

Massive Loss of Sulfur in HIV Infection

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ABSTRACT

Skeletal muscle tissue from SIV-infected macaques was previously found to contain abnormally high sulfate and low glutathione levels indicative of an excessive cysteine catabolism. We now confirm the peripheral tissue as a site of massive cysteine catabolism in HIV infection and have determined the urinary loss of sulfur per time unit. The comparison of the sulfate concentrations of the arterial and venous blood from the lower extremities of 16 symptomatic HIV⁺ patients and 18 HIV⁻ control subjects (study 1) revealed (1) that the peripheral tissue of HIV⁺ patients with or without highly active antiretroviral therapy (HAART) releases large amounts of sulfate and (2) that plasma sulfate, thioredoxin, and interleukin-6 levels are elevated in these patients. A complementary investigation of 64 asymptomatic HIV⁺ patients and 65 HIV⁻ subjects (study 2) revealed increased plasma sulfate levels in the asymptomatic patients. The analysis of the daily urinary excretion of sulfate and urea of another group of 19 HIV⁺ patients and 22 healthy HIV⁻ subjects (study 3) confirmed (1) that HIV⁺ patients experience a massive loss of sulfur and (2) that this loss is not ameliorated by HAART. The sulfur loss of asymptomatic patients was equivalent to a mean loss of about 10 g of cysteine per day. If extrapolated, this would correspond to an alarming negative balance of approximately 2 kg of cysteine per year under the assumption that the normal sulfate excretion equivalent to approximately 3 g of cysteine per day is balanced by a standard Western diet. The abnormally high sulfate/urea ratio suggests that this process drains largely the glutathione pool.

INTRODUCTION

STUDIES OF THE SKELETAL muscle tissue of SIV-infected rhesus macaques revealed that this viral infection causes a strong decrease in the muscular glutathione level and a corresponding increase in the sulfate level, indicative of excessive cysteine catabolism.^{1,2} In line with this interpretation, HIV-infected patients were found to have abnormally low plasma cystine concentrations and low intracellular glutathione (GSH) levels in peripheral blood mononuclear cells,^{3–8} although some authors failed to confirm the latter.^{9,10} Because a massive loss of cysteine may lead to a life-threatening condition, we performed a series of studies with the aim of (1) confirming the peripheral tissues (i.e., mainly the skeletal muscle) as a site of excessive sulfate production in HIV infection, (2) determining the magnitude of the sulfur loss per unit time, and (3) determining whether state-of-the-art antiretroviral therapy (ART), including treatment with a protease inhibitor (highly active ART, HAART), may ameliorate the loss of sulfur.

PATIENTS AND METHODS

Patients

The three studies described here were based on three different groups of subjects recruited on different occasions. All patients were subgroups of the Heidelberg/Mannheim metabolic cohort. This cohort was recruited for pathophysiological and interventional studies starting in 1995. In total, it includes more than 130 HIV⁺ individuals. All subjects gave their informed consent. The studies were performed in accord with the Declaration of Helsinki and approved by the Ethics Committee of the Medical Faculty in Mannheim.

Determination of peripheral exchange rates: Study 1

In a first study (study 1) we determined the exchange rates of sulfate and amino acids in the lower extremities of 16 randomly selected, clinically symptomatic and hospitalized HIV⁺

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TABLE 1. PATIENT COHORTS^a

	Study 1			Study 2			Study 3		
	HIV ⁻	Sympt. HIV ⁺	HIV ⁻	Asympt. HIV ⁺	HIV ⁻	Asympt. HIV ⁺	HIV ⁻	Asympt. HIV ⁺	Sympt. HIV ⁺
No. of patients	18	16	65	64	21	64	21	9	10
Age (years)	24.1 ± 4.4	38.7 ± 11.3	28.3 ± 7	36.0 ± 10.1	33.3 ± 10.2	36.0 ± 10.1	33.3 ± 10.2	41.0 ± 3	36.4 ± 2
Sex (F/M)	12/6	3/13	30/35	36/28	6/15	36/28	6/15	6/3	3/7
BMI (kg/m ²)	21.8 ± 0.6	20.4 ± 0.7	ND	24.0 ± 0.4	22.6 ± 0.8	24.0 ± 0.4	22.6 ± 0.8	23.4 ± 1.4	21.8 ± 0.9
Percent body cell mass	42.1 ± 1.0	41.1 ± 1.2	ND	43.5 ± 0.8	ND	43.5 ± 0.8	ND	34.4 ± 1.4	41.4 ± 1.8
Albumin (μM)	659 ± 16	669 ± 11	ND	644 ± 8	ND	644 ± 8	ND	576 ± 40	536 ± 30
Hemoglobin (mM)	7.9 ± 0.7	7.3 ± 1.3	10.7 ± 1.67	8.6 ± 1.1	ND	8.6 ± 1.1	ND	8.7 ± 0.4	7.2 ± 0.5
CD4 ⁺ cells (cells/μl)	ND	216 ± 64	894 ± 35	420 ± 25	ND	420 ± 25	ND	427 ± 66	445 ± 85
Viral load (log copies/ml)	ND	5.1 ± 0.9	ND	4.5 ± 4.0	—	4.5 ± 4.0	—	3.6 ± 0.4	4.7 ± 0.3
Treatment	—	Naive (n = 6) HAART (n = 7) Other ART (n = 3)	—	Naive (n = 29) HAART (n = 12) Other ART (n = 23)	—	Naive (n = 29) HAART (n = 12) Other ART (n = 23)	—	Naive (n = 6) HAART (n = 2) Other ART (n = 1)	Naive (n = 5) HAART (n = 5)

Abbreviations: Sympt., Symptomatic; Asympt., asymptomatic; BMI, body mass index; ND, not determined.

^aThe three studies were based on three different groups of subjects recruited on different occasions.

patients (i.e., mostly patients with opportunistic infections or diarrhea; Centers for Disease Control [CDC] 1993 stages I–IV; age >18 years; 13 males and 3 females) including 6 HIV⁺ patients without ART (256–843 CD4⁺ cells mm⁻³, 10^{2.7}–10^{5.6} HIV RNA copies ml⁻¹) and 10 HIV⁺ persons with ART (5–163 CD4⁺ cells mm⁻³, 10^{4.5}–10^{5.9} HIV RNA copies ml⁻¹). A more detailed description of the patients (Heidelberg/Mannheim metabolic cohort 1) is given in Table 1. Seven of the patients receiving ART also received highly active antiretroviral therapy (HAART) with at least one protease inhibitor. Exclusion criteria were alcohol or drug abuse as well as endocrinological diseases, liver cirrhosis, serum creatinine >2 mg/dl, and cardiorespiratory insufficiency as determined by standard laboratory tests. Eighteen randomly selected healthy persons (p24 antigen negative by enzyme-linked immunosorbent assay [ELISA]; age range 20–38 years; 6 males and 12 females) served as control subjects. The peripheral exchange rates were determined as described previously¹¹ with minor modifications. Leg blood flow was quantified by venous occlusion plethysmography,¹² using a Periquant 3500 device (J. Gutmann, Eurasburg, Germany). Blood samples were taken from the femoral artery and vein. The femoral artery was carefully punctured after digital palpation of the groin. If necessary, an ultrasound Doppler device (Handydrop; Kranzbühler, Solingen, Germany) was also used to identify the blood vessel. The femoral vein was punctured about 2 cm medially, and blood was taken by essentially the same procedure. All subjects were studied after an overnight fast. The arterial and venous plasma concentrations of sulfate, thioredoxin, and interleukin 6 (IL-6) were determined as described.^{13–15} For amino acid analysis, the plasma samples were deproteinized with sulfosalicylic acid³ and analyzed by column chromatography with a Biotronic LC 5001 instrument with Durum DC 4A (Eppendorf-Biotronic, Hamburg, Germany). The exchange rates were finally calculated on the basis of the arterial and venous plasma concentrations of sulfate and amino acids and the blood flow according to the following equation: [(arterial concentration – venous concentration) × blood flow × (1-hematocrit)] divided by the volume of leg tissue increase. The results are expressed as nanomoles min⁻¹ 100 ml⁻¹. The prospectively defined primary end point of this study was the peripheral exchange rate of sulfate.

Plasma sulfate levels of asymptomatic patients: Study 2

Plasma sulfate levels (study 2) have been determined in postabsorptive blood samples from the cubital vein of 29 clinically asymptomatic, ART-naive HIV⁺ patients (CDC 1993 stage I) with >200 CD4⁺ cells mm⁻³, 35 clinically asymptomatic HIV⁺ patients (CDC 1993 stage I, age >18 years) with 200–500 CD4⁺ cells mm⁻³ who have been under constant (HA) ART for at least 6 months before the study, and 65 HIV⁻ control subjects. Physical and biochemical details are listed in Table 1.

Urinary sulfate and urea excretion: Study 3

The urea excretion of randomly selected groups of asymptomatic (CDC 1993 stage I/II) and symptomatic HIV⁺ patients (CDC 1993 stage III/IV), and of a randomly selected group of healthy HIV⁻ control subjects (i.e., mostly staff members of the participating institutes; Table 1), was determined as de-

scribed.¹⁶ The creatinine clearance of all subjects was normal. Eight HIV⁺ patients received (HA)ART (mostly lamidivine [3TC] and/or stavudine [d4T] alone or in combination with, at most, two other nucleoside analogs and/or protease inhibitors; see Table 1). None of the patients received indinavir-sulfate (Crixivan). The sulfate in urine was determined as described.¹³ The prospectively defined primary end point of this study was the urinary excretion of sulfate per day.

Statistical analysis

Unless indicated otherwise, different groups were compared by the Wilcoxon rank sum test. For multiple group comparison, we used in addition one-way analysis of variance (ANOVA). *p* Values <0.05 were regarded as significant. Box plots, arithmetic means, and standard errors of the mean (SEM) were used as descriptive statistics.

RESULTS

Abnormal sulfate exchange rates in the peripheral tissue: Study 1

The study on the sulfate and amino acid exchange rates in the lower extremities revealed that the sulfate exchange rate of HIV⁻ persons was not significantly different from 0, whereas HIV⁺ patients showed a significant release of sulfate indicative of a massive cysteine catabolism in the peripheral tissues (Fig. 1). The healthy controls (95% confidence interval [CI], –22.9 to 63.9 nmol min⁻¹ 100 ml⁻¹) differed significantly not only

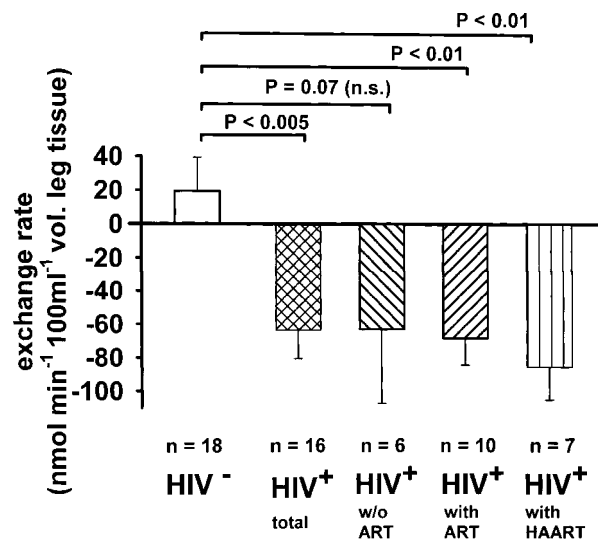


FIG. 1. Sulfate exchange rate in the lower extremities of HIV⁺ and HIV⁻ subjects. The data show sulfate exchange rates in the peripheral tissue of HIV⁻ subjects and HIV⁺ patients with and without antiretroviral therapy (ART) as indicated. A subgroup of the patients with ART received highly active antiretroviral therapy (HAART) including at least one protease inhibitor. The lower and upper 95% confidence intervals for HIV⁻, total HIV⁺, HIV⁺ with ART, and HIV⁺ with HAART were –22.8 and +64.0, –106.3 and –27.2, –108.1 and –25.7, –146.1 and –26.3 nmol min⁻¹ 100 ml⁻¹, respectively.

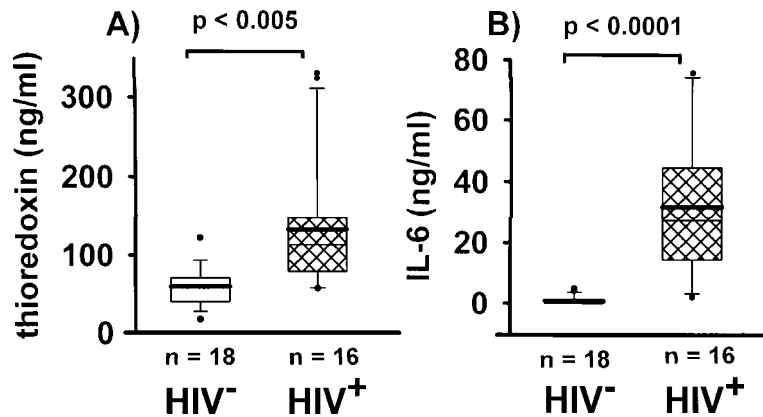


FIG. 2. Arterial plasma thioredoxin and IL-6 in symptomatic HIV⁺ and HIV⁻ subjects. (A and B) The arterial plasma thioredoxin and interleukin 6 concentration, respectively, in postabsorptive blood samples from the femoral artery (study 1).

from the total HIV⁺ group (CI, -106.3 to -27.2 nmol min⁻¹ 100 ml⁻¹) but also from the HIV⁺ subgroup with HAART (CI, -146.1 to -26.3 nmol min⁻¹ 100 ml⁻¹). Importantly, the sulfate exchange rates of patients with or without ART (including HAART) were, on average, not significantly different. It should be noted that the patients had, on average, a relatively low albumin level but an essentially normal body mass index and a normal relative body cell mass (Table 1), and that the sulfate exchange rate was not significantly correlated with the individual body mass index, body cell mass, or the albumin level (data not shown). The exchange rates of all other amino acids of HIV⁺ and HIV⁻ subjects, including that of tyrosine (-11.2 ± 2.1 and -13.6 ± 3.8 nmol min⁻¹ 100 ml⁻¹, respectively) and phenylalanine (-9.0 ± 1.7 and 9.0 ± 1.9 nmol min⁻¹ 100 ml⁻¹, respectively) were not significantly different. There was also no significant correlation between the sulfate exchange rates and the exchange rates of tyrosine or phenylalanine in both groups of patients. The exchange rates of the sulfur-containing amino acids methionine, cystine, and taurine and the exchange rates of acid-soluble thiol (i.e., mainly reduced cysteine) were, on average, close to 0 and not markedly different between HIV⁺ and HIV⁻ subjects with the exception that the postabsorptive peripheral uptake of acid-soluble thiol (i.e., mainly reduced cysteine) showed a moderate increase in HIV⁺ subjects that almost reached significance (24.6 ± 14.9 μ mol min⁻¹ 100 ml⁻¹; $p = 0.05$). The arterial and venous concentrations of thioredoxin and interleukin 6 from individual subjects were practically indistinguishable (not shown) but were significantly elevated in HIV-infected patients (Fig. 2A and B).

Elevated plasma sulfate concentrations: Study 2

The analysis of the plasma sulfate concentrations from larger groups of clinically asymptomatic HIV⁺ and HIV⁻ subjects (study 2) showed, in line with the results from the sulfate exchange studies and the plasma sulfate levels in the subjects of studies 1 and 3, that HIV⁺ patients with and without (HA)ART have significantly increased plasma sulfate concentrations (Fig. 3). Study 2 showed that healthy subjects (CI, 289.6–332.4 μ M) differed significantly from HIV⁺ subjects without ART (CI, 370.3–508.1 μ M) and HIV⁺ subjects with ART (CI, 345.6–420.0 μ M).

Excessive urinary sulfate excretion: Study 3

To determine the magnitude of the loss of nitrogen and sulfur per unit time, we analyzed in study 3 the urinary excretion per day of sulfate, urea, and amino acids of 9 randomly selected asymptomatic and 10 symptomatic HIV⁺ patients and 22 healthy HIV⁻ control subjects. Neither group was on a special (i.e., protein-rich) diet. The urinary release of amino acids did not differ significantly between HIV⁺ and HIV⁻ persons (data not shown) with the exception of methionine (37.0 ± 4.1 versus 19.4 ± 1.8 μ mol/day; $p < 0.0003$), valine (34.4 ± 4.0 versus 21.4 ± 4.2 μ mol/day; $p < 0.05$), leucine (55.3 ± 5.1 versus 34.4 ± 5.0 μ mol/day; $p < 0.01$), isoleucine (30.1 ± 5.1 versus 18.2 ± 2.6 μ mol/day; $p < 0.05$), phenylalanine (78.2 ± 8.7 versus 39.2 ± 6.0 μ mol/day; $p < 0.001$), arginine (24.0 ± 3.3 versus 12.5 ± 2.7 μ mol/day; $p = 0.01$), ornithine (15.7 ± 1.6 versus 7.7 ± 0.9 μ mol/day; $p = 0.0001$), and citrulline (5.3 ± 1.3 versus 1.1 ± 0.3 μ mol/day; $p < 0.005$). The amount of total sulfur-containing amino acids, i.e., the sum of taurine, cystine, cystathionine, and methionine, was 644 ± 178 and 657 ± 74 μ mol/day, respectively. The urinary sulfate excretion, in contrast, was quite variable and, on average, dramatically increased in HIV⁺ patients. Importantly, this increase was not ameliorated in patients receiving HAART (Table 2). In line with this finding, the sulfate excretion showed not even a marginally significant correlation with the viral load ($r = 0.23$, $p = 0.34$).

In view of the earlier findings that the muscular glutathione level is strongly decreased and that the intramuscular sulfate level is correspondingly increased in skeletal muscle tissue from SIV-infected rhesus macaques,^{1,2} and because glutathione has a higher ratio of sulfur/nitrogen atoms (i.e., 0.33) than proteins, we also determined the urinary sulfate/urea excretion ratio to determine whether the excessive excretion of sulfate may drain mainly the glutathione pool. The results of this analysis revealed that the urinary sulfate/urea excretion ratio of HIV⁺ patients was again quite variable and, on average, indeed markedly higher than that of HIV⁻ subjects (Fig. 4, middle and bottom).

The more detailed analysis revealed, furthermore, that the daily sulfate excretion and the sulfate/urea ratio of asymptomatic HIV⁺ patients are, in principle, even greater than those of clinically symptomatic patients (Table 2). On average, the

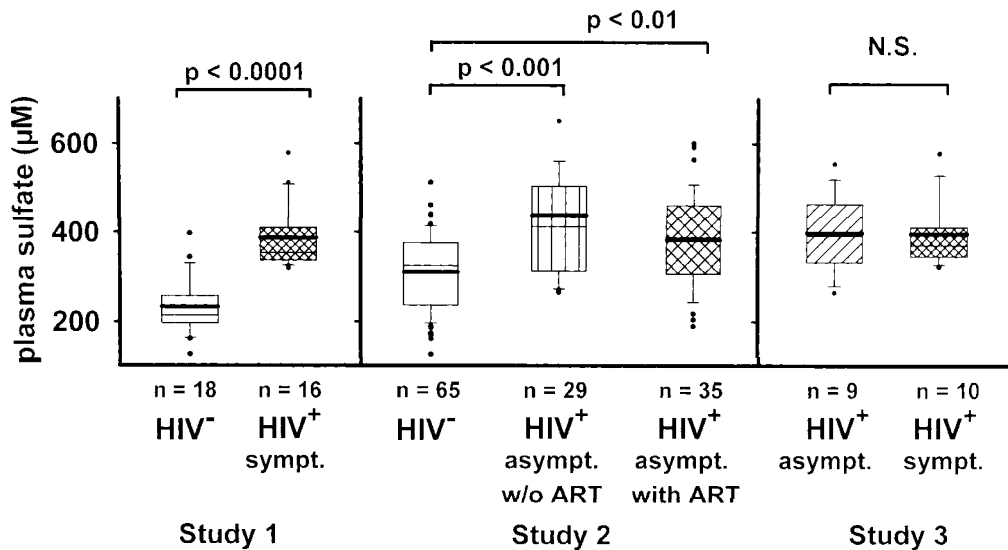


FIG. 3. Plasma sulfate concentrations in asymptomatic HIV⁺ and HIV⁻ subjects. Postabsorptive venous plasma sulfate concentrations as determined in studies 1, 2, and 3. In the case of the larger study (study 2), the lower and upper 95% confidence intervals for HIV⁻ subjects, HIV⁺ patients without ART, and HIV⁺ patients with ART were 290 and 332, 370 and 508, and 346 and 420 μM , respectively. The box plots describe the first (25%) and third (75%) quartiles of the distribution, the arithmetic mean (thick line), the median (thin line), and the outliers.

asymptomatic HIV⁺ persons showed a net increase in sulfate excretion equivalent to 7 g of cysteine per day (Table 2). The sulfate excretion of healthy subjects (CI, 21.4–26.3 mmol/day) differed significantly from that of either asymptomatic patients (CI, 57.3–104.0 mmol/day) or symptomatic patients (CI, 26.3–64.6 mmol/day).

DISCUSSION

Taken together, the results presented here, including the excessive release of sulfate from the lower extremities of HIV⁺ patients (study 1), the increase in the plasma sulfate concen-

tration (study 2), and the excessive urinary sulfate excretion (study 3), indicate that the abnormally low cysteine and glutathione levels of HIV-infected patients^{3–8} are consequences of a massive cysteine catabolism that may occur to a large extent in the peripheral tissue. This conclusion is in line with earlier findings that intracellular sulfate levels are strongly increased and intracellular glutathione levels correspondingly decreased in the skeletal muscle tissue of HIV-infected rhesus macaques.^{1,2} The sulfate exchange rates of HIV⁺ and HIV⁻ persons differed, on average, by approximately 85 $\text{nmol min}^{-1} 100 \text{ ml}^{-1}$. Under the assumption that the loss of sulfate is relatively constant throughout the day and similar in all muscle tissues, this difference would correspond to a net loss of more

TABLE 2. URINARY SULFATE EXCRETION AND UREA PRODUCTION RATE OF HIV⁺ PATIENTS: STUDY 3

	n	Urea (mmol/day)	Sulfate (mmol/day)	Equivalent amount of cysteine (g/day)
Healthy HIV ⁻ subjects	21	320 ± 12	23.8 ± 1.8	2.89 ± 0.14
HIV ⁺ total	19	424 ± 45	62.1 ± 7.6	7.52 ± 0.92
Significance versus HIV ⁻		$p < 0.05$	$p < 0.001$	
HIV ⁺ asymptomatic	9	427 ± 48	80.6 ± 10.1	9.76 ± 1.23
Significance versus HIV ⁻		$p < 0.03$	$p < 0.0001$	
Net increase ^a		107 ± 48	56.8 ± 10.1	6.87 ± 1.23
HIV ⁺ asymptomatic	10	422 ± 76	45.5 ± 8.5	5.50 ± 1.02
Significance versus HIV ⁻	NS	$p < 0.05$		
Significance versus HIV ⁺ asympt.	NS	$p < 0.01$		
Net increase ^a		102 ± 76	21.6 ± 8.5	2.62 ± 1.03
HIV ⁺ ART naive	11	384 ± 41	58.4 ± 10.9	6.04 ± 1.32
HIV ⁺ with HAART	7	483 ± 105	64.8 ± 11.9	7.84 ± 1.44

^aNet increase derived by subtraction of the mean urea and sulfate excretion rates, respectively, of the normal healthy subjects.

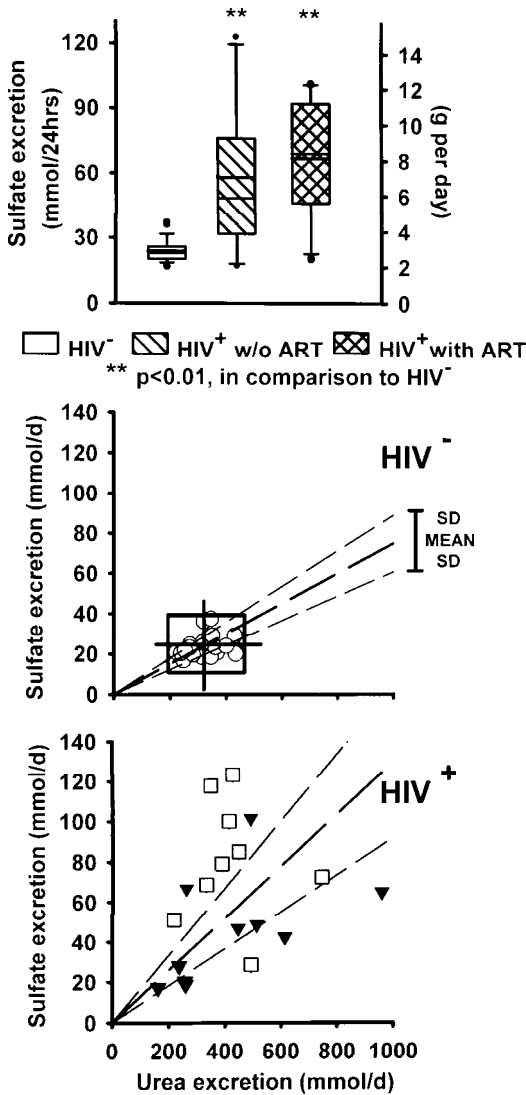


FIG. 4. Urinary sulfate and urea excretion. *Top:* Urinary sulfate excretion of HIV⁺ patients with and without ART and HIV⁻ control subjects. The box plots describe the first (25%) and third (75%) quartiles of the distribution, the arithmetic mean (thick line), and the median (thin line). *Middle and bottom:* Each circle, square, and triangle represents a single HIV⁻, asymptomatic HIV⁺, and symptomatic HIV⁺ person, respectively. Horizontal and vertical lines show the means \pm SD. The diagonal dashed lines indicate the mean \pm SD of the ratios of sulfate to urea excretion.

than 4 g of cysteine per day in the case of a person with a skeletal muscle volume of about 30 liters (i.e., about 70 kg body weight). This figure is on the same order of magnitude as the net increase in urinary sulfate excretion (Table 2), suggesting that the skeletal muscle tissue is a major site of the cysteine catabolism in HIV infection. Importantly, even the highly active antiretroviral therapy (HAART) that includes at least one protease inhibitor was found to ameliorate neither the release of sulfate from the peripheral tissue (Fig. 1) nor the urinary sulfate excretion (Table 2), indicating that the massive loss of sulfur remains a serious and potentially life-threatening problem even in patients who received

the best of the presently available antiretroviral therapies. Because healthy subjects excrete relatively small amounts of sulfate and taurine via the intestine, we would not expect the fecal route to contribute significantly to the loss of sulfur.

The excessive peripheral sulfate exchange rate as well as the excessive urinary sulfate excretion were not correlated with symptoms of a catabolic state (i.e., not significantly correlated with the individual body mass index, body cell mass, or albumin concentration) but obviously preceded the onset of symptoms. This scenario does not prove but is compatible with the hypothesis that the accumulating consequences of a steady loss of sulfur may eventually give rise to the wasting process. The striking finding that the sulfate excretion of asymptomatic patients is, on average, significantly stronger than that of clinically symptomatic patients (Table 2) makes sense in view of the progressive glutathione depletion that was found in the skeletal muscle tissue of SIV-infected macaques.¹ To obtain an estimate of the relative contribution of glutathione to the massive sulfate excretion, we calculated the net increase in sulfate and urea excretion (Table 2). The ratio of the net increase in sulfate excretion to urea excretion among the asymptomatic HIV⁺ patients, computed from these data, was 0.53, i.e., remarkably close to the ratio expected for the metabolism of glutathione only (i.e., 0.67, corresponding to one sulfur atom per three nitrogen atoms) and markedly higher than the mean sulfate/urea ratio of the HIV⁻ subjects (0.076 ± 0.004). The ratio of 0.076 corresponds to 1 sulfur per 26 nitrogen atoms and is obviously balanced by the intake of dietary protein. We, therefore, propose the hypothesis that the appearance of clinical symptoms and the concomitant decrease in sulfate excretion and sulfate/urea ratios may be, altogether, the consequence of the advanced glutathione depletion.

The marked increase in the arterial thioredoxin and IL-6 levels in HIV infection (Fig. 2) confirmed and extended earlier findings.^{14,17} In view of the abnormally high plasma thioredoxin level in these patients, we propose the hypothesis that this oxidoreductase may shuttle reducing equivalents out of the tissue into the plasma and convert thereby the plasma cystine to cysteine, which is transported more readily than cystine into the skeletal muscle cells. This effect may contribute to the decrease in the plasma cystine level and the increase in the intracellular cysteine catabolism.

The mean loss of sulfur in the asymptomatic HIV⁺ patients, equivalent to 10 g of cysteine per day, corresponds to a net loss of 7 g of cysteine per day under the assumption that the normal Western diet covers, on average, only the normal mean sulfate excretion corresponding to 3 g of cysteine per day (Table 2 and Fig. 4). Because 10 g of cysteine per day is the mean loss of sulfur in a random sample of asymptomatic HIV⁺ patients, it is suggested that the net loss, on the order of 7 g of cysteine per day or 2 kg of cysteine per year, is with good approximation the average net loss of sulfur of asymptomatic HIV⁺ patients over many months or years. Irrespective of other HIV-mediated pathogenic mechanisms, it is reasonable to assume that this massive loss of sulfur must lead to a life-threatening condition sooner or later if not compensated by cysteine replenishment. Importantly, this conclusion applies even to patients receiving HAART, i.e., the best of the presently available antiviral therapies. One study has already indicated that low serum thiol levels predict shorter times to death among

HIV⁺ injecting drug users.¹⁸ In contrast, patients with cancer,¹⁹ burn injuries,^{20,21} and cystinuria²² and fasting human subjects²³ were previously found to have a decreased rather than increased rate of urinary sulfate excretion. An abnormally high ratio of urinary sulfate versus urea (0.109 ± 0.029) was previously reported for patients with minor trauma.²⁴ The authors suggested "that some sulphur-rich tissue might be the main source of the material catabolized." This ratio, however, is not as high as that of asymptomatic HIV⁺ patients (see above), and patients with minor trauma usually return to normal conditions within a few days or weeks. The consequences of this massive and obviously continuous loss of sulfur are not known in detail but may be appreciated in the light of a recent study of the limiting order of amino acids in chicks fed a protein-free diet. This study identified cysteine as the single most limiting amino acid.²⁵

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