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Evidence that chromium is an essential factor for biological activity of low-molecular-weight, chromium-binding substance

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Biologically active Low-molecular-weight, chromium-binding substance (LMCr) has been isolated from the liver of rabbits injected with potassium bicarbonate and cow's milk. This substance enhances glucose oxidation and lipogenesis from glucose in rat adipocytes [Yamamoto A. et al. (1987) Eur. J. Biochem. 165, 627-631; (1988) J. Nut. 118, 39-45]. LMCr was shown to lose its activity almost completely as an effectant of glucose metabolism when Cr was deleted under acidic conditions and separated from LMCr by EDTA-chelation and successive molecular-sieve chromatography. Reincorporation of Cr^{3+} into apo-LMCr resulted in 50-90% recovery of its original activity. These findings suggest that the biological activity of LMCr resides in Cr.

Low-molecular-weight, chromium-binding substance (LMCr) is a trivalent chromium compound, occurring naturally and ubiqitously in mammalian organs. The substance was first noticed as a detoxifying ligand of exogenous chromium and was subsequently isolated from the liver of rabbits injected with potassium dichromate [1-3]. We have purified the substance partially, and shown it to be an aspartate- and glutamate-rich chromium-associated complex with an approximate molecular weight of 1500 [4, 5]. LMCr was found to enhance both [^{14}C]CO_2 production from [U-^{14}C]glucose and the conversion of [3-^{3}H]glucose into lipid in

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Abbreviation : LMCr, low-molecular-weight, chromium-binding substance.

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rat adipocytes in association with the action of insulin, in a manner highly similar to that of glucose tolerance factor(GTF) obtained from yeast [6,7]. These findings suggested that LMCr might play a significant role in both chromium nutrition and detoxification. In order to establish the role of chromium within LMCr for the compound's biological activity, chromium was removed from LMCr, and the effects of the resulting apo-LMCr on glucose metabolism were examined.

Materials and Methods

Analytical-grade chemicals and ion-free water were used throughout. The solutions used for purification of LMCr and depletion of Cr were passed through a column of Chelex-100 resin (Bio-Rad Lab. Richmond, CA) to remove all traces of Cr and other contaminating metal ions before the experiments. Chromium content was measured by atomic absorption spectrometry (Hitachi graphite furnace atomizer 180-30, GA2B, Tokyo, Japan).

LMCr was extracted and partially purified from the liver of a male rabbit 3 h after intravenous administration of $K_2Cr_4O_7$, as described previously [4]. The purified LMCr was used as a starting material for Cr-depletion, immediately or within 2 weeks of storage at -20°C after preparation, to minimize any loss of activity.

Bioassay of LMCr was carried out using rat adipocytes essentially as described in the previous report [8]. Briefly, rat adipocytes (4×10^4 cells/ml) were incubated for 90 min with 0.2 μCi each of [U-^{14}C]glucose and [3-^{3}H]glucose and insulin (2.5 μU/ml) with or without LMCr in Krebs-Ringer bicarbonate buffer containing 0.2% albumin and 0.5 mM glucose. The [^{14}C]CO_2 thus formed was trapped on filter paper soaked with hyamine hydroxide, then ^{3}H-labeled lipid was extracted using a scintillant. Radioactivity was assayed using a Packard model 703 liquid scintillation system enploying a toluene-based scintillator.

Results and Discussion

Preparation of Cr-depleted LMCr

Purified LMCr (2.3 μg Cr) was treated with 3.5 μmol of an aqueous solution of EDTA (total volume, 1 ml) in a Pyrex tube (10 x 60 mm), and the pH adjusted to 3.0 with HCl. Incubation was performed for 60 min with a reflux condenser on a block-bath at a controlled temperature of 110°C. The reaction mixture was then cooled, and the resulting apo-LMCr was separated from the Cr freed from LMCr on a Sephadex G-15 column using 50 mM ammonium acetate buffer, pH 6.5 (Fig. 1b). For the control experiment, LMCr was treated with HCl as above, except that EDTA was omitted and the pH was adjusted to 6.5 with 0.1 N NaOH prior to loading on the column (Fig. 1a). Pooled fractions, A and B (each of fractions No. 20-25 in Figs. 1, a and b) were dissolved in 1

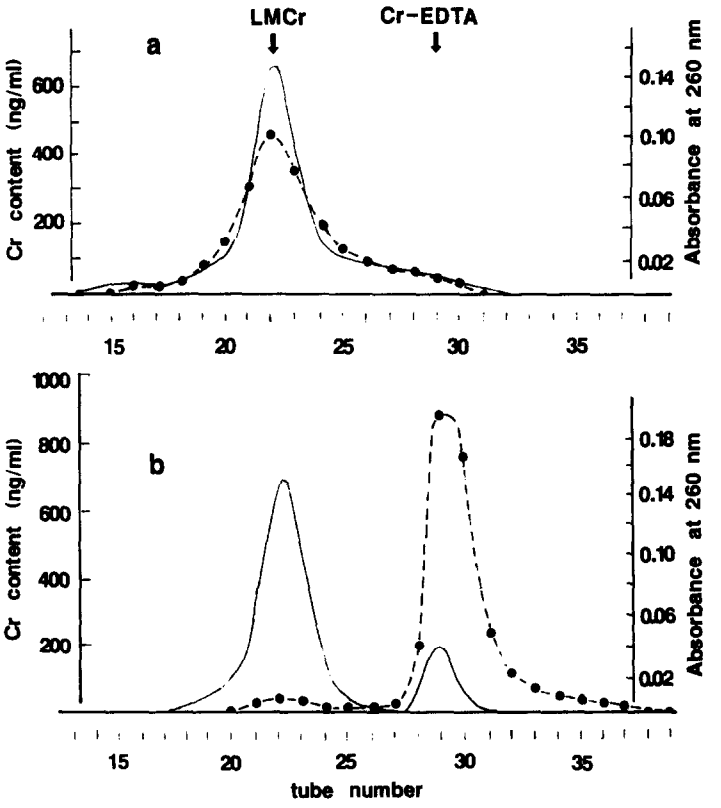

Fig. 1 Separation of Cr-deprived LMCr on Sephadex G-15 chromatography. LMCr treated with HCl (a) or HCl and EDTA (b) at pH3.0 (see text) was applied to a column (1.0x40 cm) and eluted with 50 mM ammonium acetate buffer, pH6.5,(3ml/hr). One ml of fraction was collected. Every fractions No.20-25 were pooled, lyophilized and served for the rat adipocyte assay as control LMCr (fraction A) or Cr- deprived-LMCr (fraction B). Cr content (- -) and absorbance at 260 nm(--) profiles are shown.

ml of distilled water after freeze-drying, and used as LMCr and apo-LMCr, respectively. Fraction A contained 1.92 μg of Cr, while fraction B retained only 0.22 μg of Cr. Since the recoveries of Cr from the molecular-sieve column chromatography were 92.4% and 93.7% for fractions A and B, respectively, it was calculated that 89% of Cr within the applied LMCr was depleted. Cr-EDTA thus formed was eluted in fractions No. 28-30, appearing reddish-purple under the conditions used.

Biological activity of Cr-depleted LMCr

Under the conditions used (see Materials and Methods), insulin (2.5 μU/ml) stimulated the rates of both glucose oxidation and incorporation into lipids four- to five- fold. LMCr enhanced the rates of glucose conversion in both cases by about 21% more than the values achieved with insulin alone, at a

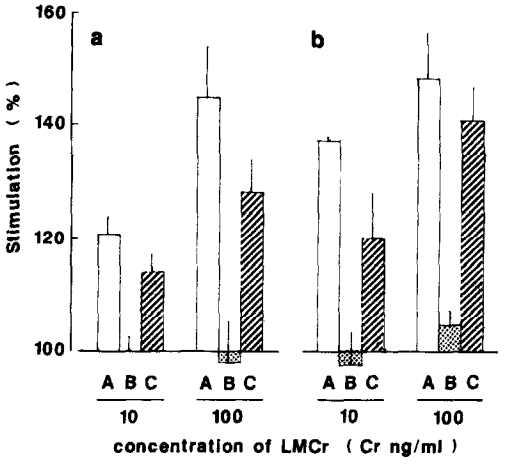

Fig. 2 Chromium dependency on the effects of LMCr on ^{14}CO production from [U-^{14}C]glucose (a) and [3-^{3}H]glucose incorporation into lipids by rat adipocytes (b). In the presence of insulin (2.5μU/ml), the cells were incubated with LMCr (fraction A from Fig. 1a) (A), Cr-deprived-LMCr (fraction B from Fig. 1b) (B) or Cr-rebind-LMCr (see in materials and Method) (C), at equivalent amounts of ligand to a concentration of Cr as indicated. The results are expressed as percentages over the control values in the absence of LMCrin each experiment and are means + SD for three or more determinations. The basal values per $4x10^4$ cells with insulin were (185.8 ± 3.5) $x10^2$ dpm and (229.1±14)$x10^2$ dpm for b, respectively. Similar results were obtained in three separate experiments.

Cr concentration of 10 ng/ml. At a Cr concentration of 100 ng/ml, 43% and 50% stimulation was observed for CO_2 production and glucose incorporation into lipid, respectively, compared to the values obtained in the presence of insulin alone (Fig. 2, a and b, column A in each). However, no stimulation was observed when Cr-depleted LMCr was added to the reaction mixtures instead of LMCr, at a concentration equivalent to LMCr, Cr 10 ng/ml (Fig. 2, a and b, column B in each). A higher concentration of apo-LMCr (equivalent to 100 ng Cr/ml) stimulated the rates of both glucose oxidation and incorporation into lipid. This might be explained as being at least partly due to Cr retained in the apo-LMCr fraction as a form of LMCr.

Prior to the addition of rat adipocytes, addition of $CrCl_3$ to the reaction mixture containing apo-LMCr, and re-binding of Cr to apo-LMCr by incubation of the mixture at 37°C for 60 min [3] resulted in increased rates of glucose conversion (Fig. 2 a and b, column C in each). The stimulation rates obtained were close to those shown with the LMCr at concentrations equivalent to the Cr-depleted LMCr used. With regard to the Cr concentrations added, at lower concentrations, restoration rates of CO_2

production and lipogenesis were much higher. This might have been due to a difference in the rate of Cr reincorporation into LMCr.

$CrCl_3$ has no effect on glucose incorporation at concentrations below 1 mg Cr/ml [9]. Cr-EDTA and EDTA were also found to be ineffective (data not shown). From these facts, it is concluded that the stimulation of glucose metabolism by LMCr depends on the concentration of Cr binding to LMCr. Apo-LMCr, which has lost Cr, no longer shows biological activity, unless Cr is reintroduced into the molecule. Clarification of the chemical structure of this substance will be required in order to obtain further information on its relationship with cell metabolism.

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