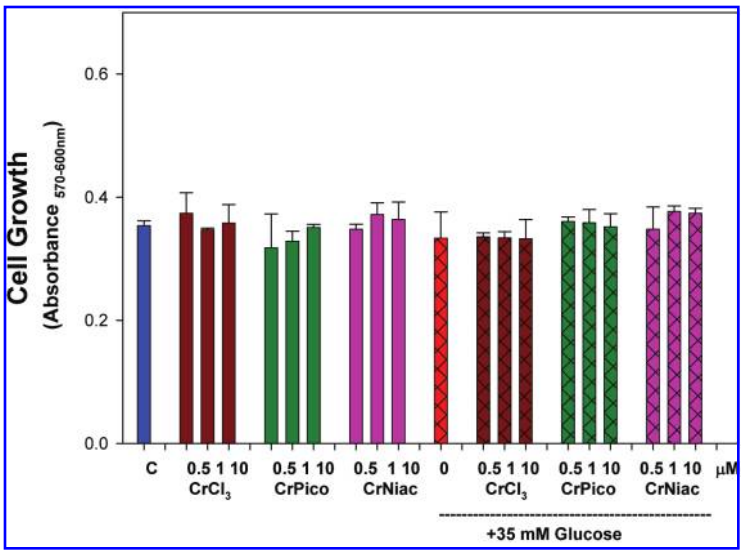

FIG. 6. Cell growth of monocytes exposed to different forms of chromium and HG. Values are mean ± SE ($n = 6$). There was no difference in cell growth between the treatments.

ples were analyzed each time to check the variation from plate to plate on different days of analyses. Cell viability was determined using the Alamar Blue reduction bioassay (Alamar Biosciences, Sacramento, CA). This method is based upon Alamar Blue dye reduction by live cells (3). Oxidative stress was determined by measuring malondialdehyde (an end product of lipid peroxidation) by its reaction with thiobarbituric acid (20, 31).

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise mentioned. Working solutions of glucose, ketones, mannitol, and chromium compounds were made sterile by filtering through 0.2 micron filters (Pall Corporation, Ann Arbor, MI). Data were analyzed statistically using the unpaired Student's *t* test between different groups with Sigma Plot statistical software (Jandel Scientific, San Rafael, CA). A p value of <0.05 was considered significant.

RESULTS

Figure 1 shows the effect of different concentrations of glucose on secretion of IL-6, IL-8, and MCP-1 in monocytes. The secretion of all three cytokines increased with increasing concentrations of glucose. Mannitol (35 m*M*) did not cause any increase in cytokine secretion in comparison with the respective controls. Figure 2 illustrates the effect of chromium supplementation on IL-6 secretion by monocytes exposed to high glucose. This shows stimulation of IL-6 secretion as a result of treatment with HG concentrations was reduced in cells pretreated with chromium. The inhibitory effect of chromium on IL-6 secretion was concentration dependent. In addition, among the three forms of chromium used, the greatest inhibitory effect was observed in cells supplemented with the niacinate form of chromium.

Figure 3 illustrates the effect of different forms of chromium supplementation on IL-8 secretion in HG-treated monocytes.

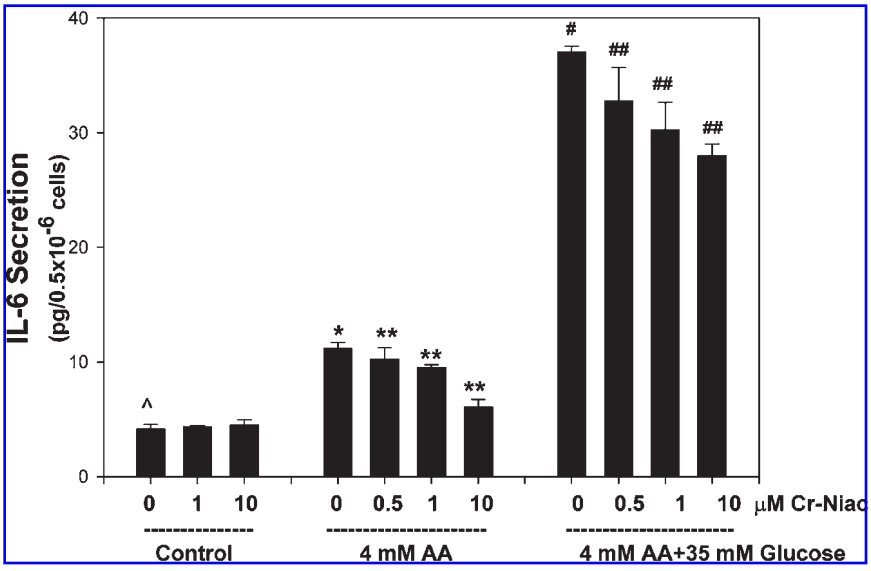

FIG. 7. Effect of chromium niacinate on IL-6 secretion in activated monocytes treated with AA + HG. Values obtained with only LPS-activated cells were considered as controls. Values are mean ± SE ($n = 4$). Differences between ^ *vs.* * and #; * *vs.* **; and # *vs.* ## are significant ($p < 0.05$).

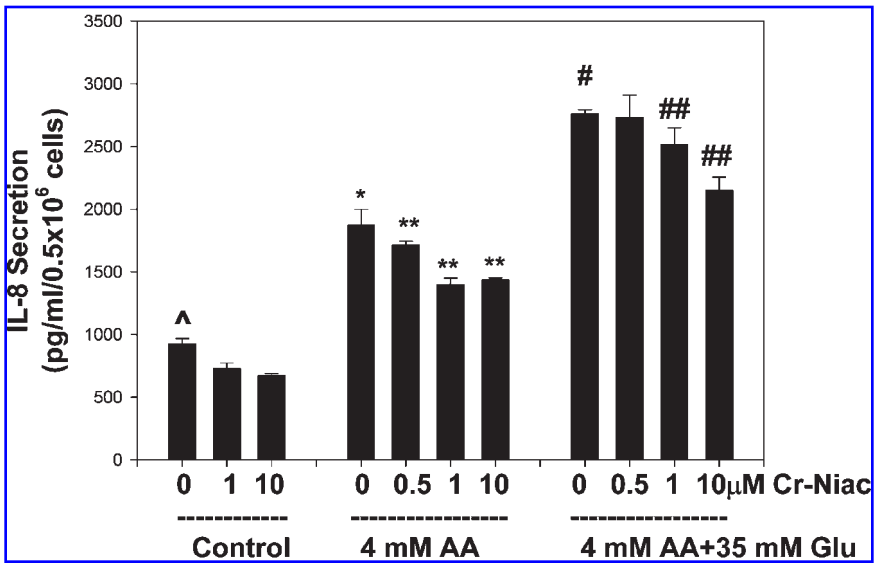

FIG. 8. Effect of chromium niacinate on IL-8 secretion in activated monocytes treated with AA + HG. Values obtained with only LPS-activated cells were considered as controls. Values are mean ± SE ($n = 4$). Differences between ^ *vs.* * and #; * *vs.* **; # *vs.* ## are significant ($p < 0.05$).

HG treatment resulted in stimulation of IL-8 secretion by monocytes, which was inhibited in a concentration-dependent manner by chromium niacinate. There was a modest decrease in IL-8 in the presence of chromium chloride and high concentrations of chromium picolinate. The effect of different forms of chromium supplementation on MCP-1 secretion in HG-treated monocytes is given in Fig. 4, which shows a significant HG-induced stimulation of MCP-1 secretion. However, neither chromium chloride nor chromium picolinate at the concentrations used showed any effect on MCP-1 secretion. Chromium niacinate affected MCP-1 secretion only at high concentrations (10 μM).

Figure 5 shows that lipid peroxidation levels were significantly higher in HG-treated monocytes compared with controls. Chromium niacinate and chromium chloride supplemented cells showed a significant reduction in lipid peroxidation compared with cells that did not receive chromium supplementation. On the other hand, chromium picolinate-supplemented cells showed an increase in lipid peroxidation levels.

Figure 6 shows that supplementation with any form of chromium had no effect on cell growth. This suggests that reductions in cytokine secretion were not due to any reduction in cell viability. Mannitol (35 mM) had no effect on secretion of IL-6, IL-8, or MCP-1, cell viability, or lipid peroxidation in comparison with the respective controls (data not given here).

As the abovementioned experiments with HG showed that chromium niacinate caused the greatest reduction in the secretion of cytokines, subsequent experiments with AA+HG focused only on the effect of chromium niacinate supplementation. Figure 7 shows that AA alone or AA+HG induces IL-6 secretion in monocytes. This effect of AA or AA+HG was reduced by chromium niacinate. The greatest reduction was seen at the highest concentrations of chromium used. Similarly, Figs.

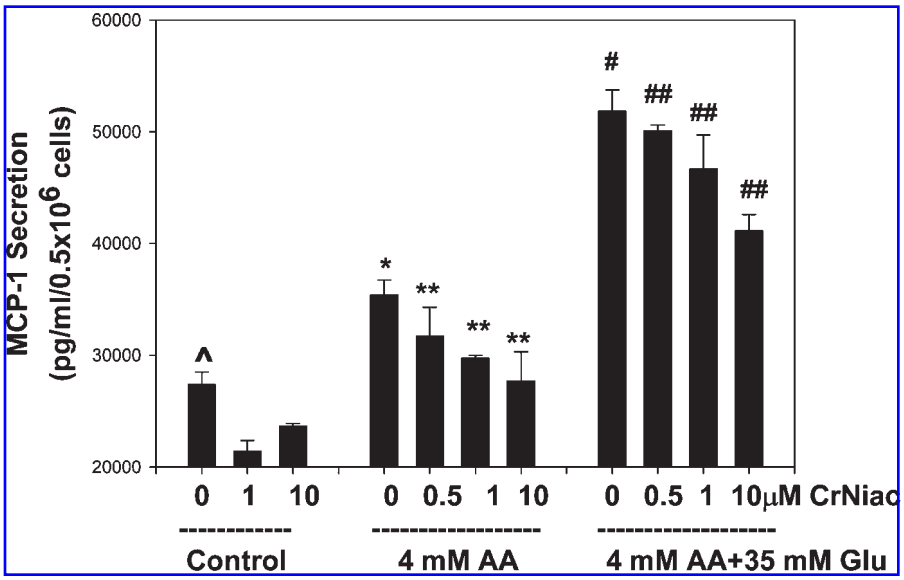

FIG. 9. Effect of chromium niacinate on MCP-1 secretion in activated monocytes treated with AA+HG monocytes. Values obtained with only LPS-activated cells were considered as controls. Values are mean ± SE ($n = 4$). Differences between ^ *vs.* * and #; * *vs.* **; # *vs.* ## are significant ($p < 0.05$).

TABLE 1. EFFECT OF CHROMIUM NIACINATE ON HYDROGEN PEROXIDE-INDUCED LIPID PEROXIDATION AND IL-6, IL-8, AND MCP-1 SECRETION IN LPS-ACTIVATED MONOCYTES

Treatment	*Lipid peroxidation (nmol MDA/10^5/cells)*	*IL-6 (pg/ml)*	*IL-8 (pg/ml)*	*MCP-1 (pg/ml)*
Control	1.29 ± 0.14*	7.76 ± 0.37*	1,301 ± 55*	13,575 ± 357*
+ Cr-N (1 μM)	1.52 ± 0.09§	8.47 ± 0.36	1,275 ± 79§	11,600 ± 600
+ H_2O_2 (40 μM)	1.79 ± 0.05†	14.05 ± 0.53†	2,048 ± 169†	16,566 ± 1146†
+ H_2O_2 + CrN	0.26 ± 0.07‡	8.88 ± 0.38	1,721 ± 58‡	11,533 ± 674‡

Values are mean ± SE ($n = 3$). Values obtained with only LPS-activated cells were considered as controls. Cr-N, chromium niacinate; MDA, malondialdehyde (a product of lipid peroxidation). Differences between values marked * *vs.* †, † *vs.* ‡, § *vs.* ‡, are significant. ($p < 0.05$).

8 and 9 show significant reduction in IL-8 and MCP-1 secretion as a result of chromium niacinate supplementation in monocytes exposed to AA and HG. Figures 7, 8, and 9 do not show values for HG alone because it is given in previous figures. In general, combination of HG and AA resulted in an additive effect on cytokine secretion (data not given here).

The effect of chromium niacinate treatment on lipid peroxidation and IL-6, IL-8, and MCP-1 secretion in peroxide-treated monocytes is given in Table I. This shows that chromium niacinate supplementation significantly reduced both lipid peroxidation and proinflammatory cytokine secretion in cells treated with the standard oxidant hydrogen peroxide. The effect of chromium niacinate was observed at 0.5 and 1 μM (data given here only with 1 μM).

DISCUSSION

Vascular inflammation and CVD are the leading causes of morbidity and mortality in the diabetic population and remain major public health issues. The risk of CVD is 3–4 times greater in subjects with Type 1 diabetes in comparison with the normal population (54). Diabetes is treated with diet, hypoglycemic drugs, and insulin administration. Intensive blood glucose control dramatically reduces the devastating complications that result from poorly controlled diabetes (52, 59). However, for many patients, achieving tight glucose control is difficult with current regimens. Thus, any adjuvant therapy that can increase insulin sensitivity, help control glycemia, and reduce vascular inflammation could significantly improve the care of diabetic patients.

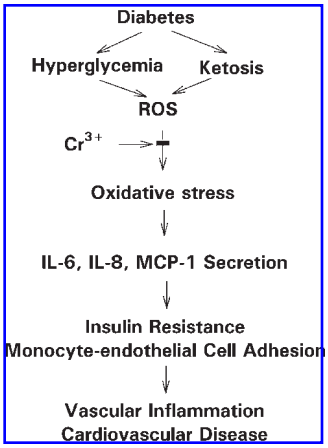

FIG. 10. Proposed model for the potential benefits of chromium niacinate supplementation to combat vascular disease in diabetes.

Trivalent chromium, the reduced form of the element, is an essential nutrient required for glucose and lipid metabolism (17, 61). No previous studies have examined the effect of trivalent chromium supplementation on blood levels of proinflammatory cytokines in diabetic patients or animals. This study demonstrates that trivalent chromium inhibits IL-6, IL-8, and MCP-1 secretion caused by HG treatment in U937 monocytes. Among the three forms of chromium used in cell culture studies, it appears that niacin-bound chromium was most effective in inhibiting cytokine secretion compared with chromium picolinate or chromium chloride. This study also found that chromium chloride and chromium niacinate completely prevented the lipid peroxidation induced by HG. In contrast, chromium picolinate significantly increased the lipid peroxidation induced by HG. This is consistent with previous studies on increased hydroxyl radical production and toxicity (55, 61, 64), DNA damage (64) and increases in lipid peroxidation on chromium picolinate-treatment (24). Previous studies have also reported that, in contrast to chromium picolinate, chromium niacinate at similar concentrations is not toxic in cell culture studies (19, 56). However, other studies by Slesinsky *et al.* showed that chromium picolinate was not toxic (9, 53). This study demonstrates that Cr^{3+} inhibits the increases in pro-inflammatory cytokines and oxidative stress levels caused by HG and AA in cultured monocytes.

The role of IL-6 in vascular inflammation has been shown in studies using IL-6 knockout mice who exhibit resistance to splanchnic artery occlusion shock, and anti-IL-6 therapy significantly prevents the inflammatory process in mice (18), as well as in studies that show increased levels of lipid peroxidation and inflammation in mice that overexpress IL-6 (12). Circulating IL-6, IL-8, and MCP-1 levels are increased in insulin-resistant states such as obesity (11), impaired glucose tolerance, and Types 1 and 2 diabetes (27, 33, 43). Studies using knockout mice lacking monocyte chemoattractant protein-1 (MCP-1) or interleukin-8 (IL-8) or their corresponding receptors show a significantly reduced progression of atherosclerosis (63). MCP-

1 and IL-8 have been reported to trigger the firm adhesion of monocytes to vascular endothelium under flow conditions (21). Recent studies have shown that overexpression of MCP-1 causes inhibition of AKT and tyrosine phosphorylation in liver and skeletal muscle, macrophage recruitment, and insulin resistance in aP2–MCP-1 mice (34). Similarly, lack of C-C motif chemokine receptor-2 (CCR2), a receptor for MCP-1, influenced insulin resistance in mice (63). IL-8 and MCP-1 play an important role in the vascular inflammation process through its multiple actions, including recruitment of neutrophils and T lymphocytes into the subendothelial space, monocyte adhesion to endothelium, and migration of vascular smooth muscle cells. Trivalent chromium supplementation can reduce elevated cholesterol and triglycerides in a dose-dependent relationship in an atherosclerotic rabbit model (2). The decrease in IL-6, IL-8, and MCP-1 secretion caused by chromium niacinate supplementation in cultured monocytes is novel.

Traditionally, clinical practice has considered hypertketonemia to be present only in Type 1 diabetic patients. However, hypeketonemia is increasingly being identified in Type 2 diabetic patients (46). Newer data indicate that hyperketonemia co-exists with hyperglycemia in ~30% of Type 2 patients, especially among older diabetic patients (65). A higher incidence of ketoacidosis has also been reported in African Americans and other minority groups with Type 2 diabetes (60). The present data, which show an inhibitory effect of chromium on the pro-inflammatory cytokine secretion caused by AA and HG in a cell culture model, indicate that trivalent chromium supplementation may be beneficial not only in Type 2 but also Type 1 diabetes.

HG can also upregulate expression of transcription factors, such as NFkB and the TNF-α gene in monocytes (23). HG and AA can result in increased oxidative stress from excessive oxygen radical production (10, 28, 32, 45). Oxidative stress can also influence the expression of multiple genes in vascular cells, including signaling molecules such as PKC, NFkB, and ERK (10, 23, 66); overexpression of these genes stimulates the secretion of proinflammatory cytokines, such as IL-6 and IL-8. Oxidative stress plays a key role in the regulatory pathway that progresses from elevated glucose to monocyte and endothelial cell activation in the enhanced vascular inflammation of diabetes. The present study demonstrates that Cr^{3+} supplementation results in a significant reduction of both oxidative stress and proinflammatory cytokine secretion in peroxide-treated monocytes. This suggests that the effect of Cr^{3+} on inhibition of HG and acetoacetate-induced stimulation of pro-inflammatory cytokines may in part be mediated by the antioxidative effect of chromium (29). However, the precise mechanism by which Cr^{3+} decreases oxidative stress is not known. Other investigators have reported that Cr^{3+}supplementation lower the blood levels of oxidative stress markers in an animal model as well as in diabetic patients (5, 8, 62). Investigations are needed to understand the molecular mechanisms by which chromium can affect proinflammatory cytokine secretion and vascular inflammation. Chromium levels of up to 0.6 μM have been reported in the blood of normal subjects (17). Therefore, the chromium concentration of ~0.5–1 μM used in this study falls within a normal physiological range; the actual chromium content in the cells after the chromium treatment was not determined. Whether the different forms of chromium being used in this study lead to similar or different amounts of chromium uptake in these monocytes is also not known.

In conclusion, Fig. 10 illustrates that hyperglycemia and ketosis can increase oxidative stress in diabetes. Trivalent chromium supplementation has the potential to decrease cellular oxidative stress and lower the secretion of proinflammatory cytokines. In addition, these results show that chromium niacinate appears to be the most effective form of chromium in inhibiting oxidative stress and proinflammatory cytokine secretion in this cell culture model. The evidence that trivalent chromium can inhibit markers of vascular inflammation needs to be explored at the clinical level to see whether widely used supplements such as chromium picolinate or chromium niacinate can lower levels of oxidative stress and proinflammatory cytokines in the diabetic patient population. If so, then chromium supplementation may be used as an adjuvant therapy for reduction of vascular inflammation and CVD in diabetes.

ACKNOWLEDGMENTS

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ABBREVIATIONS

AA, acetoacetate; Cr-N, chromium niacinate; Cr-P, chromium picolinate; CVD, cardiovascular disease; HG, high glucose; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1.

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