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Impairment of the extrusion transporter for asymmetric dimethyl-L-arginine: A novel mechanism underlying vasospastic angina

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ABSTRACT

A 37-year old male patient presented with frequent angina attacks (up to 40/day) largely resistant to classical vasodilator therapy. The patient showed severe coronary and peripheral endothelial dysfunction, increased platelet aggregation and increased platelet-derived superoxide production. The endothelial nitric oxide synthase (eNOS)-inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) reduced superoxide formation in platelets identifying "uncoupled" eNOS as a superoxide source. Oral L-arginine normalized coronary and peripheral endothelial dysfunction and reduced platelet aggregation and eNOS-derived superoxide production. Plasma concentrations of the endogenous NOS inhibitor asymmetric dimethyl-L-arginine (ADMA), representing an independent risk factor for cardiovascular disease, were normal in the patient. However, immediately after oral administration of cationic amino acid (CAA), plasma ADMA levels rose markedly, demonstrating increased ADMA efflux from intracellular stores. ADMA efflux from mononuclear cells of the patient was accelerated by CAA, but not neutral amino acids (NAA) demonstrating impairment of y^+LAT (whose expression was found reduced in these cells). These data suggest that impairment of y^+LAT may cause intracellular (endothelial) ADMA accumulation leading to systemic endothelial dysfunction. This may represent a novel mechanism underlying vasospastic angina and vascular dysfunction in general. Moreover, these new findings contribute to the understanding of the L-arginine paradox, the improvement of eNOS activity by oral L-arginine despite sufficient cellular L-arginine levels to ensure proper function of this enzyme.

1. Introduction

Vasospastic angina describes a form of angina pectoris caused by coronary artery spasm, which is brought about by a sudden occlusive vasoconstriction of a segment of the epicardial artery, resulting in a dramatic reduction of coronary blood flow [1]. In the most classical form, vasospasms occur at rest (Prinzmetal's variant angina) but may be also triggered by exercise [1]. Importantly, spasms may occur in coronary arteries with various degrees of stenosis but also in angiographic normal arteries. Vasospastic angina represents about 2.0% of hospital admissions due to unstable angina. Smoking is the only recognized risk factor and there is a 5:1 male/female ratio. The pathogenesis may involve either endothelial dysfunction due to decreased vascular NO bioavailability (for review see [2]) and/or hyperreactivity of the vascular smooth muscle to vasoconstrictors [3,4]. Interestingly, several studies have demonstrated simultaneous occurrence of endothelial dysfunction in brachial and coronary arteries pointing to a systemic vascular disease rather than a phenomenon restricted to coronary arteries [5,6]. Previous studies have shown that endothelial dysfunction in patients with vasospastic angina is associated with decreased basal NO production [7,8].

More recent reports demonstrated that concentrations of the eNOS inhibitor asymmetric dimethyl-L-arginine (ADMA) in the coronary circulation are higher in patients with vasospastic angina than in controls [9]. However, the plasma concentrations in the coronary circulation are not high enough to explain eNOS uncoupling.

Abbreviations: AA, amino acid; CAA, cationic AA; NAA, neutral AA; ADMA, asymmetric dimethyl-L-arginine; DDAH, dimethylarginine dimethylaminohydrolase; (h)CAT, (human) cationic amino acid transporter; FMD, flow mediated dilation; LAD, left anterior descending artery; L-NAME, N (G)-nitro-L-arginine methyl ester; LPI, lysinuric protein intolerance; (e)NOS, (endothelial) NO synthase; NTG, nitroglycerin; PDBU, phorbolester dibutyrate; PRMT, protein arginine methyltransferase; qRT-PCR, quantitative reverse transcription and PCR; ROS, reactive oxygen species; y^+LAT, system y^+L amino acid transporter.

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Whether ADMA accumulates in endothelial cells under pathophysiological conditions remains an open question. ADMA is formed in cells by degradation of proteins containing arginine residues that have previously been methylated by S-adenosylmethionine-dependent methyltransferases (PRMTs). There are two major routes of ADMA elimination: renal excretion and enzymatic degradation by dimethylarginine dimethylaminohydrolases (DDAH-1 and -2) [10]. Because DDAH is mainly present in liver and kidney, both elimination routes require export from the ADMA-generating (endothelial) cell to the plasma. We hypothesized that ADMA transport is mediated by the same carrier proteins as transport of other cationic amino acids (CAA). The major CAA transporters in non-epithelial cells belong to two related protein families: the CAA transporters (CAT) and the system y^+L AA transporters (y^+LAT) [11]. However, a physiological role of CAA export from non-epithelial cells has not been demonstrated so far.

Our present study identifies system y^+L as the major export route for ADMA under physiological conditions. It further indicates that a reduction in system y^+L-mediated ADMA efflux from endothelial cells may lead to intracellular ADMA accumulation and eNOS uncoupling and may account – at least in part – for systemic endothelial dysfunction and for the variant angina symptoms observed in the patient in this case report.

2. Materials and methods

For detailed protocols see online Supplement.

3. Results

3.1. Patient characteristics

The 37 year old patient without any cardiovascular risk factor experienced up to 40 attacks of angina pectoris per day despite medical treatment with isosorbide dinitrate (40 mg b.i.d.), amlodipin (5 mg b.i.d.), aspirin (100 mg q.d.), clopidogrel (75 mg q.d.) and cholesterol-lowering therapy with statins. The attacks responded to short term treatment with nitroglycerin spray only. In addition, the patient required treatment with antidepressants. There were no other concomitant diseases. All laboratory values including triglycerides and cholesterol were within normal limits. All previous diagnostic procedures including resting ECG, bicycle ergometry, echocardiogram, stress echocardiogram and diagnostic catheterization revealed no abnormalities at all. The patient was admitted to our hospital with the working diagnosis of vasospastic angina pectoris for further diagnostic evaluation and optimization of treatment.

3.2. Coronary and peripheral endothelial function before and after L-arginine treatment

Since vasospastic angina has been demonstrated to be associated with – or secondary to – endothelial dysfunction (see Section 4), we determined coronary endothelial function by an intracoronary infusion of the endothelium-dependent vasodilator acetylcholine. At an estimated intracoronary concentration of $10^{-7.3}$ M, acetylcholine caused a complete occlusion of the left anterior descending artery (LAD), which responded nicely to intracoronary nitroglycerin (NTG, 0.25 mg) (Fig. 1A–E). After L-arginine treatment for 3 mo (6 g q.d.), the patient tolerated all intra-coronary acetylcholine concentrations (from $10^{-7.3}$ M to $10^{-5.6}$ M) and even responded with a dilation of the LAD indicating good endothelial function.

In the patient, we also established severe peripheral endothelial dysfunction. He achieved only 3% flow-mediated dilation (FMD) of the brachial artery (Fig. 1F) compared to 8–12% in age-matched controls (data not shown). Upon chronic L-arginine treatment (6 g t.i.d.), the FMD of the peripheral artery increased from 3% to 13% (Fig. 1F). The FMD decreased to 8% after a 4 mo pause in the L-arginine treatment, but came back to 13% in response to a single L-arginine dose (9 g). These data demonstrate that L-arginine was very efficient in restoring and maintaining endothelial function in this patient. In contrast, endothelium-independent dilatation in response to NTG (0.8 mg, sublingual) was unchanged by the arginine treatment (Fig. 1F).

3.3. Effects of oral L-arginine treatment on platelet superoxide production and aggregatory response

Reactive oxygen species (ROS) formation measured in isolated washed platelets from the patient was significantly higher than in platelets from a healthy control subject (Fig. 1G). The NOS inhibitor L-NAME (that blocks both, eNOS-dependent NO and ROS formation) increased the superoxide signal in control platelets (due to inhibtion of NO production). In the patient's platelets it strongly decreased the signal. These observations are compatible with eNOS uncoupling. Chronic treatment with L-arginine (6 g t.i.d.) decreased oxidative stress almost to control levels. L-NAME had only a marginal effect on ROS formation in these platelets. In contrast, when the L-arginine treatment was paused, ROS formation by the patient's platelets was again very high and could be inhibited by L-NAME. This situation was reversed by a single dose of L-arginine (9 g).

In addition to ROS formation, platelet aggregation in response to the protein kinase C stimulator phorbolester dibutyrate (PDBU, 10 μM) was clearly enhanced in the patient as compared to the control subject. Treatment with L-arginine (6 g q.d. for 3 mo) markedly inhibited PDBU-induced platelet aggregation in the patient whereas platelet aggregatory responses remained unchanged in the control subject (Fig. 1H).

3.4. L-arginine handling

Initial amino acid determinations revealed reduced L-arginine and elevated L-ornithine plasma concentrations in the patient (18 and 117 μM, respectively). The good clinical and endothelial function response to acute and chronic L-arginine treatment suggested that an L-arginine deficiency might underlie the clinical symptoms of the patient. In order to monitor L-arginine resorption and metabolism, we compared the level of L-arginine and its metabolites in plasma and urine of the patient and control subjects after a single oral dose of 9 g L-arginine. The increase in plasma L-arginine was similar in both, patient and control subject, reaching a peak of 400–500 μM between 30 min and 2 h and slightly decreasing thereafter (Fig. 2A). Note that at this point, the patient had normal basic L-arginine plasma levels due to a long term L-arginine treatment (6 g t.i.d.) that was suspended for only 10 d prior to the measurement. However, after longer pauses in the L-arginine treatment, plasma L-arginine levels dropped again to 20–50 μM (data not shown). The L-arginine, L-ornithine, L-proline, L-glutamine and L-glutamate content of urine collected during three hours after L-arginine intake did not differ significantly between patient and controls (data not shown). In addition, the patient showed normal urea-derived nitrogen in plasma and urine. However, after L-arginine intake, plasma L-ornithine levels increased strikingly more then in control subjects (data not shown). In addition, the patient exhibited elevated L-glutamine concentrations in the plasma that were further increased upon L-arginine intake (data not shown). Taken together these data show normal L-arginine resorption and metabolism in the patient under L-arginine treatment, but a somehow reduced metabolism of L-ornithine and its metabolite L-glutamine.

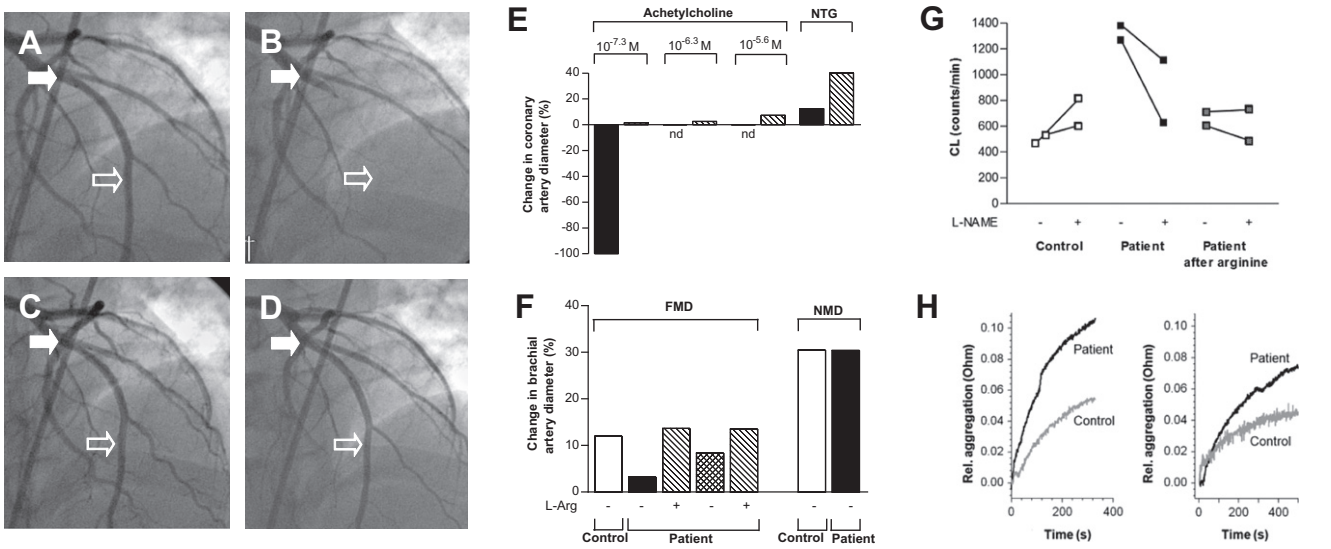

Fig. 1. Effects of oral L-arginine treatment on coronary and peripheral endothelial function as well as platelet function. (A–D) Original angiograms of the left anterior descending artery (LAD) under baseline conditions (A + C) and in response to intracoronary acetylcholine (B + D), before (A + B) and after (C + D) chronic L-arginine treatment of the patient (6 g q.d. for 3 mo). Estimated intracoronary acetylcholine concentrations: (B) $10^{-7.3}$ M; (D) $10^{-5.6}$ M. White arrow points to the infusion catheter, open arrows indicate the LAD. (E) Percent changes in the diameter of the coronary artery in response to acetylcholine (estimated intracoronary concentrations indicated) and subsequent nitroglycerin (NTG) before (black bars) and after (hatched bars) the chronic L-arginine treatment. (F) Peripheral endothelial function of the patient, and a control subject as indicated, was assessed by measuring flow-mediated dilation (FMD) of the brachial artery (left). Measurements in the patient were taken before oral L-arginine treatment (black bar), after several months chronic L-arginine treatment (6 g t.i.d., 1st hatched bar), after a subsequent 4 mo pause in L-arginine treatment (double hatched bar) and after a single L-arginine dose (9 g) following the L-arginine pause (2nd hatched bar). NTG (0.8 mg, sublingual)-mediated dilation of the same artery before and after chronic L-arginine treatment was measured as control for endothelium-independent dilation (right). (G) Superoxide production, with and without L-NAME (100 μM), by washed platelets derived from a control subject or from the patient (as indicated). Patient measurements were taken at different phases of L-arginine treatment as described above. (H) Platelet aggregation in whole blood from the patient (black lines) in response to the protein kinase C stimulator phorbolester dibutyrate (PDBU, 10 μM) was measured before (left panel) and after (right panel) a 3 mo L-arginine treatment (6 g q.d.). Platelet aggregation of a control subject was determined in parallel in both experiments (gray lines).

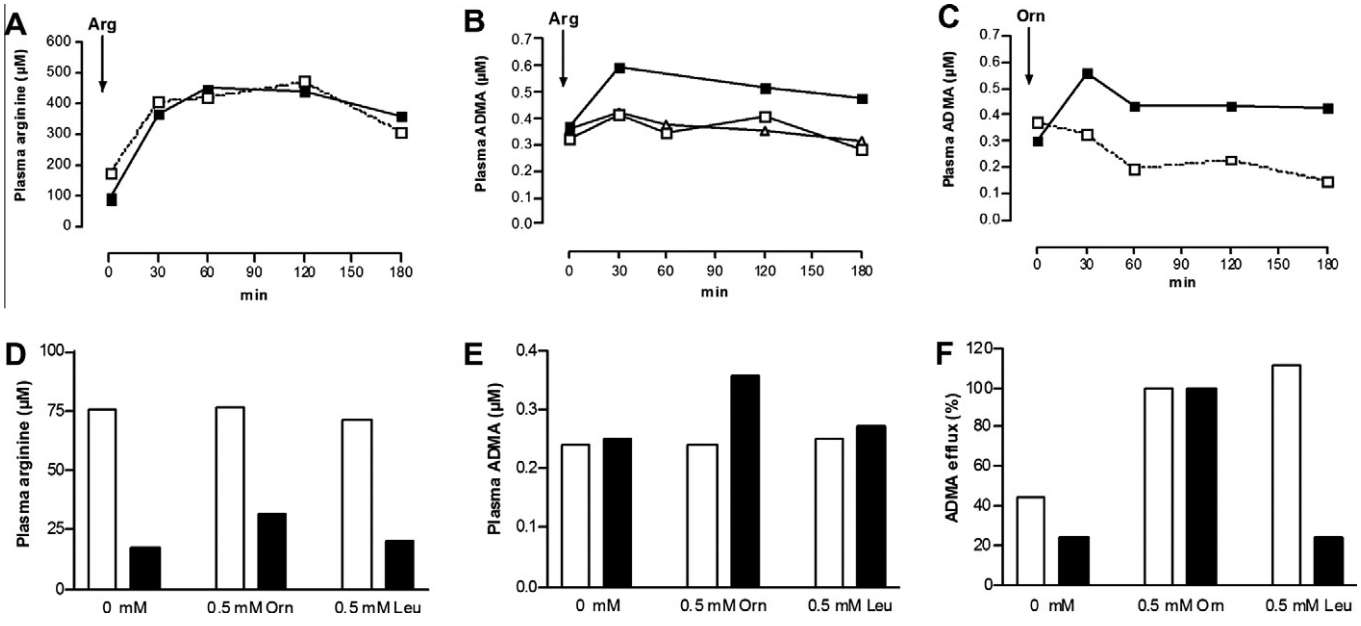

Fig. 2. Increase in ADMA plasma levels and ADMA export following an oral administration of CAA. L-arginine treatment (6 g t.i.d.) of the patient was paused for 10 d and subsequently, blood was drawn immediately before and after different time intervals (indicated) following a single oral dose of 9 g L-Arginine (A and B). Three months later, the same scheme was applied for a single dose of 9 g L-ornithine (C). Plasma concentrations of L-arginine (A) and ADMA (B and C) were determined by HPLC. Filled squares: patient; Open symbols: control subjects. (D–F) L-arginine treatment (6 g t.i.d.) of the patient was stopped for 4 weeks. Subsequently, whole blood of the patient (black columns) or control subject (white columns) was drawn and either remained untreated or supplemented with L-ornithine or L-leucine to raise the concentration of the respective amino acid by 0.5 mM. All samples were incubated for 30 min at 37 °C. Plasma concentrations of L-arginine (D) and ADMA (E) were then determined by HPLC. (F) Equal aliquots of isolated leukocytes from the patient (black columns) or control subject (white columns) were incubated for 30 min at 37 °C in buffer containing either no amino acids, 0.5 mM L-ornithine, or L-leucine as indicated. Cells were then removed by centrifugation and ADMA concentrations in the supernatants were determined by HPLC. Data are expressed as percent of efflux measured at 0.5 mM L-ornithine of the respective leukocyte preparation.

3.5. Increase of ADMA plasma levels in response to administration of CAA

Interestingly, 30 min after L-arginine intake, plasma ADMA levels in the patient were almost doubled and stayed elevated throughout the 3 h observation period (Fig. 2B). On the other hand, ADMA levels remained unchanged in control subjects. This suggested that in the patient ADMA had accumulated intracellularly and that the elevated L-arginine concentrations facilitated its otherwise impaired export. If true, ADMA export should also be enhanced by elevated plasma concentrations of another CAA, for example by L-ornithine recognized by all known L-arginine transporters with similar affinity as L-arginine. Indeed after a single dose of 9 g L-ornithine, ADMA levels in the patient's plasma increased to a similar extent as after the L-arginine intake (Fig. 2C), while the normal subject exhibited a decrease in ADMA plasma concentrations.

In a further independent experiment, we observed an increase of both L-arginine and ADMA in whole blood from the patient (collected after a four week period without L-arginine treatment) upon addition of the CAA L-ornithine, but not the NAA L-leucine (0.5 mM each) (Fig. 2D + E). This demonstrates that cellular efflux of CAA occurred preferentially via CAT and not y^+LAT in the patient. In contrast, addition of either amino acid did not change L-arginine or ADMA concentrations in blood from control subject, compatible with effective export through system y^+L at physiological high concentrations of NAA. To further prove a defect in y^+L-mediated transport in the patient, we investigated ADMA efflux from isolated leukocytes incubated in buffer containing either no amino acids, L-ornithine (0.5 mM), or L-leucine (0.5 mM). As expected, in leukocytes from the patient, ADMA efflux was only stimulated by L-ornithine, but not L-leucine, while both amino acids increased ADMA efflux from leukocytes of a control subject (Fig. 2F).

3.6. Reduced expression of y^+LAT1 in peripheral blood mononuclear cells of the patient

To investigate if the patient had a defect in his system y^+L transporters, we isolated mRNA from his peripheral blood leukocytes and cloned cDNAs encompassing the entire coding region of y^+LAT1 and the related transporter y^+LAT2, respectively. Sequence analysis revealed no mutation in the deduced amino acid sequence of either transporter (data not shown). However, the patient exhibited a significant reduction in y^+LAT1 expression compared to control subjects (Fig. 3), whereas no reduction in CAT-1 expression was found in his cells.

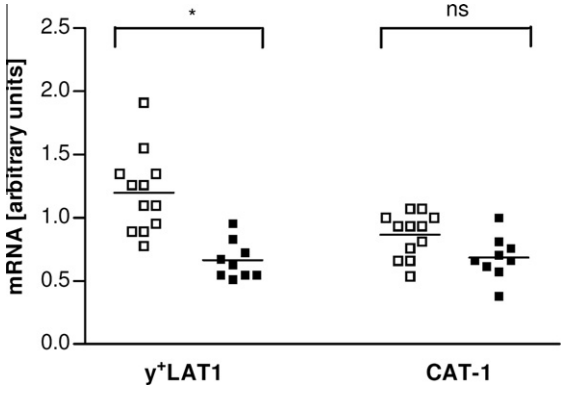

Fig. 3. Decreased expression of y^+LAT1 in peripheral mononuclear cells of the patient. Total mRNA from peripheral blood mononuclear cells was analyzed by qRT-PCR for y^+LAT1 and CAT-1 expression in the patient (closed squares) and control subjects (open squares). GAPDH was chosen as housekeeping gene for relative determinations. Data represent means ± SEM from 9 to 12 independent mRNAs prepared from blood samples collected at different days.

4. Discussion

How can eNOS uncoupling and the good response of this patient to L-arginine be explained? There are probably several factors that contribute to this particular setting: First, the low plasma L-arginine levels detected prior to L-arginine treatment may have caused an overall L-arginine deficiency in tissues. However, isolated eNOS has a very high substrate affinity (2 μM [12]) and should have been saturated with L-arginine even at the lowest plasma concentrations measured (20 μM giving rise to an estimated intracellular concentration of about 200 μM). In addition, L-arginine treatment was still beneficial for the patient after he had reached normal plasma concentrations (about 100 μM). Second, high L-glutamine concentrations may contribute to the strong L-arginine dependence of eNOS in the patient. L-Glutamine has been found to interfere with eNOS activity through depletion of the eNOS co-factor NADPH [13] and of L-citrulline (thus hindering its recycling back to L-arginine) [14]. Under high L-glutamine concentrations, eNOS may thus be especially in danger of uncoupling. Third, accumulation of intracellular ADMA may lead to eNOS inhibition or even uncoupling in the patient. The CAA-induced release of ADMA into the plasma strongly suggests that AMDA had accumulated intracellularly in the patient and that its efflux was facilitated by CAA. It is thus probably the combination of these three factors: low L-arginine, high L-glutamine and high intracellular ADMA concentrations that lead to eNOS uncoupling and contribute to the development of vasospastic angina in our patient.

We were not able to identify the cause for the low L-arginine and high L-ornithine concentrations in the patient's plasma, prior to L-arginine treatment, since after the L-arginine treatment intestinal absorption, renal resorption and urea production (a measure for L-arginine metabolism to L-ornithine) in the patient did not differ from age-matched controls. Also, arginase expression in peripheral blood monocytes was comparable to control subjects under L-arginine therapy. However, we found an increased arginase expression in these cells after a longer withdrawal of the L-arginine treatment (data not shown). In addition, L-arginine levels decreased and L-ornithine levels increased under L-arginine withdrawal. We can thus not exclude elevated arginase expression and/or activity as a cause for the disregulated L-arginine to L-ornithine ratio in the untreated patient. However the pronounced and sustained elevation in the plasma concentrations of L-ornithine and its metabolites (L-proline, L-glutamine and L-glutamate) after a single dose of either L-arginine or L-ornithine (data not shown) indicates a dysregulation in the metabolism and interconversion of these amino acids in the patient. Hyperornithinemia–hyperammonaemia–homocitrullinaemia (HHH) syndrome caused by a defect in the mitochondrial ornithine transporter-1 [15] can be excluded, because plasma ammonia was not elevated in the patient (data not shown). In addition, the elevation of L-glutamate and L-proline in response to L-ornithine intake indicates intact mitochondrial transport, because the first (common) step of these pathways takes place in mitochondria. Together with the finding, that L-arginine synthesis from L-ornithine and citrulline was normal in the patient, these findings point rather to a defect in L-arginine catabolism than synthesis.

Sole derangement of L-arginine homeostasis (as found with several defects of urea cycle enzymes and transporters) is not commonly associated with endothelial dysfunction (for review see [16]). The altered amino acid levels in the patient are thus highly unlikely to be the single cause for eNOS uncoupling and the occurrence of vasospastic angina. We thus assume that the defect in ADMA efflux detected in the patient may play a crucial role in

these processes. In this study, we establish system y^+L as an export route for ADMA. Based on the high concentration of large NAA in human plasma, we assume that exchange of intracellular ADMA against extracellular NAA efficiently extrudes the endogenous NOS inhibitor. In addition, this exchange is driven by the Na^+ gradient thus representing an active export mechanism. We thus postulate that system y^+L represents the major ADMA export pathway under physiological conditions. Given that members of this family are often asymmetric in terms of substrate recognition, it is tempting to speculate that ADMA may be recognized with higher affinity then other cationic amino acids at the intracellular substrate binding site of y^+LAT1 and/or y^+LAT2. An impairment of this transport system seems to cause intracellular (endothelial) ADMA accumulation leading to eNOS uncoupling and systemic endothelial dysfunction, and may thus represent a novel mechanism underlying vasospastic angina.

Interestingly, the loss of y^+LAT1 in LPI (lysinuric protein intolerance) patients is associated with reduced NO production and endothelial dysfunction [17,18]. This has been ascribed to the low L-arginine concentration in the plasma of these patients. However, our results suggest that reduced ADMA export may contribute considerably to the endothelial dysfunction observed in these patients. The patient described in the present study is not an LPI patient, as no mutation in either y^+LAT isoform was detected. In addition, the patient exhibited normal absorption and reabsorption of cationic amino acids. As heterozygous carrier of mutated y^+LAT1 have no conspicuous phenotype, the reduced expression of y^+LAT1 detected in peripheral blood mononuclear cells of our patient is unlikely to explain the complete lack of system y^+L activity observed in these cells. We thus assume a functional impairment of system y^+L in specific cell types and organs. The cause for this functional impairment remains elusive, however.

The finding of impaired ADMA efflux from endothelial cells as a cause for eNOS uncoupling sheds new light on the so called L-arginine paradox, the phenomenon that L-arginine often improves NO-mediated vascular function in vivo, although its baseline plasma concentration is much higher than the Michaelis–Menten constant $K(m)$ of the isolated, purified eNOS in vitro [12]. L-arginine -mediated facilitation of the export of intracellularly accumulated ADMA could explain this paradox.

In our study, we provide evidence for eNOS uncoupling as an important mechanism for endothelial dysfunction in a patient with variant angina that could be overcome with oral L-arginine treatment. We further identify system y^+LAT as the major export pathway for the NOS inhibitor ADMA and establish that decreased activity of this ADMA extrusion transporter may contribute to eNOS uncoupling in platelets and to coronary and peripheral endothelial dysfunction observed in our patient. CAT-1 is likely to represent an alternative efflux pathway for ADMA, but this requires high CAA concentrations not normally found in human plasma (see scheme Fig. 4). Finally, the present data significantly contribute to the understanding of the L-arginine paradox that is based on the observation that, under certain pathophysiological conditions, extracellular L-arginine supplementation improves endothelium-dependent vasomotor responses despite high intracellular L-arginine concentrations (sufficient for proper eNOS function).

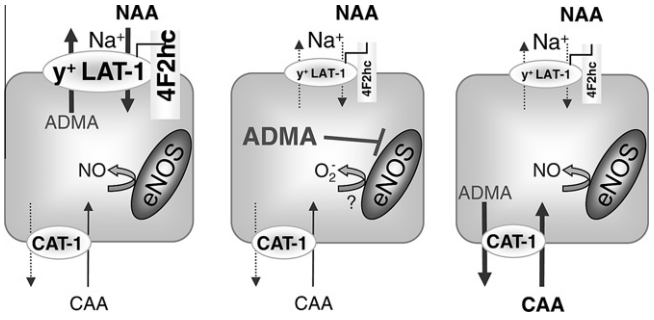

Fig. 4. Proposed scheme of ADMA efflux under normal and pathophysiological conditions. Left scheme: Under normal physiological conditions efflux of ADMA from endothelial cells occurs mainly via system y^+L (y^+LAT1 or 2/4F2hc heteromeric amino acid transporter) in exchange against extracellular large NAA present in high concentrations in human plasma. Na^+ is necessary for influx of exchange NAA through system y^+L. The Na^+ gradient thus represents the driving force for ADMA export. Middle scheme: Reduced system y^+L activity leads to intracellular accumulation of ADMA to concentrations high enough to inhibit or even uncouple NO synthesis (and maybe influence other cellular processes). Right scheme: Exchange of extracellular cationic amino acids (CAA) against intracellular ADMA via CAT proteins represents an alternative route of ADMA efflux. To run efficiently, this pathway requires however higher CAA concentrations than present in normal plasma.

5. Disclosures

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.044.

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