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Carcinogenic Chromium(VI) Compounds Formed by Intracellular Oxidation of Chromium(III) Dietary Supplements by Adipocytes

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Abstract: *Chromium(III) nutritional supplements are widely consumed for their purported antidiabetic activities. X-ray fluorescence microscopy (XFM) and X-ray absorption near-edge structure (XANES) studies have now shown that non-toxic doses of $[Cr_3O(OCOEt)_6(OH_2)_3]^+$ (**A**), a prospective antidiabetic drug that undergoes similar H_2O_2 induced oxidation reactions in the blood as other Cr supplements, was also oxidized to carcinogenic Cr^{VI} and Cr^{V} in living cells. Single adipocytes treated with **A** had approximately 1 μm large Cr hotspots containing Cr^{III}, Cr^{V}, and Cr^{VI} (primarily Cr^{VI} thiolates) species. These results strongly support the hypothesis that the antidiabetic activity of Cr^{III} and the carcinogenicity of Cr^{VI} compounds arise from similar mechanisms involving highly reactive Cr^{VI} and Cr^{V} intermediates, and highlight concerns over the safety of Cr^{III} nutritional supplements.*

Chromium(III) supplements are widely consumed for the purported treatment of metabolic disorders, such as insulin resistance and type 2 diabetes.[1] However, controversy exists about both the essentiality of Cr^{III} for humans, and the efficacy and safety of Cr^{III} supplements.[2–5] $[Cr^{III}_3O(OCOEt)_3(OH_2)_3]^+$ (**A** in Figure 1)[6] was proposed as a structural and functional model of the poorly defined Cr^{III} binding peptide, chromodulin, and as an antidiabetic drug.[7] Conversely, we found that chromodulin was an artefact of isolation,[8] and that the biological activity of **A** and other Cr^{III} supplements involved oxidation to genotoxic Cr^{V} and Cr^{VI} in the blood under biologically relevant conditions of oxidative stress.[4,9] We proposed[4] that the insulin-enhancing activities of Cr^{VI} and Cr^{V} have similar mechanisms to those of antidiabetic V^{V}/V^{IV} complexes,[10] namely reversible and/or irreversible binding to cysteines at the active sites of protein tyrosine phosphatases (PTPs) to enhance the insulin signaling cascade.[4,9] The hypothesis that the genotoxicity and carcinogenicity of Cr^{VI}[11] and the controversial antidiabetic activity of Cr^{III}[2—5,12] are based on similar reactive intermediates[4,9] raises safety concerns over Cr^{III} nutritional supplements,[2–4] but evidence for Cr^{VI} in insulin-sensitive cells has not been reported. Herein, we used X-ray fluorescence microscopy (XFM) elemental mapping of single chromium-treated 3T3-L1 adipocytes at submicrometer resolution in combination with microfocus X-ray absorption near-edge structure (μ-XANES) analysis[13–15] of micrometer-sized Cr hotspots to show directly that intracellular oxidation of Cr^{III} does occur.

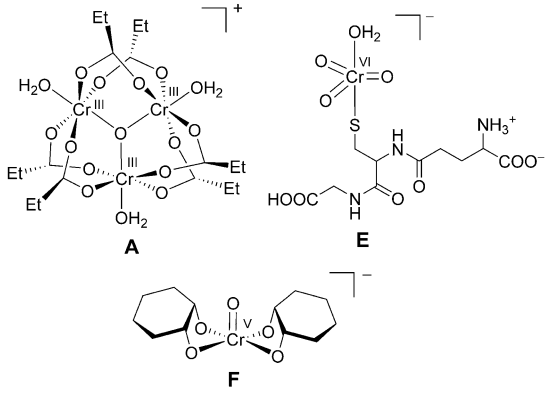

Figure 1. Structures of **A**[6] and the model Cr^{VI} (**E**)[19] and Cr^{V} (**F**)[21] complexes used for XANES data fitting (Figure 3 and Figure 4).

Adipocytes grown on Si_3N_4 substrates[15e] were treated with **A** (100 μM, 20 h, 310 K), then fixed (methanol for ca. 5 s, 253 K), and dried in air. XFM and XANES data were collected at beamline 2-ID-D of the Advanced Photon Source (see the Supporting Information). XFM maps of mature cells showed a relatively low Cr background, punctuated by approximately 1 μm sized hotspots of high Cr intensity (Figure 2; see also the Supporting Information, Figure S1). The maximal density of Cr in the hotspots was 0.17 $\mu g\,cm^{-2}$. By contrast, untreated control samples (Figure S2) showed low background Cr levels (< 0.01 $\mu g\,cm^{-2}$) and no Cr hotspots. Chromium K-edge XANES spectra (spot size: 1×1 μm^2; energy range: 5950–6050 eV; step size: 0.25 eV)[8,9c,15c] were

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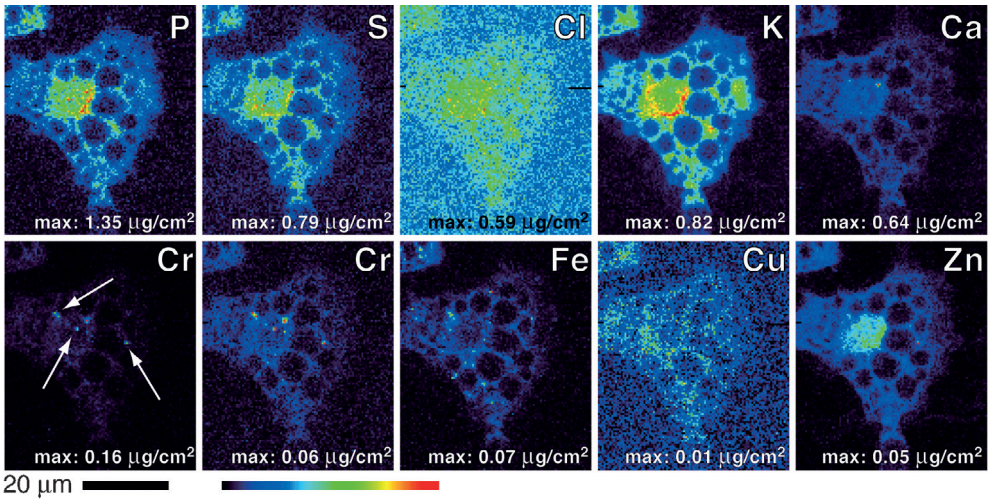

Figure 2. XFM elemental maps (295 K, He atmosphere) of a Cr^{III}-treated (100 μM **A**, 20 h at 310 K) adipocyte with Cr punctate structures (arrows) of unknown identity (maximum concentrations, $\mu g\,cm^{-2}$). A second Cr map is shown with the maximum scaled to 40% to show low-concentration features. The "holes" are X-ray-transparent fat globules that were observed under a microscope.

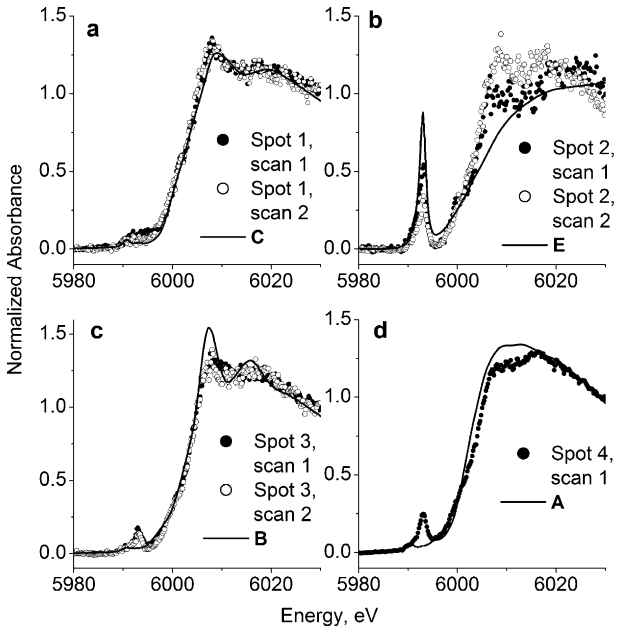

Figure 3. Splined and normalized[17,18] XANES spectra of Cr hotspots ($1\times1\ \mu m^2$, Figure 2, 295 K) in Cr^{III}-treated adipocytes compared with data[8,19] for typical model Cr^{III} and Cr^{VI} complexes (Table 1 and Figure 1). For a color version, see Figure S5.

collected on Cr hotspots from different Cr^{III}-treated cells. The samples were scanned repeatedly to check for photodamage.[16] Figure 3 shows splined and normalized[14,17,18] XANES spectra for single cells, and published[8,19] XANES data for model Cr^{III} and Cr^{VI} complexes (Table 1). All XANES spectra from hotspots had pre-edge bands (symmetry-forbidden $1s\rightarrow3d$ transitions)[14a] that were more intense than those of octahedral Cr^{III} complexes (Figure 3). This finding unambiguously confirmed the presence of high oxidation states of Cr ($Cr^{VI}/Cr^{V}/Cr^{IV}$).[14a] The decrease in the pre-edge peak intensities in the second scans (Figure 3a–c) showed X-ray photoreduction of higher oxidation states,[16] and excluded photooxidation of Cr^{III}. Hence, the initial levels of Cr^{VI} and Cr^{V} in the hotspots were higher than those measured.

XANES[8] data from Cr hotspots were fitted to a XANES library of biologically relevant Cr complexes (Table 1).[8,9c,19–24] The best fits (Figure 4; Table S1, Figure S4) included XANES spectra of **A**, its likely hydrolysis products (the Cr^{III} aqua and hydroxido complexes **B** and **C**),[8,9c] and Cr^{III} cysteinato complex **D**.[25] The XANES spectra of other Cr^{III} complexes with amino acid ligands were rejected computationally. The best fits for all single-cell XANES analyses, except for the second scan at spot 1 (Figure 3a), had significant contributions (8–60%, see Table S1) corresponding to the XANES spectrum of a five-coordinate Cr^{VI} glutathione complex (**E**; Table 1, Figure 4),[19] whereas XANES spectra from chromate species[19] were rejected during the fits. The XANES spectrum of a Cr^{V} complex with 1,2-diolato ligands (**F**)[21] contributed only slightly ($\leq10\%$, Table S1) to the best fits for spots 3 and 4 (Figure 4 and Table S1). Complex **F** serves as a model of Cr^{V} sugar complexes[26] that have been observed in Cr^{VI}-treated

Table 1: Model Cr complexes used for the XANES fits.

Compound[a]	Ref.[b]	Fit[c]
$[Cr^{III}_3O(OCOEt)_6(OH_2)_3](NO_3)\cdot3H_2O$	[8]	**A**
$Na_9[Cr^{III}(OH)_6]_2(OH)_3\cdot6H_2O$	[8]	**B**
$[Cr^{III}(OH_2)_6](NO_3)_3\cdot3H_2O$	[8]	**C**
$Na[Cr^{III}(cys)_2]\cdot H_2O$	[8]	**D**
$Na[Cr^{VI}O_3(LH_4)(OH_2)]$	[19]	**E**
$Na_2Cr^{VI}O_4\cdot4H_2O$	[19]	–
$Na_3[Cr^{V}O(LH_2)_2]$	[20]	–
$K[Cr^{V}O(chd)_2]$	[21]	**F**
$Na[Cr^{V}O(ehba)]\cdot H_2O$	[22]	–
$[Cr^{IV}O(ehbaH)_2]$	[22]	–
$K[Cr^{V}O(bha)_2]\cdot Me_2CO$	[23]	–
$K_n[Cr(cat)_3]$ $(n=1–3)$[d]	[24]	–

[a] cys = L-cysteinato^{2-}, LH_5 = glutathione;, chd = 1,2-cyclohexanediolato^{2-}, ehba = 2-ethyl-2-hydroxybutanoato^{2-}, bha = benzhydroxamato^{2-}, cat = catecholato^{2-}. [b] References for XANES data. See Table S2 for the references for synthesis and characterization. Published XANES data were re-splined by the method of Penner-Hahn and co-workers[18] as described previously.[9c] [c] Designations of models **A**–**F** used in Figure 4; other model XANES data were rejected computationally.[8] [d] Electrochemically generated reduced and oxidized Cr tris(catecholato) complexes. Oxidation states are ambiguous because of the delocalization of electron density between the Cr center and the ligands.[24]

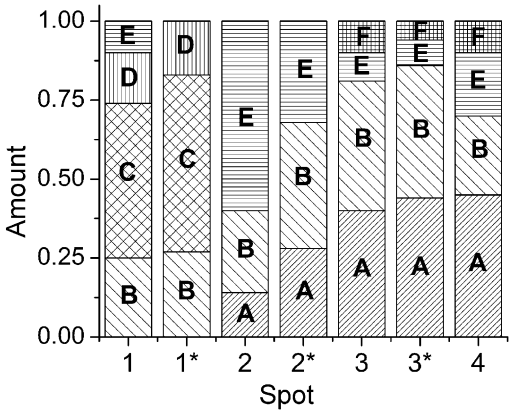

Figure 4. Summary of the best multiple linear regression fits[8,14a] of the XANES spectra from Cr hotspots in Cr^{III}-treated adipocytes (Figure 3; Table S1; Figure S4). The model structures **A**–**F** correspond to those in Table 1 and Figure 1. Asterisks designate a second scan at the same spot (Figure 3). For a color version, see Figure S6.

cells, plants, and animals by EPR spectroscopy.[8,27] Other biologically relevant Cr^{V}[20,22,23] and Cr^{IV} complexes with 2-hydroxycarboxylato[22] or catecholato[24] ligands were rejected computationally during the fitting (Table 1). The oxidation of Cr^{III} to Cr^{VI} in individual adipocytes (Figure 3) is in marked contrast to the reduction of Cr^{VI} to Cr^{III} in other mammalian cells.[8,15a–d] Although intracellular environments are generally reducing, significant local concentrations of strong oxidants, such as H_2O_2, are formed during cell signaling, including insulin signaling,[28] which could be responsible for the observed oxidation of Cr^{III} to Cr^{V} and Cr^{VI} species.[4,9] The formation of Cr^{VI} thiolato species (modelled by compound **E**)[19] as the most abundant product of Cr^{III} oxidation in adipocytes (Figure 4) is consistent with the binding of Cr^{VI} to cysteine residues in the active centers of PTPs.[4,9] XANES fits (Figure 4) indicated the presence of significant amounts of **A**, despite its low stability in cell culture medium.[9c] These results may point to the rapid uptake of **A** by endocytosis,[29] which could explain the observed punctate Cr distributions in cells (Figures 2 and S1), but the nature of these structures is unclear. As observed previously,[30] there was punctate distribution of Fe (Figures 2, S1, and S2), possibly owing to transferrin-mediated uptake of Fe^{III} into endosomes. Deposits of xenobiotic elements in punctate areas have previously been observed in XFM studies of Cr^{VI}-treated cells,[15d] and with supraphysiological concentrations of Se^{IV}, Ti^{IV}, and V^{IV} species.[31,32] In spot 1 (Figures 3 and 4), **A** was replaced with Cr^{III} hydrolysis products (**B** and **C**)[8] and with a Cr^{III} cysteinato complex (**D**), which is a model of Cr^{VI} thiolato reduction products.[33] As spot 1 was more photoreduced than the other three spots (Figure 3), it is likely that the hydrolysis of **A** with formation of **B** and **C** was catalyzed by partial photoreduction of kinetically inert Cr^{III} to reactive Cr^{II} species.[34] Lower concentrations of hydrolysis product **B** were also present in other hotspots (Figure 4).

In summary, XFM and XANES data from chromium(III)-treated adipocytes provide strong support for the hypothesis that the antidiabetic activity of Cr^{III} complexes is based on the formation of reactive, and carcinogenic, Cr^{V} and Cr^{VI} intermediates.[4,9] This raises concern over the possible carcinogenicity of Cr^{III} compounds[2,35] and the risks of long-term Cr^{III} nutritional supplementation.[4] Although animal experiments have yet to provide conclusive evidence for Cr^{III} carcinogenicity,[11] these studies cannot be extrapolated to human exposure because of the long latency time of chromium-induced cancer in humans,[11] and the long-term exposure of patients with diabetes to the oxidative stress that facilitates Cr oxidation in both the blood[9] and cells. Animal studies that mimic long-term oxidative stress have yet to be conducted. In light of these findings, there is a need for epidemiological studies to ascertain whether Cr^{III} supplements alter cancer risk.

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