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Vanadyl as a catalyst of human lipoprotein oxidation

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Abstract

Lipoprotein oxidation, which is relevant to atherogenesis, can be induced by redox-active transition metals, such as copper. Vanadium is a metal usually used as vanadyl to improve metabolic control in diabetic patients; given its redox-active properties, we have investigated possible oxidative effects of the metal on lipoproteins from healthy and diabetic subjects. Beginning from 10 μM, vanadyl, but not vanadate, induced oxidation of the non-HDL fraction, which was inhibited by EDTA, butylated hydroxytoluene and Vitamins E and C, but not by mannitol, SOD and catalase. Differently from copper, vanadyl could oxidize directly lipoprotein lipids, although it showed a lower oxidant activity against critical tryptophan residues of the lipoprotein protein moiety. Moreover, the non-HDL fraction of diabetic patients was more susceptible to vanadyl-dependent oxidation than that of controls. Thus, vanadium, in its reduced form which may be used in humans, can oxidize the non-HDL fraction through oxidative effects exerted especially on lipoprotein lipids; the specific pro-oxidant activity of vanadyl is more evident with lipoproteins of diabetic patients. Given also the tissue accumulating capacity of vanadium conceivably in a reduced form, its prolonged administration to humans, especially to diabetic patients without adequate antioxidant supplementation, needs caution.

Keywords: Vanadium; Diabetes mellitus; Lipoprotein oxidation; Oxidative stress; Lipoperoxidation; Atherogenesis

1. Introduction

In recent years, evidence has accumulated indicating that oxidatively damaged lipoproteins may play a major role in atherogenic processes [1,2].

Redox-active transition metals, such as copper and iron, are capable of catalysing oxidant damage of biologically relevant molecules, including lipoproteins [1–4]. Vanadium is a catalytic metal, which has been reported to induce reactive oxygen species generation *in vitro*, as well as lipid peroxidation and oxidative damage in experimental models [4–7].

Given the redox-active properties of vanadium, it is possible that the metal could also foster oxidation of human lipoproteins. This appears a relevant aspect, considering that vanadium, usually as VS, may be administered to diabetic patients because of its insulin-like therapeutic properties [8,9]; moreover, VS is used to improve performances especially in weight-training athletes [10].

In the present paper, we have investigated whether vanadium, in the vanadyl and vanadate form, was capable of inducing human lipoprotein oxidation. The results show that the former can readily oxidize apo B-containing lipoproteins, especially those of diabetic patients, with direct oxidative effects exerted also on the particle lipid moiety.

2. Materials and methods

2.1. Materials

Reagents were from Sigma Aldrich, including the diagnostic kits for total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides and apo B; the commercial kit for

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Abbreviations: BHT, butylated hydroxytoluene; EDTA, ethylendiaminetetraacetic acid; SOD, superoxide dismutase; CD, conjugated dienes; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; FDPL, fluorescent damage products of lipid peroxidation; VS, vanadyl sulfate.

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phospholipid assay was from Roche Diagnostics. Solutions were prepared in bidistilled deionized water and buffers were stirred with Chelex 100 resin to prevent contamination by adventitious transition metals.

2.2. Lipoprotein isolation and oxidation

The non-high-density lipoprotein (non-HDL) fraction was obtained from EDTA plasma of healthy adults (age 35–65 years) as previously reported [11,12], using dextrane sulfate (MW 500,000) plus $MgCl_2$ to precipitate the fraction itself and remove EDTA. Briefly, 0.4 mL of precipitation reagent (formed by mixing equal volumes of 20 g/L solution of dextrane sulfate with 2 M $MgCl_2$) was added to 4.0 mL of twice diluted plasma, followed by vortexing and centrifugation at 1500 *g* for 10 min. In order to remove EDTA from the non-HDL fraction, the non-HDL pellet was suspended in 4.0 mL of 0.9% saline and reprecipitated by adding 0.2 mL precipitation reagent, vortexing and centrifuging [11,12]. The reprecipitated non-HDL pellet was dissolved in 4% saline, and aliquots of this concentrated non-HDL solution were then added to PBS, pH 7.4, for lipoprotein oxidation experiments. It is noteworthy that the non-HDL fraction contains both LDL and VLDL, which are apo B-containing lipoproteins with an intrinsic oxidizability and atherogenic potential. Non-HDL protein was measured by Lowry method [13].

Potential oxidative effects of vanadium(IV) and vanadium(V) as, respectively, VS and sodium orthovanadate, on the non-HDL fraction were at first investigated through continuous spectrophotometric monitoring of absorbance increase at 234 nm, reflecting CD formation during lipid peroxidation [1,12]. Reactions were carried out at 37° in quartz cuvettes containing 0.1 mg non-HDL protein per mL, in PBS, pH 7.4; vanadium(IV) or (V) concentrations ranged from 5 to 30 μM. Reference cuvettes contained lipoproteins in PBS. Molar extinction coefficient of CD was considered to be 29,500 at 234 nm [1,12].

In other specific experiments, the effects of various antioxidants on vanadium-mediated lipoprotein oxidation were investigated. Thus, the non-HDL fraction (0.1 mg protein/mL) was oxidized by 24 hr incubation at 37° with 40 μM VS, in PBS pH 7.4, with and without the following antioxidants: BHT (0.06 mM), Vitamin E (0.06 mM), ascorbic acid (0.12 mM), mannitol (0.25 mM), EDTA (0.4 mM), SOD (15 μg/mL) or catalase (50 μg/mL). VS was used here at 40 μM concentration to allow an adequate TBARS yield. Lipoprotein oxidation was indeed evaluated spectrophotometrically by TBARS assay, as previously reported [12]. Briefly, to a suitable aliquot of the lipoprotein sample, 1.3 mL of 20% acetic acid, pH 3.5, 1.3 mL aqueous solution of 0.6% TBA, 0.1 mL of 8.1% sodium dodecyl sulfate, 0.25 mM EDTA and 2.0 mM BHT in absolute ethanol were added, followed by 30 min heating at 95°. After cooling and centrifugation, the chromogen was extracted with *n*-butanol and read at 532 nm against an appropriate blank. Results were calculated as nmol TBARS/mg non-HDL protein, using a molar extinction coefficient of 154,000.

2.3. Mechanicistic aspects of vanadyl-induced lipoprotein oxidation

In a first set of experiments, capability of VS to oxidize directly the lipid phase of the non-HDL fraction (namely, in the absence of the protein moiety) was evaluated. To 1.0 mL of lipoprotein sample containing 0.96 mg non-HDL protein/mL, 0.5 mL of methanol plus 1.5 mL of ethyl acetate were added, followed by vortex and centrifugation at 3000 *g* for 5 min. The upper layer was removed and the lower one re-extracted twice with 1.0 mL of ethyl acetate. The extracted volumes were combined and dried under a stream of argon. The residue was resuspended in 3.2 mL of PBS and incubated with and without 10 and 25 μM VS for 2 hr at 37°; in some experiment, incubation was instead with 25 μM $CuCl_2$. Lipid peroxidation was assessed by the TBA-test, as described above, and results were calculated as nmol TBARS/mL.

In a second set of experiments, we investigated whether vanadium was able to exert some direct oxidant damage against the particle protein moiety. In this regard, quenching of the intrinsic fluorescence of critical apo B tryptophan residues was evaluated [14]. Optimal operative conditions were found using 5.5 μg non-HDL protein/mL; after addition of VS at concentrations of 10, 20 and 30 μM, tryptophan-related fluorescence emission at 335 nm after excitation at 280 nm was rapidly recorded, expressing the results as units of relative fluorescence/μg non-HDL protein. The same experiment was also performed with 10 μM $CuCl_2$.

2.4. Vanadyl-induced lipoprotein oxidation in diabetic and control subjects

VS may be used in patients with diabetes mellitus to improve their metabolic control [8,9]; thus, we investigated whether lipoproteins of diabetic patients were more susceptible to vanadyl-dependent oxidant damage than those of healthy controls. The non-HDL fraction was isolated from seven diabetic patients (four insulin-independent and three insulin-dependent), and from seven matched controls. Clinical characteristics of the study subjects are reported in Table 1. There was no evidence of hyperuricaemia, nor of renal, hepatic, cardiovascular and inflammatory pathological conditions in the entire study population. Moreover, all subjects were from the same geographical area (Chieti, Abruzzo, Italy), and had a similar dietary pattern (the so-called mediterranean diet); no subject used compounds with a recognized antioxidant activity. The non-HDL fraction was subjected to oxidation for 24 hr with 40 μM VS, in PBS, pH 7.4. Lipoprotein oxidation was evaluated measuring TBARS, as described

Table 1
Clinical characteristics and serum metabolic parameters of study subjects

	Controls	Diabetic patients
Number (male/female)	2/5	2/5
Age (years)	62.3 ± 4	62.7 ± 4.3
HbA_{1C} (%)	4.3 ± 0.35	9.8 ± 1.6*
Total cholesterol (mM)	4.7 ± 0.6	5 ± 0.8
HDL cholesterol (mM)	1.2 ± 0.3	1.05 ± 0.25
LDL cholesterol (mM)	3 ± 0.4	3.15 ± 0.5
Triglycerides (mM)	1.13 ± 0.4	1.5 ± 0.55

Means ± SD of seven controls and seven diabetic patients.
*$P < 0.0001$ vs. controls.

above, as well as FDPL, which result from reaction of lipoperoxidation aldehydes, such as 4-hydroxy-nonenal, with free amino groups of critical lysine residues of apo B [1]. FDPL were assessed at 430 nm emission after excitation at 360 nm using a Kontron SFM 25 spectrofluorimeter standardized with quinine sulfate (1.0 μg/mL in 0.1N H_2SO_4) to give a fluorescence intensity of 200 at 430 nm after excitation at 360 nm. Results are expressed as units of relative fluorescence (URF)/mg non-HDL protein.

Moreover, the non-HDL fraction of control and diabetic subjects was assayed for the content of total cholesterol, triglycerides, phospholipids and apo B by specific commercially available kits.

3. Statistics

Results were calculated as means ± SD and analyzed by one-way ANOVA plus Student–Newman–Keuls test [15]. Data from diabetic and control subjects were analyzed by Student's t test for unpaired data [15]. $P < 0.05$ was regarded as statistically significant [15].

4. Results

4.1. Lipoprotein oxidative activity of vanadium

As depicted in Fig. 1, VS, beginning from 10 μM, did promote oxidation of the non-HDL fraction, with an apparently typical metal-related oxidation kinetics characterized by lag, propagation and decomposition phases [1,12]. In particular, although lag time of oxidation tended to be shorter with increasing metal concentrations, this phenomenon was not statistically significant (Table 2). Oxidation rate and maximal CD amount were instead significantly higher with 20 and 30 than with 10 μM VS (Table 2). Vanadium(V), i.e. vanadate, was apparently ineffective as catalyst of lipoprotein oxidation (Fig. 1).

Table 2
Oxidative parameters of the kinetics of vanadyl-dependent oxidation of human non-HDL fraction

	Lag time (min)	Oxidation rate (nmol CD/min/mg non-HDL protein)	Max CD amount (nmol CD/mg non-HDL protein)
10 μm VS	37 ± 6	2.2 ± 0.75	73.2 ± 9.5
20 μm VS	34 ± 8.5	4.05 ± 1.1*	130.5 ± 26*
30 μm VS	32 ± 8	5.3 ± 2.1*	160 ± 33.3*

Means ± SD of six different experiments.
*$P < 0.05$ vs. 10 μm VS.

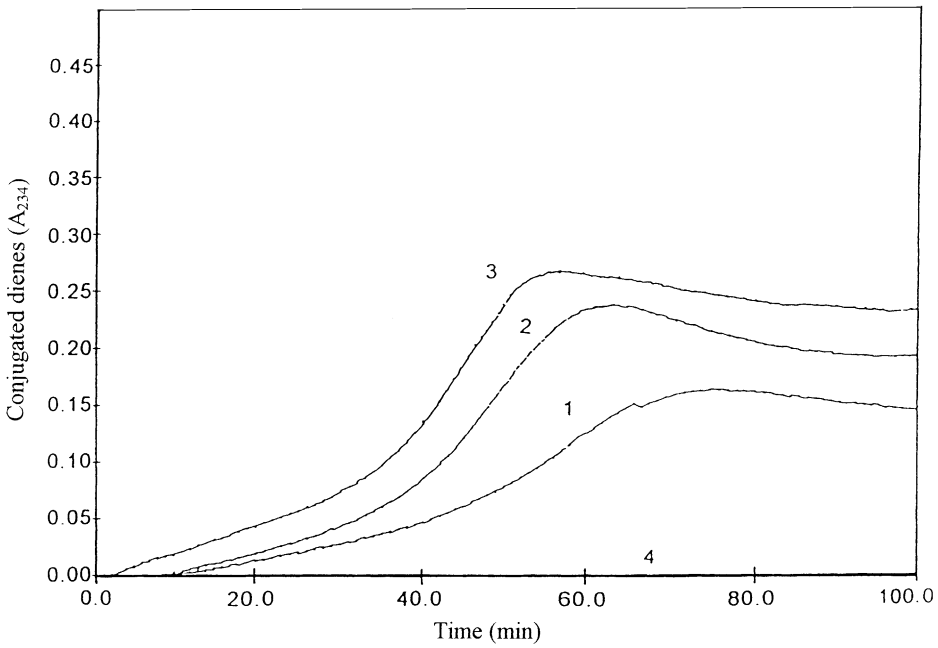

Fig. 1. Oxidative effects of VS on human non-HDL fraction evaluated as kinetics of absorbance increase at 234 nm (A_{234}) due to CD formation. Trace 1, 2 and 3: 10, 20 and 30 μM VS; trace 4: 30 μM sodium orthovanadate. The results shown are representative of six similar experiments. See Sections 2 and 3 for further explanations.

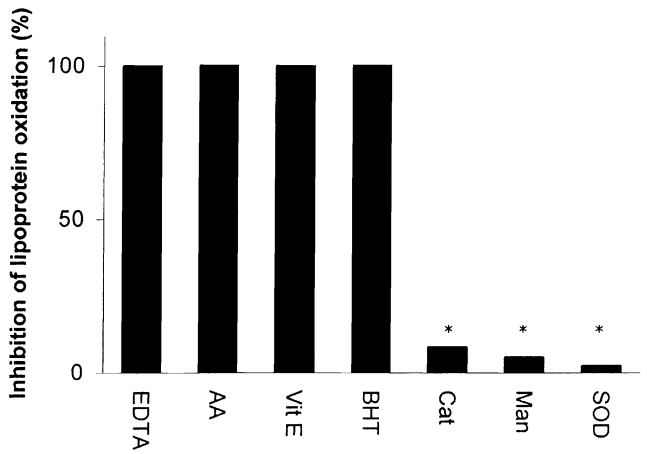

Fig. 2. Effects of EDTA (0.4 mM), ascorbic acid (AA, 0.12 mM), Vitamin E (Vit E, 0.06 mM), BHT (0.06 mM), catalase (Cat, 50 μg/mL), mannitol (Man, 0.25 mM) and SOD (15 μg/mL) on vanadyl-dependent oxidation of human non-HDL fraction assessed as TBARS formation (which was 30 ± 6 nmol TBARS/mg non-HDL protein in control experiments). Bars represent the means percentage inhibition of lipoprotein oxidation calculated from four independent experiments. Standard deviations are less than 3, 2 and 1%, respectively, in the case of catalase, SOD and mannitol, while they cannot be calculated for all the other antioxidants which resulted in undetectable TBARS values (total inhibition of lipoprotein oxidation). The asterisk (*) P = NS vs. control experiments (ANOVA plus Student–Newman–Keuls test). See Section 2 for further explanations.

Vanadyl-dependent lipoprotein oxidation was totally inhibited by EDTA, BHT and Vitamins C and E, whilst SOD, catalase and mannitol gave slight and insignificant inhibition (Fig. 2).

4.2. Mechanicistic aspects of vanadyl-induced lipoprotein oxidation

VS was capable of oxidizing directly the lipids extracted from the non-HDL fraction. Indeed, 1.52 ± 0.5 and 3.3 ± 0.9 nmol TBARS/mL were generated with 10 and 25 μM VS, respectively, whereas TBARS were undetectable without the metal. Similar to a previous report [16], copper was instead ineffective at promoting peroxidation of isolated lipoprotein lipids.

As shown in Table 3, the intrinsic lipoprotein fluorescence was significantly quenched by VS, which was, however, considerably less active than copper. In fact, both at 10 μM concentration, VS and $CuCl_2$ resulted in about 16 and 64% decrement of tryptophan-related fluorescence, respectively (Table 3). It should be noted that this decrement was very rapid and preceded the initiation of lipid peroxidation, ruling out the possibility that quenching of tryptophan fluorescence could be due non-specifically to by-products of lipoperoxidation. Unlike copper, vanadyl has a lower capacity to induce direct oxidant damage of critical tryptophan residues of the lipoprotein protein moiety, whereas it can oxidize directly the lipid moiety.

Table 3
Quenching of tryptophan fluorescence of the non-HDL fraction by VS and copper chloride ($CuCl_2$)

	URF/μg non-HDL lipoprotein
Control (lipoprotein alone)	17.5 ± 1
Control plus	
10 μM VS	$14.7 \pm 1.9^{*}$
20 μM VS	$13.5 \pm 1.5^{*}$
30 μM VS	$12.8 \pm 2^{*}$
10 μM $CuCl_2$	$6.2 \pm 0.9^{*,**}$

Means ± SD of six different experiments. URF: units of relative fluorescence.
$^{*}P < 0.05$ vs. control.
$^{**}P < 0.05$ vs. all VS concentrations. See Sections 2 and 3 for further explanations.

4.3. Vanadyl-induced lipoprotein oxidation in diabetic and control subjects

The non-HDL fraction of diabetic patients was more susceptible to vanadyl-mediated oxidative injury than that of controls. Indeed, after lipoprotein incubation with VS, both TBARS and FDPL were significantly higher in the diabetic than in the control subjects (Table 4), while no significant oxidation was apparently detectable without the metal. Associated with these findings, the non-HDL fraction of diabetic patients had a content of total lipids (namely, cholesterol plus triglycerides and phospholipids) significantly higher than that of controls (Table 5). Regarding instead each lipid class, although the concentration of total cholesterol, triglycerides and phospholipids was higher by about 15, 50 and 30%, respectively, in the non-

Table 4
Vanadyl-induced oxidation of the non-HDL fraction of control and diabetic subjects

	Controls	Diabetic patients
TBARS	31 ± 5.2	$44.7 \pm 7^{*}$
FDPL	96 ± 16	$131 \pm 18^{*}$

Means ± SD of seven controls and seven diabetic patients.
$^{*}P < 0.01$ vs. controls. TBARS and FDPL are expressed, respectively, as nmol/mg non-HDL protein and units of relative fluorescence/mg non-HDL protein. See Section 2 for further explanations.

Table 5
Composition of the non-HDL fraction from control and diabetic subjects

	Controls	Diabetic patients
Total cholesterol (mM)	3.6 ± 0.55	4.1 ± 0.65
Triglycerides (mM)	0.9 ± 0.5	1.35 ± 0.62
Phospholipids (mM)	1.7 ± 0.3	2.2 ± 0.4
Total lipids (mM)	6.1 ± 1	$7.6 \pm 0.7^{*}$
Apo B (mg/dL)	102 ± 17	106 ± 20

Means ± SD of seven control and seven diabetic subjects. Total lipids: total cholesterol + triglycerides + phospholipids.
$^{*}P < 0.01$ vs. controls.

HDL fraction of diabetic patients than in that of controls (Table 5), the level of statistical significance was not reached partly as a result of the relatively small number of subjects studied.

5. Discussion

The present study shows that vanadyl has a specific lipoprotein oxidative activity. Even though the kinetics of vanadyl-dependent lipoprotein oxidation resembles that of copper, nonetheless some differences seem to exist. In this regard, it is known that lag time, oxidation rate and maximal CD amount remain virtually constant after reaching a determined copper concentration (i.e. 5 μM with 0.05 mg LDL protein/mL) [1,17]; this phenomenon suggests that LDL are saturable by copper, which needs to bind to specific apo B aminoacidic sites to favor lipid peroxidation [1,14,16]. Similarly, the non-HDL fraction, which is formed by the apo B-containing lipoproteins LDL and VLDL, is oxidized by copper. The oxidative behavior of vanadyl is apparently different; in fact, while lag time is only slightly decreased increasing VS concentrations from 10 to 30 μM, oxidation rate and maximal CD amount are instead significantly increased. A possible explanation may be related to the direct oxidant activity of vanadyl on lipoprotein lipids, resulting in a metal concentration-dependent increase of lipid peroxidation; indeed, we observed that isolated lipoprotein lipids are oxidized dose dependently by VS. A similar phenomenon has been reported for vanadyl-driven oxidation of lipid micellar systems [5].

The ineffectiveness of SOD, catalase and mannitol against vanadyl-induced lipoprotein oxidation suggests no involvement of pro-oxidant species such as free superoxide, H_2O_2 and hydroxyl radical ($OH^{\bullet}$); however, it is possible that some ''crypto'' $OH^{\bullet}$ generated by the metal directly at lipoprotein phospholipid level could be involved in specific oxidative processes [3,5]. Lipoprotein oxidation is instead counteracted by the metal chelator EDTA, as well as by chain-breaking antioxidants, such as the physiological compounds Vitamins E and C. The capability of vanadium to induce ascorbate depletion *in vivo* [18], and the intrinsic deficient status of Vitamins E and C of diabetic patients [19], may therefore favor vanadium-dependent oxidative stress *in vivo* especially in the clinical setting of diabetes mellitus. In such a context, it is of note that the non-HDL fraction of diabetic patients is more susceptible to vanadyl-driven oxidant damage than that of controls. Basically in line with previous reports [20,21], total lipid concentration of the non-HDL fraction is significantly higher in the diabetic than in the control subjects (Table 5). Moreover, the LDL content of arachidonate (a high oxidizable polyunsaturated fatty acid) has been shown to be more elevated in diabetic patients than in controls [22]. Thus, not only more total lipids but also more oxidizable lipids, such as arachidonate, seem to be present in apo B-containing diabetic lipoproteins, eventually resulting in their enhanced susceptibility to oxidation by vanadyl, which has a direct lipid oxidant activity. Indeed, plasma lipids have been reported to be more prone to oxidant-driven lipoperoxidation in diabetic than in control subjects [23,24].

Our data shows that vanadium is able to induce lipoprotein oxidation only in the reduced form, namely as vanadyl, suggesting that human lipoproteins, which can bind and reduce copper before initiation of oxidative processes [25], may be instead incapable of binding and/or reducing the metal added as vanadate. In this regard, vanadium, which is usually used as VS in humans, is conceivably present *in vivo* in its reduced state owing to specific physiological reductants [4,5,26,27]. Consistently, while vanadate *per se* does not induce lipid peroxidation *in vitro* [5], its administration *in vivo* has significant lipoperoxidative effects [4,6], which are directly correlated with tissue vanadium concentrations [6].

Our data also shows that, under the experimental conditions used, lipoprotein oxidation is evident with 10 μM or more VS; on the other hand, serum vanadium levels are very low in humans, thus questioning the potential relevance of vanadyl-dependent lipoprotein oxidation *in vivo*. However, vanadium is characterized by a tissue-accumulating capacity; indeed, serum metal concentrations of 10 μM may be reached after 6 weeks intake of 300 mg/day VS [28]. Furthermore, in diabetic animals chronically treated with the metal, serum vanadium levels average 20 μM [6,8,29], with concentrations even higher in tissue compartments [6,8,29]. This aspect appears relevant, considering that lipoprotein oxidation conceivably occurs in the arterial tissue and not in the bloodstream because of the high antioxidant capacity of plasma [1,2].

Notably, Goldfine and associates [28] have recently reported that 6 weeks VS administration to diabetic patients does not increase significantly serum TBARS levels, apparently suggesting that short-term metal intake is not associated with lipoperoxide burden in humans. However, TBARS, when measured in whole serum or plasma and not in isolated lipoproteins, are unspecific and inadequate indicators of lipid peroxidation [3,30]. Moreover, also vanadium-dependent lipoprotein oxidation is expected to happen *in vivo* in the vascular tissue (where the metal may accumulate), so that it could not be revealed by serum peroxidation assessment. Thus, given the herewith reported capability of vanadium(IV) to oxidize human lipoproteins at *in vivo* achievable metal concentrations, possible pro-oxidant/proatherogenic effects of vanadyl appear feasible and warrant further clinical study. Diabetic patients after prolonged metal intake without adequate antioxidant supplementation and, possibly, weight-training athletes, who can undergo VS abuse to improve specific performance, may be at risk of vanadyl-dependent lipoprotein oxidation.

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