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Mechanism of erythrocyte accumulation of methylation inhibitor S-adenosylhomocysteine in uremia

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Mechanism of erythrocyte accumulation of methylation inhibitor S-adenosylhomocysteine in uremia. We have recently demonstrated that methyl esterification of erythrocyte membrane proteins, a reaction involved in recognition and repair of specifically damaged proteins, is impaired in uremia. This is accompanied by a significant increase in intracellular S-adenosylhomocysteine (AdoHcy), a potent inhibitor of methyltransferases. AdoHcy accumulation is normally prevented by its enzymatic hydrolysis to homocysteine (Hcy) and adenosine, a reversible reaction catalyzed by AdoHcy hydrolase. To assess the contribution that Hcy offers in the elevation of AdoHcy, we measured plasma and red blood cell Hcy, AdoHcy, adenosine, and S-adenosylmethionine (AdoMet) intracellular concentrations, as well as RBC AdoHcy hydrolase specific activity, in standard hemodialysis patients and normal subjects. Plasma and red blood cell Hcy levels are significantly higher in the dialysis group, and are positively correlated to AdoHcy levels. Adenosine and AdoMet levels, and AdoHcy hydrolase specific activity are not significantly different between the two groups. The enzymatic formation of labeled AdoHcy from Hcy and tracer adenosine appears to be significantly increased, *in vitro*, in erythrocytes from both control and uremic patients, when 50 μM Hcy (concentration comparable to plasma levels actually found *in vivo* in uremic patients) is added to the incubation medium. When erythrocytes from uremic patients are incubated *in vitro* in absence of Hcy, a significant reduction of intracellular AdoHcy is observed with time compared to identical samples incubated in presence of 50 μM Hcy, with a $T_{1/2}$ of approximately 270 minutes. The results allow us to conclude that plasma and red cell Hcy levels actually found in uremia can be effectively responsible for the intracellular accumulation of the toxic compound AdoHcy.

We have demonstrated that carboxyl methyl esterification of erythrocyte membrane proteins is impaired in chronic renal failure (CRF) patients treated conservatively, or on hemodialysis therapy [1]. This enzymatic reaction is involved in the recognition and repair of specific protein damages induced by spontaneous deamidation, racemization and isomerization reactions at the level of asparaginyl and aspartyl residues [2–8]. Decrease in enzymatic protein methylation in uremia is accompanied by a significant increase in the intracellular concentration of S-adenosylhomocysteine (AdoHcy) [1], a potent competitive inhibitor of most, if not all, methyltransferases [9–12]. No change in S-adenosylmethionine (AdoMet) concentration was noted; thus, the resulting inhibitory effect of the altered [AdoMet]/[AdoHcy] ratio brings about the observed reduction in membrane protein carboxyl methyl esterification. However, the mechanisms responsible for this elevation in AdoHcy and the consequent change in [AdoMet]/[AdoHcy] ratio have not yet been elucidated.

The intracellular concentration of AdoHcy is normally maintained at low levels through its enzymatic hydrolysis, catalyzed by AdoHcy hydrolase (E.C. 3.3.1.1) [9, 10, 13]. However, AdoHcy breakdown to its hydrolysis products adenosine and homocysteine (Hcy) is a readily reversible reaction [11]:

$$\text{Adenosine} + \text{Hcy} \rightleftarrows \text{AdoHcy}$$

It has been shown that the reaction *in vivo* proceeds in the direction of hydrolysis since the products are rapidly removed, while *in vitro* thermodynamics actually favor synthesis of AdoHcy [9, 10].

It is well known that plasma Hcy is significantly increased in uremic patients [14–18], and it has been reported that it is able to cross the cellular membrane barrier [11]. Such a process would induce an increase of erythrocyte Hcy, and this in turn would favor AdoHcy production, and therefore be responsible of the accumulation of AdoHcy in uremia. An inhibition of AdoHcy hydrolysis in uremia could also cause an elevation in AdoHcy. The present investigation examined the mechanism responsible for the intracellular elevation of AdoHcy in uremic patients, with the aim to elucidate if plasma levels of Hcy in the range observed in uremia can bring about an increase of erythrocyte AdoHcy.

Methods

Patients

Two groups of subjects were studied: (1) a control group of healthy individuals age and sex matched with patients of group 2; (2) patients on standard bicarbonate hemodialysis therapy thrice weekly, clinically stable at the time of investigation, age range 38 to 62 (mean 50 ± 3), treated with erythropoietin i.v. to reach a 30% hematocrit. This group in particular was selected because our previous work [1] showed that hemodialysis patients with no residual renal function displayed consistently high levels of erythrocyte AdoHcy and reduced membrane protein methyl esterification. Causes of renal failure were chronic glomerulonephritis, chronic obstructive nephropathy, or polycystic renal disease.

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Blood pressure and phosphate levels were kept under pharmacological control. Patients with systemic pathologies (hypertension, cardiac disease, diabetes mellitus, vasculitis) who were under treatment with inhibitors of cyclic nucleotide phosphodiesterases [19] were excluded from the study. Selected patients stopped vitamin or folate supplementation for at least three months prior to the study. All patients gave informed consent.

On the day of the study, routine biochemical tests (Hitachi 911 Automatic Analyzer) and blood cell count, were performed. In the dialysis group, blood was drawn immediately before the dialysis session. Blood samples were collected in the fasting state by venipuncture, using EDTA (1 mg/ml of blood).

S-adenosyl-L-[methyl-^{3}H]methionine (sp act 80 Ci/mmol) and [8-^{14}C]adenosine (sp act 46 mCi/mmol) were obtained from Amersham International, UK. D,L-Homocysteine was from Sigma Chemical Co. (St. Louis, Missouri, USA). Leibovitz L-15 modified cell medium was from ICN Biomedicals (Milan, Italy).

HPLC determination of plasma and erythrocyte Hcy

Determination of total Hcy in plasma and red blood cells (RBCs) was carried out according to the method of Ubbink et al [20], with some modifications. Whole blood was centrifuged and deprived of leukocytes and platelets, as described by Galletti et al [21], with further modifications [22]. Erythrocytes were processed along with plasma samples without any previous washings to prevent Hcy leakage from the intracellular space. Thirty microliters of a 10% solution of tri-*n*-butil-phosphine in dimethylformamide (vol/vol) were added to 0.27 ml of fresh plasma, 0.3 ml fresh packed RBCs, or Hcy standard. The mixture was incubated at 4°C for 30 minutes to obtain reduction of homocystine and the mixed disulfide cysteine-Hcy, and the release of protein-bound Hcy. Subsequently, 0.3 ml of a 10% trichloroacetic acid solution containing 1 mM EDTA, was added. After centrifuging for 10 minutes at 10000 × *g*, 100 μl of the supernatant were added to 20 μl of 1.55 M NaOH, 250 μl of 0.125 M borate buffer, pH 9.5, containing 4 mM EDTA, and 100 μl of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F, a thiol specific fluorogenic probe) solution, dissolved in borate buffer (1 mg/ml). The mixture was vortexed and incubated for one hour at 60°C to reach complete derivatization of thiols, with consequent formation of fluorescent products. Fifty microliters for plasma or 200 μl for RBC sample aliquots were utilized for isocratic HPLC analysis. A Beckman pump system Model 100A (Beckman Instruments, San Ramon, California, USA) was fitted with a Supelco (Bellefonte, Pennsylvania, USA) LC-18-DB analytical column (150 mm × 4.6 mm I.D.) provided with a Supelguard pre-column. Fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm, using a Shimadzu RF-535 fluorescence detector (Shimadzu Co., Kyoto, Japan) equipped with a Shimadzu Chromatopac C-R6A data processor. Detection conditions were optimized for Hcy. The mobile phase was 0.1 M KH_2PO_4, pH 2.1, containing 4% acetonitrile (ACN), with a flow rate of 2.0 ml/min. Retention time of Hcy was four minutes, and recovery, assessed by running samples containing an Hcy internal standard, was complete. Micromolar concentrations of Hcy are referred to 1 liter of packed erythrocytes, or to 1 liter of whole plasma.

HPLC determination of intracellular AdoHcy, AdoMet and adenosine

Erythrocytes were processed as previously described [1], and stored at −80°C for later use. In preparation for HPLC analysis, frozen packed red cells were thawed, processed and analyzed as outlined earlier [1]. Retention times were 13 minutes for adenosine, 15.1 minutes for AdoHcy, and 17.2 minutes for AdoMet. Micromolar concentrations of the indicated compounds are referred to 1 liter of packed erythrocytes.

Determination of AdoHcy hydrolase activity

AdoHcy hydrolase specific activity was determined with a radioenzymatic method coupled to HPLC analysis, by measuring the formation of AdoHcy from [8-^{14}C]adenosine and Hcy, according to the method of Kaminska and Fox [23], Hershfield et al [24], and Chiang [25], with modifications. Packed RBCs stored at −80°C were thawed and either immediately diluted 1:19 in 10 mM KH_2PO_4, pH 7.4, or diafiltrated prior to dilution. Diafiltration was performed against 20 volumes of 1 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA buffer, at 4°C, utilizing Centricon-30 Amicon microconcentrators, and repeated twice. The incubation medium contained in a final volume of 50 μl, 20 μl of diluted hemolysate, 25 mM KH_2PO_4, 1 mM dithiothreitol, 1 mM Na_2-EDTA, 5 μM erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), 10 mM Hcy, and 161 μM Ado. EHNA, an adenosine deaminase inhibitor, was added to prevent adenosine hydrolysis. The mixture was incubated for 20 minutes at 37°C, and the reaction stopped by the addition of 50 μl of 20% perchloric acid. After centrifuging for five minutes at 4000 rpm, the clear supernatant was processed in the same system described above. Radioactivity of the AdoHcy peak was collected and counted with a Beckman liquid scintillation spectrometer, model LS 7800. Results are expressed as nmol/hr/mg hemoglobin (Hb). Hb concentration was determined by measuring ultraviolet absorbance at 280 nm wavelength, as previously reported [1].

Glutamic oxalacetic transaminase (GOT) activity

GOT (E.C. 2.6.1.1) specific activity in erythrocyte cytosol was measured at 37°C, on the diluted hemolyzed samples utilized for AdoHcy hydrolase assay, obtained as follows: erythrocyte samples were diluted 1:9 with a stabilizing solution, consisting of 2.7 mM EDTA, pH 7.0, and 0.7 mM β-mercaptoethanol, followed by rapid freeze-thawing, according to Beutler [26]. The assay method described by Beutler was employed, and results expressed as U/g Hb (μmol of substrate converted/min/g Hb).

In vitro experiments

Fresh RBCs, isolated from control subjects or from uremic patients, where incubated *in vitro* under either condition A or B [modified from 11].

Condition A (short incubation). Fresh packed RBCs from control and hemodialysis patients were incubated for five minutes with and without 50 μM Hcy (a concentration comparable to that found in the plasma of uremic patients), and a tracer amount (1.3 × 10^5 cpm/ml cell suspension) of [8-^{14}C]adenosine, plus 5 μM EHNA, at a 28% hematocrit. After centrifuging to eliminate the supernatant, packed red cells were analyzed for AdoHcy as described above, and radioactivity of the AdoHcy peak was

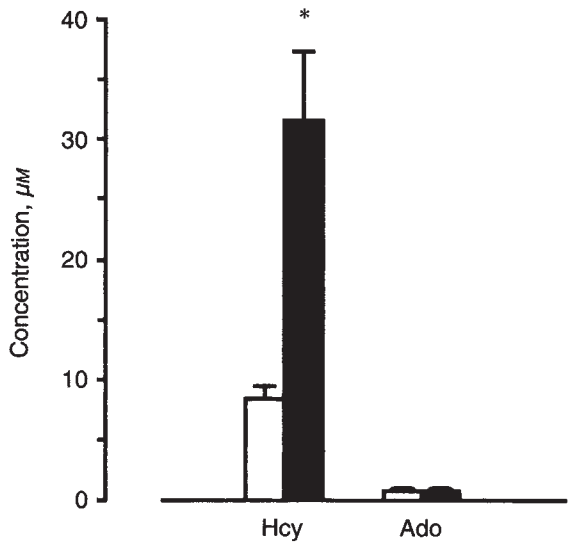

Fig. 1. *Erythrocyte Hcy and adenosine concentrations.* Determination of total Hcy in RBCs was carried out according to the method of Ubbink et al [20], with some modifications. Detection conditions were optimized for Hcy. Retention time of Hcy was 4 minutes, and recovery, assessed by addition of Hcy to samples, was complete. Adenosine RBC concentration was determined by HPLC analysis, as described under **Methods**. * $P <$ 0.001. Ado = adenosine. Symbols are: (□) control; (■) uremic.

collected and counted with a Beckman liquid scintillation spectrometer, model LS 7800.

Condition B (longer incubation). Cells from hemodialysis patients were rapidly diluted at a 10% hematocrit in the Leibovitz L-15 modified cell medium. Hcy, at a 50 μM concentration in the medium, was added, while the control was represented by cells incubated in the absence of Hcy. Cells were then incubated at 37°C for the indicated time in a shaking water bath. Cell aliquots were taken at definite times (0, 60, 120, 200, and 300 min), centrifuged to eliminate the supernatant, and AdoHcy concentration determined by HPLC as above described. In the *in vitro* experiments, hematocrit of sample aliquots was checked by means of centrifugation to ensure homogeneity among the various samples processed.

Statistical analysis

Statistical analysis was performed employing Student's unpaired *t*-test. Linear regression analysis was done to assess the independent effects of the different variables [27]. All calculations were performed using the software package StatWorks (Cricket Software, Inc., Philadelphia, Pennsylvania, USA), running on an Apple Macintosh IIfx personal computer. All results are presented as the mean ± SEM.

Results

Plasma and erythrocyte Hcy concentration, RBC AdoHcy, AdoMet, and adenosine levels

Plasma levels of Hcy in the control group are 11.78 ± 1.328 μM (N = 11); while in the patient group are 40.56 ± 6.884 μM (N = 17), a concentration significantly higher than the control, with $P <$ 0.003. Results referring to RBC Hcy and adenosine levels are shown in Figure 1 (RBC Hcy: Control N = 11, Hemodialysis N = 17; adenosine: Control N = 8, Hemodialysis N = 15). RBC concentrations of Hcy were significantly higher in the hemodialysis group compared to control. Interestingly, plasma levels were higher than RBC levels, a gradient which was detectable in both control and patient groups. A good linear correlation was present between plasma Hcy levels and red cell Hcy determined on the same blood samples (r = 0.740, $P <$ 0.01). Adenosine levels in uremic patients were not significantly different from control.

Table 1. RBC AdoHcy, AdoMet micromolar concentrations, and AdoHcy hydrolase specific activity, expressed as nmol/h/mg Hb

	Controls	Uremic patients
AdoHcy μM	0.77 ± 0.086 (N=8)	6.67 ± 1.309[a] (N=15)
AdoMet μM	2.68 ± 0.293 (N=8)	2.69 ± 0.267 (N =15)
AdoHcy Hydr Diafiltr (N = 10)	5.71 ± 0.506	6.99 ± 0.921
Nondiafiltr (N = 10)	5.81 ± 0.229	5.96 ± 0.562

Abbreviations are: AdoHcy Hydr; AdoHcy hydrolase specific activity; Diafiltr, Diafiltrated samples; Nondiafiltr, nondiafiltrated samples. AdoHcy and AdoMet concentrations were determined as previously described [1]. Micromolar concentrations of AdoHcy and AdoMet are referred to 1 liter of packed erythrocytes. AdoHcy hydrolase specific activity was determined with a radioenzymatic method coupled to HPLC analysis, by measuring the formation of AdoHcy from [8-^{14}C]adenosine and Hcy, according to the method of Kaminska et al [23], Hershfield et al [24], and Chiang [25], with modifications.

[a] $P <$ 0.005 vs. control subjects

AdoHcy levels were significantly higher in uremic patients compared to control, while AdoMet did not differ significantly from normal subjects (Table 1). Since AdoHcy inhibitory mechanism is competitive with respect to AdoMet, it is the relative concentrations of the two compounds, expressed as concentration ratio, that will determine the extent of methylation inhibition [9, 11, 28, 29]. The calculated [AdoMet]/[AdoHcy] ratio is significantly reduced in the dialysis group (Control 3.57 ± 0.381, Dialysis 0.78 ± 0.205, $P <$ 0.001), which is in agreement with the concept that the intracellular microenvironment present in these patients is not suitable for correct methyl esterification [1].

To evaluate the correlation between plasma Hcy and red cell AdoHcy, the concentrations relative to the indicated compounds were determined in identical blood samples and plotted. Figure 2 shows that a significant positive correlation between plasma Hcy and red cell AdoHcy is present (r = 0.710, $P <$ 0.001). Similarly, a significant correlation between RBC Hcy and AdoHcy (r = 0.706, $P <$ 0.01) was found.

AdoHcy hydrolase activity

The results of AdoHcy hydrolase specific activity are shown in Table 1, indicating that no significant difference was present in AdoHcy hydrolase activity between healthy control patients and uremic patients. No significant difference was also noted when the enzymatic assays were performed using diafiltrated samples as enzyme sources. Diafiltration was meant to deplete erythrocytes of inhibiting compounds possibly present in the samples, and/or of endogenous substrates.

It has been reported that AdoHcy hydrolase activity in younger erythrocytes is higher than older erythrocytes [23]. Since it is well known that in uremia red cell age can be shorter than average [30], due to increased hemolysis and red cell fragility [31, 32], we

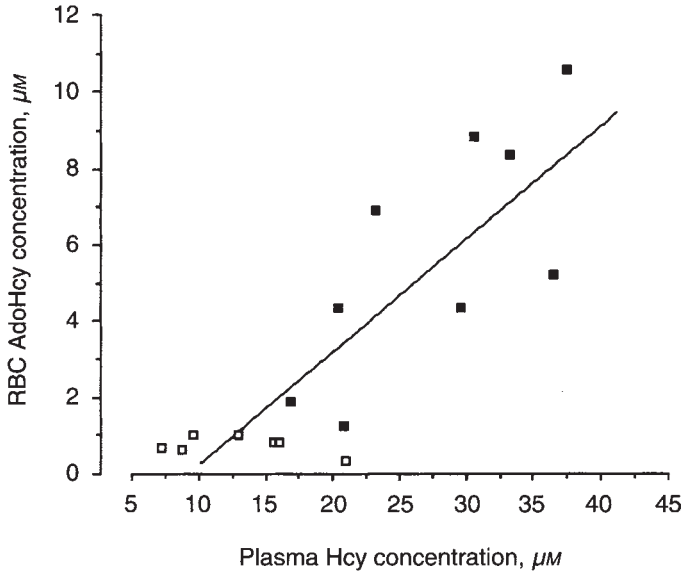

Fig. 2. *Correlation between plasma Hcy and AdoHcy.* AdoHcy RBC concentration was determined by HPLC analysis, as previously described [1]. Micromolar concentrations of the indicated compounds are referred to 1 liter of packed erythrocytes. A scatter plot of Hcy values (μM), in the abscissa, relative to the respective AdoHcy levels (μM), in the ordinate, is shown. Open symbols represent control, while closed squares represent hemodialysis patients. A positive simple correlation between plasma Hcy and AdoHcy concentrations, $r = 0.71$, $P < 0.001$, is documented.

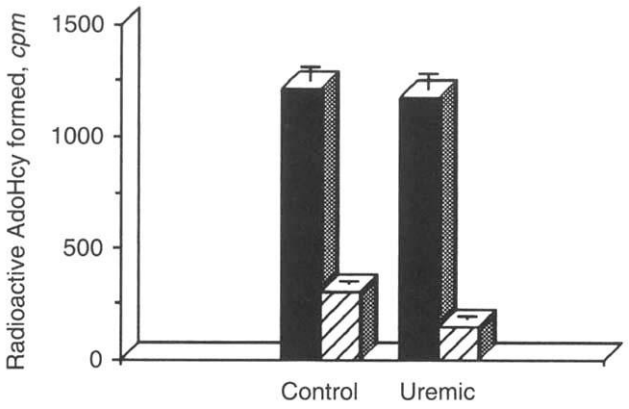

Fig. 3. *AdoHcy formation in RBCs of control and hemodialysis patients in vitro.* RBCs from control and hemodialysis patients were shortly incubated *in vitro* under condition A (**Methods**), with [8-^{14}C]adenosine, either in the presence (■) or in the absence (▨) of 50 μM Hcy. At the end of the treatment cells were rapidly processed, AdoHcy isolated by HPLC, and radioactivity coeluting with AdoHcy was measured by liquid scintillation counting. The hematocrit of sample aliquots was checked by means of centrifugation to ensure homogeneity among the various samples processed, and found to be not different from 26% in all samples. Error bars show the range of triplicate measurements. Condition A produced a marked rise in incorporated radioactivity measured as AdoHcy only in the set of samples incubated with Hcy.

measured GOT activity as a marker of red cell age [33], in order to check if enzyme activity correlated with RBC life span. No significant correlation was found between AdoHcy hydrolase activity and GOT (data not shown), and there was no significant difference in GOT activity (Control 5.03 ± 0.29, Hemodialysis 5.66 ± 0.40 U/g Hb, P = NS).

Hcy effects on AdoHcy biosynthesis and intracellular accumulation in vitro

To verify the ability of Hcy to induce and subsequently maintain high AdoHcy intracellular levels at a concentration equal to that present chronically in hemodialysis patients, the following experiments were performed.

To put in evidence the possible formation of AdoHcy from Hcy under conditions similar to those of dialysis patients, we treated RBCs from control and hemodialysis patients under condition A, characterized by the presence of tracer amounts of labeled adenosine in the medium (**Methods**). In this experiment parallel samples were incubated *in vitro* with or without 50 μM Hcy. The concentration of this compound was chosen on the basis of plasma concentrations we actually found *in vivo* in hemodialysis patients. Results in Figure 3 show a marked rise in radioactive AdoHcy only in the set of samples incubated with Hcy, both in the control, and in the hemodialysis patient group.

A time course experiment was performed by incubating pooled RBCs from hemodialysis patients under condition B (**Methods**). Erythrocyte samples from hemodialysis patients were incubated either in the presence or in the absence of 50 μM Hcy. Aliquots were taken at 0, 60, 120, 200 and 300 minutes, centrifuged, and the packed RBCs thus obtained were processed for AdoHcy HPLC assay. Results are shown in Figure 4. AdoHcy concentrations in the samples incubated in the absence of Hcy were expressed, at each time point, as percentage of the corresponding values observed in identical samples incubated in the presence of Hcy. It can be noted that a time dependent decrease of intracellular AdoHcy took place when Hcy was removed from the medium, with a $t_{1/2}$ of approximately 270 minutes.

Discussion

The present study shows that both plasma and RBC Hcy levels are significantly higher in uremic patients undergoing hemodialysis therapy, with respect to normal healthy controls. Hcy, which is known to easily cross the red cell membrane [11], builds up in red cells, and the actual RBC levels found in uremic patients are almost four times higher than normal cells. A significant correlation between plasma Hcy levels and red cell Hcy is present.

Conversely, adenosine content is not different between the two groups, indicating that this compound plays no apparent role in the elevation of intracellular AdoHcy detected *in vivo* in hemodialysis patients.

Furthermore, the levels of red blood cell AdoHcy, which are shown to be increased in these patients compared to control, correlate significantly with both plasma and intracellular Hcy.

Specific activity of AdoHcy hydrolase is not significantly different compared to control, either in the extensively diafiltrated or in the nondiafiltrated samples. Diafiltration was performed in order to reduce the impact of any possible inhibitory substance on this enzyme, or the effects of endogenous substrates.

In the end, our data do not support the view that the accumulation of AdoHcy is caused by a decreased enzyme function in hemodialysis patients.

The *in vitro* experiments that involve the use of concentrations of Hcy in the range present in uremic plasma (Fig. 3), thus closely resembling an *in vivo* situation, demonstrate that, in RBCs from control and from hemodialysis patients incubated with tracer

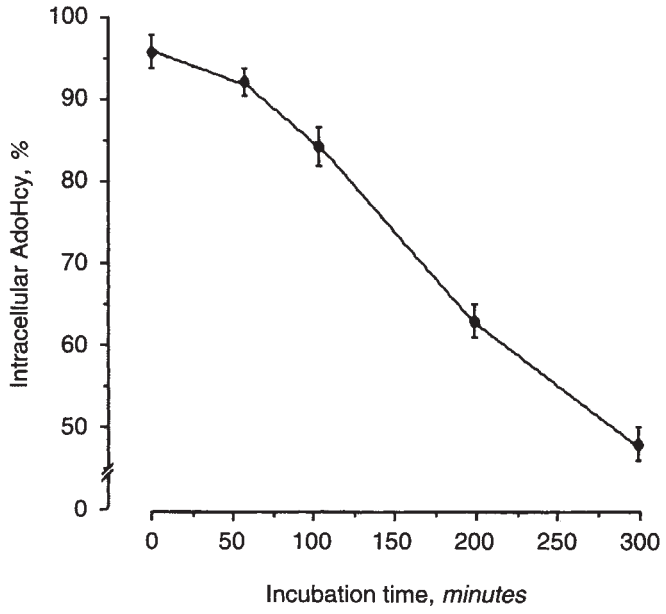

Fig. 4. *Time dependent Hcy effects on RBC AdoHcy content of hemodialysis patients in vitro.* RBCs from hemodialysis patients were incubated *in vitro* under condition B, as detailed under **Methods**, either in the absence, or in the presence of 50 μM Hcy, a concentration comparable to what actually found *in vivo* in hemodialysis patients' plasma. At the indicated time points cell aliquots were withdrawn and intracellular AdoHcy determined by means of HPLC. AdoHcy concentrations in the RBCs incubated with Hcy were always significantly lower than those found in the corresponding samples incubated in parallel in the presence of Hcy. Results are expressed as percentage of AdoHcy content measured in the "minus Hcy" sample, compared to the value measured in the "plus Hcy" sample. Error bars show the range of triplicate measurements. Initial intracellular AdoHcy was 7.6 ± 0.3 μM. The hematocrit of sample aliquots was checked by means of centrifugation to ensure homogeneity among the various samples processed, and found to be not different from 9% in all samples.

radioactive adenosine and 50 μM Hcy, increased Hcy can induce net formation of AdoHcy intracellularly.

Moreover, the time course experiment shows that when erythrocytes from hemodialysis patients are kept in an environment where Hcy is absent, the AdoHcy intracellular concentration falls with time, if compared with the set of RBCs that were incubated with Hcy.

The latter experiment substantiates the view that even in an environment of chronic exposure to high Hcy, such as uremia, the intracellular accumulation of this compound and its effects can be at least partially reversed by effective removal of the extracellular compound. In this respect it should be pointed out that Wilcken and coworkers [17] reported a significant decrease of plasma Hcy in dialysis patients treated with folic acid as co-factor in the enzymatic conversion of Hcy to methionine.

AdoMet is the universal methyl donor of enzymatic methylation reactions [34]; thus it is involved in the biosynthesis, modification, or regulation of the function of phospholipids, proteins, nucleic acids, small molecules, etc. AdoHcy, the demethylated product of AdoMet, is a natural inhibitor of all these metabolic processes [9–12, 28, 29].

We have demonstrated that in CRF a significant increase of RBC AdoHcy is present, while AdoMet concentration is not different from control [1]. This pattern is not modified in patients requiring maintenance hemodialysis therapy, but on the contrary it is enhanced [1]. Furthermore, the intracellular ratio [AdoMet]/[AdoHcy], an indicator of the inhibition potential of AdoHcy on AdoMet-dependent enzymatic reactions, decreases 5- to 10-fold in the patient population [1].

Type II MTase (D-aspartyl/L-isoaspartyl protein carboxyl methyltransferase, E.C. 2.1.1.77) is an ubiquitous enzyme implicated in the repair of specific structural damages, due to asparaginyl deamidation and aspartyl isomerization mechanisms spontaneously occurring in proteins [3, 35–40], and leading to less active or potentially toxic protein derivatives [7, 8, 39]. A significant decrease in erythrocyte membrane protein methyl esterification, catalyzed by Type II MTase, accompanied the noted increase in AdoHcy [1]. In particular, the reduction was significant for band 2.1 [1], a cytoskeletal component involved in membrane stability and integrity [41]. In the end, it appears that damaged membrane proteins accumulate in CRF and are not adequately repaired, and that this is due to the high levels of AdoHcy present.

The results presented in this paper allow us to trace the abnormal build up of the inhibitor AdoHcy to the abnormal elevation of Hcy occurring in these patients.

The intracellular accumulation of AdoHcy is normally prevented by the action of the enzyme AdoHcy hydrolase, which catalyzes the reversible hydrolysis of AdoHcy to adenosine and Hcy [9, 10, 12, 29]. As a result, under normal conditions (that is, low Hcy present), transmethylation reactions are not likely to be affected by AdoHcy [9, 10]. The equilibrium constant for AdoHcy hydrolysis is highly unfavorable, so Hcy must be further metabolized, along with adenosine, in order to insure AdoHcy cleavage, and prevent its accumulation. On the other hand, Hcy can be converted to AdoHcy, if in excess, through the action of the same enzyme.

Hyperhomocysteinemia is a common feature in CRF patients, either treated conservatively or necessitating dialysis. It has been reported for the latter group a three- to fourfold increase of Hcy [14–16], which is in agreement with our present findings.

According to Laidlaw et al [15], elevated plasma Hcy can only be partially lowered during a hemodialysis session. High plasma Hcy can be a consequence of decreased renal excretion caused by nephron loss, decreased metabolism to cystathionine, or to impaired remethylation to methionine, due to relative folic acid deficiency [17, 18].

It is widely acknowledged that elevated plasma Hcy is an independent risk factor for occlusive arterial disease, and it is significantly more common in asymptomatic adults with carotid artery wall thickening, in a recent case-control study [42, 43]. Since chronic uremic patients exhibit a higher incidence of cardiovascular disease, high plasma Hcy is regarded among the risk factors that play a role in the genesis of premature atherosclerosis in these patients [16, 17].

In our opinion, the information provided in this work broadens the scope of studies concerning the effects of moderately high Hcy in blood. Actually, it appears that a conspicuous increase of Hcy is not needed in the high micromolar or millimolar range for Hcy to exert detectable biological effects, but even a modest increase, such as it is present in hemodialysis patients, can determine tangible *in vivo* effects that can be quantified. This last notation requires some comment. Published experiments on Hcy biological and pathological effects are often designed to evaluate possible actions of Hcy present in high plasma concentrations

specifically as it is found in a condition such as homocystinuria [44–46]. Untreated patients with homocystinuria usually have fasting Hcy levels of 0.2 mM; their non fasting levels may be higher [46].

In summary, this study supports the proposal that accumulation of AdoHcy is due to the rise in Hcy levels present in these patients. First, plasma and erythrocyte levels of Hcy correlate with AdoHcy intracellular levels. Second, control cells incubated with Hcy present at levels comparable to those of uremic subjects show a net formation of AdoHcy. Third, red cells from hemodialysis patients when incubated with Hcy give way to a rise in intracellular AdoHcy, like control subjects. Fourth, the absence of Hcy from the incubation medium induces a time dependent decrease in erythrocyte AdoHcy. Fifth, the enzyme AdoHcy hydrolase specific activity is normal. Moreover, no difference was found in the intracellular concentration of adenosine in RBCs of hemodialysis patients compared to control, indicating that *in vivo* the increase in AdoHcy concentration is solely due to Hcy accumulation.

In conclusion, our results suggest that in uremia AdoHcy represents a preferential deposit form of Hcy in the intracellular compartment, at least in erythrocytes. Consequently, AdoHcy can be considered a prospective highly toxic metabolite of Hcy, since it is able to interfere with many AdoMet-dependent pathways involved in the regulation of crucial cell functions [47–53].

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